CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

Pregnancy, also known as gestation, is the time during which one or more offspring develops inside a woman (Shriver, 2015). A multiple pregnancy involves more than one offspring, such as with twins (Wylie, 2005). Pregnancy can occur by sexual intercourse or assisted reproductive technology (Shehan, 2016). Childbirth typically occurs around 40 weeks from the last menstrual period (LMP) (Abman, 2011 and Shriver, 2015). Pregnancy is typically divided into three trimesters. The first trimester is from week one through 12 and includes conception, which is when the sperm fertilizes the egg. The second trimester is from week 13 through 28, while the third trimester is from 29 weeks through 40 weeks (Shriver, 2015).

Normal pregnancy is a state characterized by many physiologic haematological changes, which appear to be pathological in the non – pregnant state (Chandra, 2012). The haematologic system must adapt in a number of ways such as provision of vitamins and minerals for foetal haematopoiesis (iron, vitamin B12, folic acid) which can exacerbate maternal anaemia, and preparation for bleeding at delivery, which requires enhanced haemostatic function, while these changes facilitate healthy pregnancy, they also increase the risk of some conditions (e.g. venous thromboembolism) (Mohammed *et al*., 2016). Pregnancy is influenced by many factors, some of which include culture, environment, socioeconomic status, and access to medical care (Yip, 2000). Haematological profile is measured all over the world to estimate general health, because, it is a reliable indicator and is a simple, fast and cost-effective test (Shen *et al.,* 2010). During pregnancy, changes occur and can be observed in haematological indices such as Red Blood Cell (RBC) count, haemoglobin (Hb) concentration platelet (PLT) count, and white Blood cell (WBC) count. For example, the RBC and PLT counts are decreased, partly as a result of the physiological haemodilution that occurs in pregnancy (Dhariwal *et al.*, 2016), while others are increased, such as the WBC count (Akingbola *et al.*,2006). Many of the haematological indices are also influenced by many factors such as sex, seasonal variation, lactation, healthy and nutritional status (Smith, 1993). Some studies such as Osonuga *et al.,* (2011) and Shaw *et al.,* (2010), have also identified the haematological indices as being affected by pregnancy.

Pregnancy is a physiologically immunocompromised state during which alterations in T – lymphocyte subsets may occur (Tanjong *et al*., 2012). It requires physiologc adaptations in all

maternal systems including the immune system. This process is complex and includes modifications at different levels and compartments of the maternal immune system. Although many of these changes are only partially explored and understood, recent investigations have shown that during pregnancy, maternal circulating immune cells undergo modifications in cell counts, phenotypes, functions, and ability to produce soluble factors, such as cytokines. The ultimate goal is to establish and maintain a successful pregnancy, which involves a state of selective immune tolerance, immune suppression and immunomodulation in the presence of a strong antimicrobial immunity. The mammalian immune system has evolved to co-exist with these needs by down-regulating potentially dangerous T-cell-mediated immune responses, while activating certain components of the innate immune system, such as monocytes and neutrophils. This unique dysregulation between different components of the immune system plays a central role in the maternal adaptation to pregnancy (Luppi, 2003).

Cytokines are signaling proteins, usually less than 80 kDa in size, which regulate a wide range of biological functions including innate and acquired immunity, hematopoiesis, inflammation and repair, and proliferation through mostly extraellular signaling. They are secreted by many cell types at local high concentrations and are involved in cell - cell interactions,have an effect on closely adjacent cells, and therefore function in a predominantly paracrine fashion. They may also act at a distance by secretion of soluble products into the circulation (endocrine or systemic effect) and may have effects on the cell of origin itself (autocrine effect) (Chung, 2009). Despite the unique ability of the immune system to highly specific antigenic recognition, its susceptibility to cytokines allows these molecules to dominate all kinds of immune reactions. These proteins, produced in an autocrine or paracrine fashion, bind to specific receptors initiating thus a cascade of reactions on different targets having beneficial or harmful effects since their redundancy and/or pleiotropic nature may account for all possible reactions (Vassiliadis *et al*., 1998). With T-helper (Th) lymphocytes being the major producers of cytokines, it is believed that the equilibrated balance of Th1 versus Th2 cytokines defines the welfare of the organism. If Th1 type cytokines are indeed deleterious to pregnancy, T- helper 2(Th2) type cytokines may be conducive to pregnancy and Th2 type immunity has been proposed to be the normal profile in successful pregnancy (Raghupathy *et al*., 2000).

In Pregnancy, Th1-1mediated responses were shown to induce abortion (Wegman *et al*., 1993) and therefore Th1 was regarded as ‘the bad’ immune reaction. Accordingly, all cytokines countering Th1 responses were regarded as Th2, like IL-4 and IL-10. Although IL-10 was originally launched as a cytokine produced by Th2 cytokine, it is not strictly a Th2 cytokine, but

rather a more general immunomodulating cytokine. Indeed IL-10 inhibits Th1, but in fact it also inhibits Th2 immunity, as it does inhibit several other inflammatory mediators (Commins *et al*., 2010). IL-10 is better classified as an anti-inflammatory cytokine. In the simplified Th1/Th2 model, pro-inflammatory cytokines are often regarded as belonging to the Th1 subset in fields where Th1 is the disease-promoting response, for example, in organ-specific autoimmunity and in pregnancy. Of note, inflammation is a hallmark of other T helper subsets like the Th2. Consequently, pro-inflammatory cytokines like tumor recrosis factor (TNF) are also present in Th2-mediated pathology such as allergic inflammation, and the pro-inflammatory cytokine IL-6 is, for example, involved in the development of Th17. Therefore, it is important to view cytokines from different perspectives and distinguish the T helper (Th1/Th2/Th17) perspective from the pro-versus anti-inflammatory perspective. However, the most relevant approach is to denote the referred cytokine by name rather than to its belonging to a certain group. Here, IL-2 deserves some extra attention because, it is often referred to as a Th1 cytokine, although it is a growth factor necessary for the activation of all the T helper subsets (Commins *et al*., 2010).

The immune system, in order to ensure protection from microbial infections, auto-immune reactions, graft rejection, allergies etc, shifts the balance towards one of the other family of cytokines (Th2). Pregnancy is a natural example of an immune reaction occurring for a determined time period in the organism which opposes the rules of graft rejection. The semi – or allogenic fetal components growing in the privileged site of uterus, not only escape maternal immune attack but are supported by the maternal immune system (Vassiliadis *et al*., 1998).

Normal pregnancy is a hypercoagulable state, a physiological safety valve aimed at preventing excessive maternal blood loss at delivery (Ibeh *et al.*, 2015). Pregnancy and the puerperium are well-established risk factors for venous thromboembolism (VTE), a disease that includes pulmonary embolism (PE) and deep venous thrombosis (DVT).Approximately 30% of apparently isolated episodes of PE are associated with silent DVT, and in patients presenting with symptoms of DVT, the incidence of silent PE raises from 40-50%. VTE is both more common and more complex to diagonose in those patients who are pregnant than in those who are not (Marik and Plante, 2008).). Women are up to 5times more likely to develop DVT during pregnancy than when not pregnant (Jacobsen *et al*., 2008).

Estimates of the incidence of Venous Thrombo Embolism(VTE) vary between 1 in 1000 and 1 in 2000 deliveries (Hui and Lili, 2012) and the incidence is higher during the third trimester of pregnancy, and in the puerperium. Pregnancy is normally associated with significant changes in all aspects of the classic triad of Virchow: Venous stasis, endothelial damage and enhanced

coagulation, to the extent that the procoagulant effect becomes dominant (O'Riordan and Higgins, 2003). In late pregnancy the concentrations of many coagulation factors are increased to twice their levels in non-pregnant women (Bremme, 2003). The modifications of the coagulation system result from hormonal changes and are part of a complex physiological adaptation of the human female organ to pregnancy. Its purpose is to ensure rapid and effective control bleeding from the placental site and prevent fetal haemorrhage during delivery and the puerperium. Placental separation is a severe and acute challenge to haemostasis (Bremme, 2003).

This study was undertaken to determine the CD4+ cell count, and levels of some cytokines and haematological parameters of apparently healthy pregnant women attending Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra state.

1.2 Statement of the Problem

Previous studies have shown that normal pregnancy is associated with profound changes in haematological profile which appears to be pathological in the non-pregnant state (Chandra, 2012), coagulation profile, which may lead to excessive maternal bleeding during delivery (Ibeh *et al*., 2015) and immunological parameters, which may cause complications in pregnancy (Chatterjee *et al.,* 2014). Studies conducted by Akinbami *et al*., (2013), showed that in normal pregnancy, some haematological parameters such as PCV, Hb, RBC and platelets counts were decreased, partly as a result of haemodilution, while some like WBC counts were increased due to leukocytosis associated with pregnancy, and the body building immunity for the foetus. Ibeh *et al*., (2015), stated that the PT and APTT were shortened in pregnancy to prevent excessive maternal bleeding during delivery. According to Ufelle *et al*., (2017), the CD4+ counts were decreased in pregnancy, may be due to the fact that pregnancy is an immunocompromised state which alters T-lymphocyte subsets and the presence of hormones which locally suppress immune response. Felicano *et al*., (2014), showed that Th2 cytokines are increased in pregnancy, and that the balance aids in the explanation about the environment of cytokines underlying a successful pregnancy.

In recent past, maternal mortality has been remarkably attributed to pregnancy - related conditions. Most complications in pregnancy are associated with certain immunological and haematological dysfunctions. The consistency of this distribution has scarcely been studied in our environment for various gestational periods of pregnancy. A timely approval of parameters, will to a great extent curtail the untoward consequences arising from the parameters.

Therefore, the need to investigate these parameters in pregnancy is most expedient in order to monitor and follow-up pregnancies at risk, to prevent adverse outcomes.

1.3 Justification of the Study

Pregnancy is characterized by changes in haematological and immunological profiles, but the influence of pregnancy on these profiles is scarcely highlighted.Most of the studies concentrated more on few of the haematological profiles without the need for longitidunal study (Osonuga *et al.*, 2011**,** Akinbami *et al*., 2013 and Obeagu *et al*., 2014). There is dearth **of** longitudinal studies on immunological aspect of normal pregnancy in Nnewi and its environs, and most of the studies concentrated on pregnancy in diseased state (Igwegbe *et al*., 2010, Umeononihu *et al*., 2013, Chikwendu *et al*., 2015 and Okonkwo and Okaka, 2017). Hence, the need for this study.

This Study will determine the CD4+ cell count, and levels of some cytokines and haematological parameters of pregnant women, help in the management of pregnancies at risk, and reinforce the need for early booking and iron supplementation.

1.4 Aim and Objectives of the Study

1.4.1 Aim

The study was aimed at determining the CD4+ cell count, and levels of some cytokines and haematological parameters of apparently healthy pregnant women attending NAUTH, Nnewi.

1.4.2 Specific Objectives

1. To determine the concentrations of IL – 2, IL – 4, IL – 10, TNF –α and CD4+ cell count in pregnancy at different trimesters and in non – pregnant women.
2. To evaluate the Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) at different trimesters and in non – pregnant women.
3. To determine the levels of serum ferritin and serum iron in pregnancy at different trimesters and in non – pregnant women.
4. To evaluate the levels of PCV, Hb, RBC, WBC, PLTs, MCV, MCH, MCHC and differential white cell counts in pregnancy and in non – pregnant women.
5. To compare these parameters across the trimesters.

1.5 Research Questions

1. Can Pregnancy affect haematological and Immunological profiles?

2. Is there a relationship between haematological and immunological parameters in pregnancy?

1.6 Research Hypothesis

Ho: (Null)

H1: (Alternative)

Ho - Haematological parameters of pregnant women are not different from that of non-pregnant females.

H1 - Haematological parameters of pregnant women are different from that of non-pregnant females.

Ho - Immunological profiles of pregnant women are not different from that of non-pregnant females.

H1- Immunological parameters of pregnant women are different from that of non-pregnant females.

Ho - There are no relationships between immunological and haematological profiles at different trimesters.

H1 - There are relationships between immunological and haematological profiles at different trimesters.

CHAPTER TWO
LITERATURE REVIEW

2.1 Haematology of Pregnancy

In normal pregnancy, there is an increase in erythropoietin activity. However, at the same time, an increase in plasma volume occurs, and this results in a progressive decrease in Haemoglobin (Hb), Haematocrit (HCT) and Red Blood Cell (RBC). The level returns to normal about a week after delivery. There is a slight increase in mean cell volume (MCV) during the 2ndtrimester. Serum ferritin decreases in early pregnancy and usually remains low throughout pregnancy, even when supplementary iron is given (Bain *et al.,* 2012).

Pregnancy places extreme stress on the haematological system and an understanding of the physiological changes that result is obligatory in order to interpret any need for therapeutic intervention. (Hoffbrand and Moss, 2011). Changes in haematological profile affect pregnancy and its outcome. Anaemia is the most common haematological profile, followed by thrombocytopenia. Leukocytosis is always associated with pregnancy (Akinbami *et al*., 2013 and Hay *et al*., 2016*).* During pregnancy, the plasma volume increases by 50% and red cell mass by 20 – 30% (Guyton and Hall, 2005). The haematocrit is decreased particularly in the last trimester due to increase in plasma volume (Chaudari and Bodat, 2015). Conversely, there is a significant decrease in platelets count with gestational age (Azab *et al*., 2017). During pregnancy, the uterine wall continuously expands to accommodate fetal growth. This causes laceration of blood vessels at the uterus leading to massive hemorrhage. The primary hemostatic plug where these tears occur is formed by platelets (Kaur *et al*., 2014). A pregnant woman will also become hypercoagulable, leading to increased risk for developing blood clots and embolisms, due to increased liver production of coagulation factors, mainly fibrinogen and factor VIII. This hypercoagulable state along with the decreased ambulation (exercise involving legs) causes an increased risk of both Deep Vein Thrombosis (DVT) and Pulmonary Embolism (PES). Women are at highest risk for developing clots, or thrombi, during the weeks following labour. Clots usually develop in the left leg or the left iliac artery. The increased flow in the right iliac artery

after birth compresses the left iliac vein leading to an increased risk for thrombosis (clotting) which is exacerbated by the aforementioned lack of ambulation following delivery. Both underlying thrombophilia and caesarean section can further increase these risks. Edema or

swelling of the feet is common during pregnancy, partly because the enlarging uterus compresses veins and lymphatic drainage from the legs (Guyton and Hall, 2005).

During pregnancy, Activated partial Thromboplastin Time (APTT) is normal or slightly decreased, but prothrombin Time (PT) is markedly shortened (Abbassi- Ghanavati *et al.,* 2009). Changes in the variables of coagulation are more significant in the third trimester of pregnancy (Han *et al*., 2014), and are probably due to hormonal changes and especially to the increasing Oestrogen levels as pregnancy progresses.The changes in haemostatic parameters can explain why women who use oral contraceptives are exposed to an increase risk of various thrombosis and why the risk is further increased in third-generation oral contraceptive users (Tchaikovski *et al*., 2006). The use of oral contraceptives is associated with an increased risk of venous thrombosis. It is now generally accepted that women who use oral contraceptives that contain so-called third-generation progestins (desogestrel or gestodene) are exposed to a two-fold higher risk of venous thrombosis than women who use oral contraceptives that contain the second-generation progestin levonor-gestre l(Tchaikovski *et al*., 2006).

2.1.1 Physiological anaemia

Physiological anaemia is the term often used to describe the fall in haemoglobin (Hb) concentration that occurs during normal pregnancy. Blood plasma volume increases by approximately 1250ml or 45%, above normal by the end of gestation, and although the red cell mass itself increases by some 25%, this leads to fall in Hb concentration. Values below 10gl/dl are probably abnormal and require investigation (Hoffbrand and Moss, 2011).

Anaemia is a widely identified haematological abnormality, and it is also associated with adverse pregnancy outcome. It is generally defined as a state in which the amount of haemoglobin or the number of red blood cells in the circulation is reduced significantly below that which is normal for the age and sex of the individual in a given environment (Sifakis and Pharmakides, 2000). This is referred as physiological anaemia, which is due to haemodilution, resulting from the disproportionate increases in the plasma volume and red cell mass of the pregnant woman (Llwellyn- Jones, 2000).During the past few years, the relation between anaemia early in pregnancy and an increased risk of preterm delivery has been suggested. Likewise, the relation of adverse pregnancy outcomes with high haemoglobin and increased iron stores has been documented. However, the risks and benefits of prophylactic iron supplementation in pregnant women who are not iron deficient remains a source of controversy (Scanlon *et al.,* 2000).

The diagnostic work-up of iron deficiency includes red cell indices, serum iron, serum total iron binding capacity, serum transferrin saturation, serum transferrin receptors level and serum ferritin level. Microcytosis is a sensitive index of iron deficiency but its value is limited by physiological increase in MCV that often occurs during pregnancy. Serum iron transferrin is frequently abnormal in pregnancy. Low sensitivity of transferrin saturation and day to day and even hour to hour fluctuation of serum iron levels renders it less efficient than serum ferritin level for diagnosing iron deficiency which is the only condition associated with decreased serum ferritin concentration (Fai and Lao, 1999 and Namama, 2015).

Previous studies have proven that serum ferritin is the single best non-invasive test and is a very useful and reliable index of iron stores especially during pregnancy, with low levels indicating iron deficiency (Hou *et al.,* 2000 and Mast, 2001). It is the best indicator of discrimination between iron replete and iron deficient subjects and except for bone marrow biopsy, is the best measure of body iron stores. There is only one limitation with serum ferritin, as it is an acute phase reactant protein and is a sensitive indicator of iron stores in those suffering from an infection, inflammation or cancer. Serum ferritin concentration shows a marked decrease after 12 weeks of gestation with relatively constant values after 32 weeks. This decline is more in women who start pregnancy with inadequate stores and in those who had three or more pregnancies (multiparous) (Punnonen *et al*., 1997). Routine daily iron supplementation during pregnancy has been found to improve the iron status of both mother and baby, and also the outcome of pregnancy (Gomber *et al.,* 2002).

Gomber *et al.,* (2002), have shown that serum ferritin values continue to remain low during pregnancy irrespective of supplementation, since the demands for iron outstrips the supply, and supplementation helps in preventing the depletion of iron stores. Thus, iron replacement in deficient mothers, detected by low serum ferritin concentration, seems most appropriate (Fai and Lao, 1999).

2.1.2 Iron deficiency anaemia

Iron deficiency anaemia (IDA) is a common problem in many developing countries. It is still considered the most common nutritional deficiency worldwide. Apart from its direct haematologic importance, IDA affects cellular and humoral immunity and predisposes the host to infections (Ekiz *et al.,* 2005).

Pregnant women are highly prone to IDA (Ekiz *et al.,* 2005). Controversial results are reported in studies targeting this group of patients. Tang *et al.,* (2006), showed a direct association

between haemoglobin concentration and the count of $CD4^+$ T-cell lymphocytes, serum levels of IL-2 and lgG, and an inverse association with susceptilibility to infection (Zhou *et al.*, 2006).

Up to 600mg iron is required for the increase in red cell mass and a further 300mg for the fetus. Despite an increase in iron absorption, few women avoid depletion of iron reserves by the end of pregnancy. In uncomplicated pregnancy, the mean corpuscular volume (MCV) typically rises by approximately 4fl. A fall in red cell MCV is the earliest sign of iron deficiency. Later, the mean corpuscular Haemoglobin (MCH) falls and finally anaemia results. Early iron deficiency is likely if the serum ferritin is below 15ug/l together with serum iron <10 umol/l and should be treated with oral iron supplements. The use of routine iron supplementation in pregnancy is debated, but iron is probably better avoided until Hb falls below 10gldl or MCV below 82fl in the third trimester (Hoffbrand and Moss, 2011).

2.1.3 Folate deficiency

Folate requirements are increased approximately two-fold in pregnancy and serum folate levels fall to approximately half the normal range with a less dramatic fall in red cell folate. In some parts of the world, megaloblastic anaemia during pregnancy is common because of a combination of poor diet and exaggerated folate requirements. Given the protective effect of folate against neural tube defects, folic acid 400ugl per day should be taken periconceptually and throughout pregnancy. Food fortification with folate is now being practiced in many countries. Vit B_{12} deficiency is rare during pregnancy although serum vitamin B_{12} levels fall to below normal in 20 – 30% of pregnancies and low values are sometimes the cause of diagnostic confusion (Hoffbrand and Moss, 2011).

2.1.4 Thrombocytopenia

The platelet count typically falls by approximately 10% in an uncomplicated pregnancy. In approximately 7% of women, this falls more severe and can result in thrombocytopenia (Platelet count $<140x10^9/_L$). In over 75% of cases, this is mild and of unknown cause, a condition referred to as incidental thrombocytopenia of pregnancy. Approximately 21% of cases are secondary to a hypertensive disorder and 4% are associated with immune thrombocytopenic purpura (Hoffbrand and Moss, 2011).

2.1.4.1 Incidental thrombocytopenia of pregnancy

This is a diagnosis of exclusion and is usually detected at the time of delivery. The platelet count is always >70 x 10^9/L and recovers within 6 weeks. No treatment is required and the infant is not affected (Bain *et al.*, 2012).

2.1.4.2 Thrombocytopenia of hypertensive disorders

This is variable in severity, but the platelet count rarely falls to <40 x 10^9/L. It is more severe when associated with pre-eclampsia and if severe, the primary treatment is as rapid delivery as HELLP syndrome (Haemolysis Elevated Liver Enzymes and low platelets) is a subtype of this category (Hoffbrand and Moss, 2011).

2.1.4.3 Idiopathic thrombocytopenic purpura (ITP)

In pregnancy, ITP represents a particular problem, both to the mother and to the fetus, as the antibody crosses the placenta and the fetus may become severely thrombocytopenic, like all adult, pregnant women with ITP and platelet counts <10 x 10^9/L who are in their second or third trimester or who are bleeding. Treatment is with steroids, intravenous immunoglobulin GI(IgG) and splenectomy as appropriate (Hoffbrand and Moss, 2011).

At delivery, umbilical vein blood sampling or fetal scalp vein sampling to measure the fetal platelet count may be offered although their exact role is unclear, in general, caesaraean section is not indicated when the maternal platelet count is >50 x 10^9/L unless the fetal platelet count is known to be <20 x 10^9/L. Platelet transfusion may be given to mothers in labour with very low platelet counts or who are actively bleeding. Newborns of mothers with ITP should have a blood count measured for the first four days of life as the platelet count may progressively drop. A count greater than 50 x 10^9/L is reassuring. Cerebral ultrasounds may be performed to look for intracranial haemorrhage (Hoffbrand and Moss, 2011).

2.1.5 Changes in red blood cell in pregnancy

In pregnancy, plasma volume increases 25 – 80% between 6^{th} and 24^{th}week of gestation (Abbassi – Ghanavati *et al.*, 2006). However, the increase in RBC mass has been found to be approximately 30% between the 12^{th} and 36^{th} week of gestation when iron and folate are supplemented (Abbassi – Ghanavati *et al.*, 2006). The discrepancy between the rate of increase

in plasma volume and that in RBC mass leads to physiological anaemia. In late pregnancy, plasma volume increases at a slower rate, inducing a slight rise in haematocrit level. These

physiological changes during pregnancy make it difficult to define normal haematological reference intervals for pregnant women (Shen *et al.*, 2010).

Anaemia is the most common haematological problem in pregnancy (CDC, 2005). In iron – affluent pregnant women, "anaemia" is defined as Hb <ll0gll or less than the fifth percentile of the distribution, based on age and stage of pregnancy (Akingbola *et al.*, 2006). Anaemia contributes to low birth weight and miscarriages and is a primary cause of low immunity in both the mother and the child, which makes them vulnerable to several infections (RCOG, 2010). Malaria infection causes 3 – 5% of maternal anaemia, and worldwide, about 50 million women are exposed to malaria especially in highly endemic regions like Nigeria (Adeyemi *et al.*, 2007).

Akinbami *et al.*, (2013), found a progressive decline in Hb concentration from the first to the third trimester, but a drop from first to the second trimester. There was a slight rise in the PCV in the third trimester. These findings corroborate those of a similar study undertaken in Ibadan, South-Western Nigeria, by Akingbola *et al.*, (2006), which reported exactly the same pattern. The progressive decline in Hb concentration from the first to third trimester may be due to an increased demand for iron as pregnancy progresses. More iron is required to meet the expansion of maternal Hb mass and the needs of fetal growth. The additional progesterone and estrogen that are secreted by the placenta during pregnancy cause a release of rennin from the kidneys. Rennin stimulates the aldosterone – rennin angiotension mechanism, leading to sodium retention and increased plasma volume. The increase in plasma volume is relatively greater than the increase in red cell mass, which results in a fall in maternal Hb, hence the physiological anaemia that occurs in pregnancy (Akinbami *et al.*, 2013). Despite the physiological haemodilution associated with pregnancy, which also contributes to the drop in PCV in the first and second trimester, in late pregnancy, plasma volume increases at a slower rate, inducing a slight rise in haematocrit that may account for the slight rise in PCV in the third trimester (Shen *et al.*, 2010). MCV declined from the first to the third trimester, while MCH remained relatively stable through all trimesters. MCHC was stable in the first and second trimesters, but dropped in the third. These findings may be a reflection of iron deficiency anaemia (Akinbami *et al.*, 2013). Table 2.1 shows normal reference ranges of some haematological parameters in non – pregnant women and at different trimesters of pregnancy.

Parameter	**Non-Pregnant women**	**First Trimester**	**Second Trimester**	**Third Trimester**

PCV (%)	38.8-40.5	35.9-39.4	34.8-36.	35.4-36.6
Hb (g/dl)	12.4-13.1	11.1 -12.5	11.3 – 11.8	11.7 – 12.1
WBC ($x10^9/_L$)	4.4 – 4.82	5.2 -6.6	6.4 – 7.4	6.3– 6.9
RBC ($X10^9/_L$)	4.5 – 5.3	4.5 –5.0	4.0- 4.3	4.5 – 5.0
Percentage Neutrophil (%)	49.1 – 52.3	56.9 – 63.1	62.3 – 65.8	62.8 – 65.6
Percentage Lymphocyte (%)	39.0 – 42.1	26.8 – 33.0	25.8 – 29.0	25.3 – 28.1
Monocytes (%)	6.5 – 7.5	7.1 – 9.3	6.6 – 7.8	6.7 – 7.6
Eosinophils (%)	1.32 – 1.91	0.8 – 3.0	0.8 – 1.8	1.6 – 2.5
Basophils (%)	0.06 – 0.18	0.07 – 0.38	0.00 – 0.2	0.00 – 0.10
Platelet ($x10^9/_L$)	229.3 – 251.2	221.8 – 277.8	212.9 – 256.1	190.1- 212.9
MCV (fl)	84.8 – 86.5	77.4-84.5	84.1-88.0	83.8-86.6
MCH (pg/cell)	27.1-28.9	23.4-26.7	27.2-28.8	27.0-28.8
MCHC (g/dl)	31.8 – 32.3	30.5 – 32.1	32.2 – 32.9	32.8 – 33.4

Table 2.1: Normal Reference Ranges of some Haematological Parameters in Non-pregnant women and at different Trimesters of Pregnancy. (Timzing *et al*., 2014).

2.1.6 Platelet changes in pregnancy

The platelet count is slightly lower in pregnant women than in non-pregnant women (McCrae, 2003). Most studies report an approximately 10% lower platelet level at term compared with pre-pregnancy (Oladokun *et al.,* 2010). Large cross sectional studies done in pregnancy of healthy women, have shown that the platelet count does decrease during pregnancy, particularly in the third trimester. This is termed as "Gestational thrombocytopenia". It is partly due to haemodilution and partly due to increased platelet activation and accelerated clearance (Chandra *et al*., 2012) However, van Buul, (1995), reported an increase in platelet count in pregnancy. The majority of pregnant women still have levels within the normal range. However, if the pregnancy level is borderline or there is a more severe reduction, this may fall below the normal range. The mechanisms for this are thought to be due to dilution effects and accelerated destruction of platelets passing over the often scarred and damaged trophoblast surface of the placenta (Jensen *et al.,* 2011). Platelet counts may also be lowered in women with twin compared with singleton pregnancies, possibly due to greater thrombin generation (Tsunoda *et al*., 2002). Although most cases of thrombocytopenia in pregnancy are mild, with no adverse outcome for mother or baby, occasionally, a low platelet count may be part of a complex disorder with significant morbidity and be (rarely) life threatening (Tsunoda *et al*., 2002).

Overall about 75% of cases of platelet changes are due to gestational thrombocytopenia, 15 – 20% secondary to hypertensive disorders, 3-4% due to an immune process, and the remaining 1 – 2% comprises rare constitutional thrombocytopenias, infections and malignancies (Bochlen *et al*., 2000). Akinbami *et al*.,(2013), reported a gradual reduction in platelet count as pregnancy advanced, which was also consistent with Akingbola *et al's* study (Scholl, 1998). Due to haemodilution secondary to expansion of plasma volume, the platelet count in normal pregnancies may decrease by approximately 10%, with most of this decrease occurring during the third trimester (Sloan *et al*., 2002), although the absolute platelet count tends to remain within the normal reference range in most patients. Thrombocytopenia is the second most common haematologic abnormality that occurs during pregnancy after anaemia (Chen and Scholl, 2003), and it's classically defined as a platelet count of less than 150,000 x 10^9/L (Hemminki and Rimpela, 1991). Counts from 100,000 to 150,000 x 10^9/L are considered mildly depressed, from 50,000 to 100,000 x 10^9/L, moderately depressed and of less than 50,000 x 10^9/L, severely depressed. The overall incidence of thrombocytopenia in pregnancy is 8%, but when patients with obstetric or medical conditions are excluded, the incidence drops to 5.1% (Chen and Scholl, 2003).

2.1.7 White blood cell changes in pregnancy

White Blood Cell count (WBC) is increased in pregnancy with the lower limit of the reference range being typically 6,000/cumm. Leukocytosis, occurring during pregnancy is due to the physiologic stress induced by the pregnant state (Abramson and Melton, 2000).

Neutrophils are the major type of leukocytes on differential counts (Jessica *et al.*, 2007). This is likely due to impaired neutrophilic apoptosis in pregnancy. The neutrophil chemotaxis and phagocytic activity are depressed, especially due to inhibitory factors present in the serum of a pregnant female. There is also evidence of increased oxidative metabolism in neutrophils during pregnancy (Jessica *et al*., 2007). Immature forms as myelocytes and metamyelocytes may be found in the peripheral blood film of healthy women during pregnancy and do not have any pathological significance (Karalis *et al*., 2005). They simply indicate adequate bone marrow response to an increased drive for erythropoesis occurring during pregnancy. Lymphocyte count decreases during pregnancy through the first and second trimesters and increases during the third trimester. There is an absolute monocytosis during pregnancy, especially in the first trimester, but decreases as gestation advances. Monocytes help in preventing fetal allograft rejection by in filtrating the decidual tissue (7^{th} – 20^{th} week of gestation) possibly, through PGE 2 mediated immunosuppression (Kline *et al*., 2005). The monocyte to lymphocytes ratio is markedly increased in pregnancy. Eosinophil and basophil counts, however, do not change significantly during pregnancy (Eldestam *et al.,* 2001). The stress of delivery may itself lead to brisk leucocytosis. Few hours after delivery, healthy women have been documented as having a WBC count varying from 9,000 to 25,000/cumm. By 4 weeks post – delivery, typical WBC ranges are similar to those in healthy non-pregnant women.

Previous studies have reported that pregnancy is usually accompanied by leukocytosis, but the full sequential changes of the various cell types responsible for this observed leucocytosis have not been clearly determined in all geographical locations and physiological conditions (Okunola *et al.,* 2006). As such, the establishment of reference values of haematological indices in pregnancy is considered important. According to Akinbami *et al.,* (2013), there was an increase in WBC count from the first to the third trimester, and this finding was consistent with the findings of Akingbola *et al.,* (2006). The increase is primarily due to an increase in neutrophils and may represent a response to stress due to redistribution of the WBC, between the marginal and circulating pools. Pain, nausea, vomiting and anxiety have been reported to cause leukocytosis in the absence of infection (Onwukeme and Uguru, 1990 and Akinbami *et al.,* 2013). A rising WBC count in pregnancy is not a reliable indicator of infection in subclinical

chorioamnionitis, rather, clinical methods of detection such as maternal pyrexia, offensive vaginal discharge, and fetal tachycardia are better indicators, especially of preterm labour and membrane rupture (Akinbami *et al.,* 2013).

2.1.8 Iron status during pregnancy

Iron is by mass the most common element on earth, forming much of earth's outer and inner core. It is the fourth most common element in the earth's crust (MIC, 2016).The body of an adult human contains about 4 grams (0.005% body weight) of iron, mostly in hemoglobin and myoglobin. These two proteins play essential roles in vertebrate metabolism, respectively oxygen transport by blood and oxygen storage in muscles. To maintain the necessary levels, human iron metabolism requires a minimum of iron in the diet (MIC, 2016).

Iron requirements are greater in pregnancy than in the non-pregnant state. Although iron requirements are reduced in the first trimester because of the absence of menstruation, they steadily increase thereafter. The total requirement of a 55kg woman is approximately 1000mg. Translated into daily needs the requirement is approximately 0.8mg Fe in the first trimester, and greater than 6mg in the third trimester. Absorptive behavior changes accordingly, a reduction in iron absorption in the first trimester is followed by a progressive rise in absorption throughout the remainder of pregnancy. The amounts that can be absorbed from even an optimal diet, however, are less than the iron requirements in later pregnancy and a woman must enter pregnancy with iron stores of >/ = 300mg if she is to meet her requirements fully (Bothwell, 2000)

Iron deficiency continues to be the leading single nutritional deficiency in the world, despite considerable efforts to decrease its prevalence (WHO, 2001).Women in developing countries are always in a state of precarious iron balance during their reproductive years. Their iron stores are not well developed because of poor nutritional intake, recurrent infections, menstrual blood loss and repeated pregnancies (Mukherji, 2002). During the first two trimesters of pregnancy iron deficiency amaemia increases the risk for preterm labour, low - birth weight babies, and infant mortality and predicts iron deficiency in infants after 4 months of age (Brabin *et al*., 2001). It is estimated that anaemia accounts for 3.7% and 12.8% of maternal deaths during pregnancy and childbirth in Africa and Asia, respectively (Khan *et al*., 2006)

2.2 Maternal Anaemia and Iron Supplementation

It is not certain if the effect of anaemia on pregnancy outcome is casual and could be prevented by supplementation with iron. Observational data on anaemia imply that iron supplementation should be started early in pregnancy, if not before, to prevent preterm delivery. If this is true, then iron supplementation started after mid pregnancy, the usual time for most women, is unlikely to reduce risk. In a clinical trial conducted by Cogswell *et al.,* (2003), prophylactic iron supplementation from entry to week 28 did not increase maternal serum ferritin or hemoglobin, reduce risk of maternal anaemia or reduce any other measures of maternal iron status in iron supplemented women compared with controls. However, after adjustment was made for 2 factors that differed initially between the groups (pregravid weight and serum ferritin concentration) the proportions with absent iron stores (ferritin <12ug/L) at week IDA (Hb < 119g/L, ferritin <12ug/L) at week 28 were significantly lower among the iron supplemented. Supplemented women had significantly longer gestation durations (+ 0.6 wk), and increased infant birth weight and preterm low birth weight. Risk of preterm delivery was not reduced by supplementation but had been reckoned solely from the mother's last menstrual period (LMP) based on her recall. Failure to confirm or modify the mother's LMP by ultrasound would introduce an unknown amount of error into an estimate of preterm birth (Cogswell *et al.,* 2003).

2.3 Outcome of Pregnancy with increased Iron Status and Stores

Randomized trials of iron prophylaxis during pregnancy have demonstrated positive effects on reducing low haemoglobin and haematocrit, and increasing serum ferritin, serum iron and other measures, including bone marrow iron (Sloan *et al.,* 2002). A study of iron containing supplement utilization from NHANES, 1988 – 94, showed that 72% of pregnant and 69% of lactating women used iron supplements during month before they were surveyed. However, median consumption of supplemental iron was in excess of the tolerable upper limit of 45mg/day in pregnant (58mg/d) and lactating women (57mg/d) (Cogswell *et al* ., 2003).

Overall, < 15% of reproductive age women, pregnant and non-pregnant alike, who took iron supplements, had or were being treated for anaemia within the past 3 months. Thus, there is a potential concern that some women who are not anaemic may be taking large doses of supplemental iron during pregnancy. It has been suggested that such use may build up the mother's iron stores and increase blood viscosity so that utero-placental blood flows impaired or that the excess iron intake could cause other toxic reactions (Rush. 2000).

In addition to their work on anaemia, Scanlon and Colleagues considered high levels of haemoglobin during the 1st and 2nd trimesters (Scanlon *et al.,* 2000). They found that high

haemoglobin was associated with an increased risk (5% - 79%) of small for gestation (SGA) births, but not with preterm delivery. Levels that were SD unit or more above the mean marked the threshold for increased risk and were equivalent to 131g/l at week 12 and 126g/L at week 18.

2.4 Blood Ferritin Levels in Normal Pregnancy

Ferritin is a protein that performs an iron storage function in mammals. It is found mainly in the liver, spleen and bone marrow and to a lesser extent throughout the tissues. The concentrations of serum ferritin present a close correlation with total reserves of iron in the body. This protein can therefore be used as a reliable estimator of iron reserves in the organism. During gestation, levels of serum ferritin fall by 50%. This is a consequence of the normal heme-iron dilution process during pregnancy, and also of the extraction of iron by the foetus (Jones *et al.,* 2000). The utility of ferrotherapy during gestation is still a matter of controversy. Some studies have found beneficial effects for the mother including lower rate of anaemia (Karimi *et al.,* 2000), and for the foetus where higher levels of ferritin have been found in newborns when the mothers received iron supplements during their pregnancy (Casanova *et al.,* 2005). Serum ferritin is considered as a better parameter to detect latent iron deficiency especially before the change of red cell morphology and red cell indices. A high degree of correlation has been shown between serum ferritin concentration and bone marrow iron stores. In the stage of latent iron deficiency (absence of storage iron), as assessed by marrow iron content, serum ferritin concentration is decreased, but the transferrin saturation, serum iron, and Hb levels may remain unchanged (Hyder *et al.*, 2004).

During pregnancy, low serum ferritin concentrations in the presence of normal Hb indicate deficient iron stores. Such females are prone to develop overt iron deficient anaemia. In pregnancy, serum ferritin concentration is the maximum at 12 – 16 weeks of gestation, and then he levels start decreasing as the pregnancy advances. Low serum ferritin levels during second and third trimesters predict low haemoglobin levels in late pregnancy (Naghmi *et al*., 2007) showed that serum ferritin levels showed significantly lowest values in second trimester, with slight increase again in third trimester. Increasing Gravidity had no significant effect on serum ferritin levels (Naghmi *et al*., 2007).

Iron stores that are elevated in pregnancy are associated with preterm delivery, pre-eclampsia and gestation diabetes mellitus. Women with ferritin levels that are elevated for the third trimester of pregnancy (>41ng/ml) have a greatly increased risk of preterm and very preterm

delivery that has been attributed to intrauterine infection. Another plausible mechanism for high ferritin levels is failure of the maternal plasma volume to expand. In Camden, increased IDA and lower levels of folate were found in women who went to have high third trimester ferritin. In the third trimester, the situation reversed, thus implicating plasma volume expansion. Ferritin production also is increased with infection, produce and inflammation as part of the acute phase response (Scholl, 1998).

In the presence of infection, macrophages produce inflammatory cytokines that generate reactive oxygen species, releasing free iron from ferritin.The normal reference ranges for ferritin (ng/ml) in non-pregnant, first, second and third trimesters are 10 – 150, 6 – 130, 2 – 230, and 0 – 116 respectively (Milman *et al.,* 2007).

2.5 Blood Coagulation

The coagulation process is a complex series of enzymatic reactions involving the proteolytic activation of circulating coagulation factors, (Zymogens) and activity of co-factors (V, VIII), leading to the production of thrombin which converts soluble plasma fibrinogen into fibrin. The fibrin enmeshes the platelet plug, forming a stable thrombus which prevents further blood loss from the damaged vessel (Cheesbrough, 2000).

2.5.1 The extrinsic and intrinsic pathways

The terms intrinsic and extrinsic have traditionally been used in describing the clotting process in the classical blood coagulation theory. It is now known that the intrinsic pathway (involving factor XII and Kallikrein) is of importance in the in vitro process (laboratory tests), but is less significant in the vivo (in the body) clotting process. The intrinsic pathway in vivo begins with the activation of factor IX by VIIa. Factor XI in vivo is activated by thrombin, calcium and the co-factor HMWK (high molecular weight kininogen). In the test tube, the initiation of clotting via the intrinsic system begins with the activation of factor XII when it is exposed to the glass surface (Cheesbrough, 2000).

2.5.2 Screening tests for blood coagulation

The following screening tests are used for blood coagulation: Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and Thrombin Time (TT).

2.5.2.1 Activated partial thromboplastin time (aptt)

The APTT is a screening test of the intrinsic clotting system. It will detect the inhibition or deficiency of one or more of the following factors: Prothrombin, V, VIII (antihaemophilic factor), IX, X, XI, XII and fibrinogen. The APTT is also used to monitor patients being treated with heparin (Cheesbrough, 2000).

Normal ranges of APTT in pregnancy and non-pregnancy states are as follows: Non – pregenancy (26.3 -39.4) 1st trimester (24.3 -38.9), 2nd (24.2 – 38.1) and 3rd (24.7 – 35.0) (Abbasi – Ghanavati *et al*., 2009). But it is also acknowledged that these values are to be interpreted in the light of ethnicity, race and socio-economic background (Hui and Lili, 2012). Singh *et al.,*(2016), showed a very high prevalence of shortened PT and APTT during third trimester of pregnancy and labour when chances of bleeding are highest.

2.5.2.2 Prothrombin time (PT)

The PT is a screening test for the extrinsic clotting system, i.e. factor VII. It will also detect deficiencies of factors, prothrombin, V, X, and fibrinogen. It is mainly used to monitor patients receiving warfarin anticoagulation (Cheesbrough, 2000).

Phrothrombin time (PT), which accesses the factors in the extrinsic pathway, was reduced in pregnancy when compared with the value in non – pregnant controls (Durotoye *et al.,* 2012). Okungbowa *et al.,* (2015), identified a significant increase in PT in pregnant women when compared with non – pregnant women. Endogenous thrombin generation and increased PT are associated with pregnancy (Hellgreen, 2003).Changes in coagulation system could be due to increased synthesis or increased activation by coagulation factors. These changes protect the mother from the hazard of bleeding imposed by placentation and delivery, but they also carry the risk of an exaggerated response, localized or generalized (Bijoy *et al.,* 1999). It has been observed that pregnant state results in significant increase in some coagulation parameters showing that pregnancy is a risk for hyper–coagulability and should be managed and monitored in order to reduce maternal and neonatal morbidities.

Normal ranges of PT in pregnancy and non – pregnancy are: Non- pregnant adult (12.7 – 15.4), 1st trimester (9.7 – 13.5), 2nd trimester (9.5 – 13.4) and 3rd (9.6 – 12.9). (Abbassi – Ghanavati *et al.,* 2009).

2.6 Haemostasis and Pregnancy

During normal pregnancy, the haemostatic balance tilts in the direction of hypercoagulability which helps to reduce bleeding complications during delivery (Hellgreen, 2003). The changes in the coagulation system during normal pregnancy are consistent with a continuing low grade process of coagulant activity. The hormones which are necessary for the maintenance of pregnancy i.e. estrogen and progesterone increase several folds and these especially estrogen stimulate hepatocytes thereby increasing the production of virtually all coagulation factors. Progesterone has been found to increase decidual tissue factor and also increase the synthesis of plasminogen activator inhibitor type 1 (Uchikova and Ledgev, 2005). Elevation of the levels of certain coagulation factors and the fibrinolytic inhibitors occur in practically all healthy pregnant women which is most likely the result of small amounts of procoagulant factors such as tissue thromboplastin which could cause direct and slow systemic activation of the coagulation cascade. In a study carried out by Durotoye *et al.*, 2012, the prothrombin time which assesses the factors in the extrinsic pathway, was reduced when compared with the value in non-pregnant controls.

The findings in the study are also similar to those of Hellgren in 2003, who observed increase in prothrombin complex level (Prothrombin Time) expressed as international normalized ratio (INR) of less than 0.9, similarly, Uchikova and Ledgev, in 2005, reported prothrombin time as being significantly shortened in pregnancy compared with control. By contrast however, the work of Adediran *et al.*, in 1999 in Ile-Ife, showed prolongation of prothrombin time in the face of what was otherwise known as a hypercoagulable state. Durotoye *et al*, 2012, stated that the mean prothrombin times in the 1^{st}, 2^{nd} and 3^{rd} trimesters of pregnancy showed that production of these coagulation factors increases as pregnancy advanced, as there was statistically significant reduction in prothrombin time from the 1^{st} to the 3^{rd} trimesters of pregnancy (P value 0. 001). Significant difference was also noticed in the Activated Partial Thromboplastic time with Kaolin (APTT) among the pregnant and control subject, which shows that levels of factors in the intrinsic pathway are also increased in normal pregnancy. There was no statistically significant difference in the result of APTT in various trimesters of pregnancy. This may probably be due to the fact that the eostrogen induced stimulation of factors VIII and IX production is much less than for the extrinsic pathway factors. Furthermore, estrogen has less effect in stimulating endothelial cells and macrophages, which are also sites of production and storage of factor VIII. Normal pregnancy is associated with haemostatic changes which have been linked to a complex physiological adaptation, but these changes return to that of non-pregnant state at about 4 weeks of post delivery. Durotoye *et al*., 2012 confirmed the development of a transient hypercogulable state in normal pregnant women in Nigeria of which the evidence is found in the significant

shortening, compared to the non-pregnant state, of the prothrombin time and Activated partial thromboplastin time with kaolin.

2.7 Cluster of Differentiation Four (CD4)

CD4+ cells / T-helper cells are white blood cells that fight infection. They are made in the spleen, lymph nodes and thymus gland, which are part of the lymph or infection – fighting system. CD4+ cells move throughout the body, helping to identify and destroy germs such as bacteria and viruses. The CD4+ count measures the number of CD4+ cells in a sample of blood. Along with other tests, the CD4 + cell count helps tell how strong the immune system is, indicates the stage of HIV disease, guides treatment and predicts how disease may progress. CD4+ counts are reported as the number of cells in a cubic millimeter of blood. A normal CD4+ count is from 500 to 1500 cells per cubic millimeter of blood (Chama *et al.,* 2009).

Cluster of differentiation four (CD4+) T – lymphocytes and other lymphocytes synchronize the immune systems response to pathogens. CD4+ cell count, provides a picture of immune system health with higher CD4+ counts typically signifying healthier immune systems in human immunodeficiency virus (HIV) negative individuals (Tsegaye *et al.,* 2003). CD4+ counts are widely used as prognostic markers to assess the degree of immune impairment in HIV antiretroviral treatment (Mernard *et al.*, 2003, Bussann *et al*., 2004, Ching *et al*., 2004, and Tanjong *et al*., 2012). Children have high CD4+ cell counts which decline slowly through adolescence and then plateau (Tsegaye *et al.,* 2003).

Demographic features, gender, genetic factors in pregnancy, current exposures to infectious diseases and behavioural factors are associated with variations in CD4+ cell counts in HIV – negative population (Tanjong *et al.,* 2012). It has been reported that healthy African and Asian populations typically have lower CD4+ lymphocyte counts than their Western European and Caucasian counterparts (Clerici *et al.,* 2000). Cigarette smoking has ironically been associated with higher CD4+ counts in several studies (Abuye *et al.,* 2005). Infectious diseases, like pneumonia and Tuberculosis (TB) have been associated with decreased CD4+ levels (Aldrich *et al.,* 2000). Commercial sex workers (CSW), who are exposed typically to a wide variety of sexually transmitted infections, have somewhat lower lymphocyte counts than females who are not involved in the sex trade (Messele *et al*., 2001). Black race in Western populations, low body mass index (BMI) and injection drug use, have also been associated with lower CD4+ lymphocyte counts (Aldrich *et al.,* 2000) and women tend to have levels 1-200 cells are higher than men with comparable demographic and behavioural patterns.

2.7.1 CD4+ counts in pregnancy

Pregnancy requires physiologic adaptations in all maternal systems, including the immune system. This process is complex and includes modifications at different levels and compartments of the maternal immune system. Recent investigations have shown that during pregnancy, maternal circulating immune cells undergo modifications in cell counts, phenotypes, functions and ability to produce soluble factors, such as cytokines. The ultimate goal is to establish and maintain a successful pregnancy, which involves a state of selective immune tolerance, immunosuppression and immunomodulation in the presence of a strong antimicrobial immunity. The mammalian immune system has evolved to coexist with these needs by down – regulating potentially dangerous T – cell mediated immune responses, while activating certain components of the innate immune system, such as monocytes and neutrophils. This unique dysregulation between different components of the immune system plays a central role in the maternal adaptation to pregnancy (Luppi, 2003). Immune function is suppressed. The state of pregnancy represents an extreme challenge for the immune system. The hormonal and immunological changes that occur over the course of pregnancy are necessary to support a healthy pregnancy, but also dramatically affect female susceptility to autoimmune and infectious diseases (Raghupathy, 1997).The maternal immune system during pregnancy is altered to actively tolerate the semi – allogenic fetus. These alterations include changes in local immune responses, that is in the uterine mucosa (Decidua) and changes in peripheral immune responses (Chen *et al*., 2012). The change in the hormonal environment of pregnancy contributes to local suppression of cell – mediated immunity at the maternal fetal interface (Bakalor *et al.,* 2001).

Several studies have been published on CD4+ cell counts during normal pregnancy. In a study in Maiduguri, Nigeria the normal CD4+ T - lymphocyte baseline in healthy HIV negative pregnant woman was determined, the mean CD4 +count of the pregnant woman was 751.4 cells/µl which was significantly lower than the mean CD4+ count of 869 cells/µl for the non- pregnant woman(Burns *et al*., 1996). Primigravidas had a lower mean CD4+ count, than both multiparas and grand multiparas. The mean CD4+ count was higher in the first trimester than in the later parts of pregnancy. There was no significant difference in the mean CD4+ count across all age groups (Akinbami *et al.,* 2014). There was a slight fall in the mean CD4+ count in pregnancy, which was more in the first trimester of pregnancy and in primigravidas (Burns *et al.,* 1996). CD4+ count decreased in pregnancy compared to non – pregnant females and decreased significantly as pregnancy progressed (Ufelle *et al*., 2017)

Aina *et al.,* 2005, also reported a lower mean CD4+ count of 771cells/µl in pregnancy compared with 828cells/µl for men and non- pregnant women. A similar study in the U.S examined the changes in CD4+ and CD8+ cell levels during Pregnancy and post partum in women seropositive and seronegative for HIV and concluded that the percent CD4+ cell levels declined steadily during pregnancy and post – partum among HIV – seropositive women indicating that HIV disease continues to progress during this period. Akinbami *et al.,* (2014), reported an insignificant association between CD4+ count and gestational age. There was a slight variation in CD4+ cell count by trimester, the highest in first trimester, then the third and lastly the second. Gestational age may or may not affect CD4+ cell count, while some authors reported an increase in CD4+ count as gestational age increases. Others reported no relationships exist between gestational age and CD4+ cell count in HIV – negative women (Gomo *et al.,* 2004). (Gomo *et al.*, 2004) reported a decline by 25 for each weeks increase in gestation, among women week's low serum retinol. Parity (< 4 versus > 4) was found to be insignificantly related to the odds of having a low CD4+ cell count (Oladepo *et al.,* 2009). Akinbami *et al.,* (2014) reported no association exists between parity and CD4+ cell count. Similar to Akinbami, educational level was not significantly associated with CD4+ cell count in Cameroun (Nanzigu *et al.,* 2011) and Uganda (Dayama *et al.,* 2003). CD4+ count significantly correlates with lymphocyte percentage and number and this may be due to the fact that CD4+ cell is a subset of T- lymphocyte and generally function as helper (Induces) T- cells (Akinbami *et al.,* 2014). CD4+ T cell count was significantly decreased when compared with the control and decreases as the gestational age increases (Ufelle *et al.,* 2017). The decrease in CD4+ cell counts at different gestational ages could be attributed to increasing physiological demand during pregnancy and the changes in the hormonal environment of pregnancy contribute to local suppression of cell mediated immunity at the maternal fetal interface (Bakalor *et al*., 2001). The results of Ufelle *et al.,* (2017) confirmed that CD4+ varies based on gestational age and showed decreased CD4+ T- lymphocyte count during pregnancy.

CD4+ levels in pregnant women are significantly lower than those in non-pregnant women. Several studies have been published on CD4+ cells counts during normal pregnancy. Similarly, an earlier study among African women demonstrated reduced absolute values of CD4+, CD8+ and total lymphocytes in pregnancy (Dayama *et al.,* 2003). According to Oladepo et al., 2009), immunity in pregnancy is physiologically compromised and may affect the CD4+ cell count, as lower CD4+ cell count was reported in pregnancy compared with non – pregnant females.

2.8 Cytokines

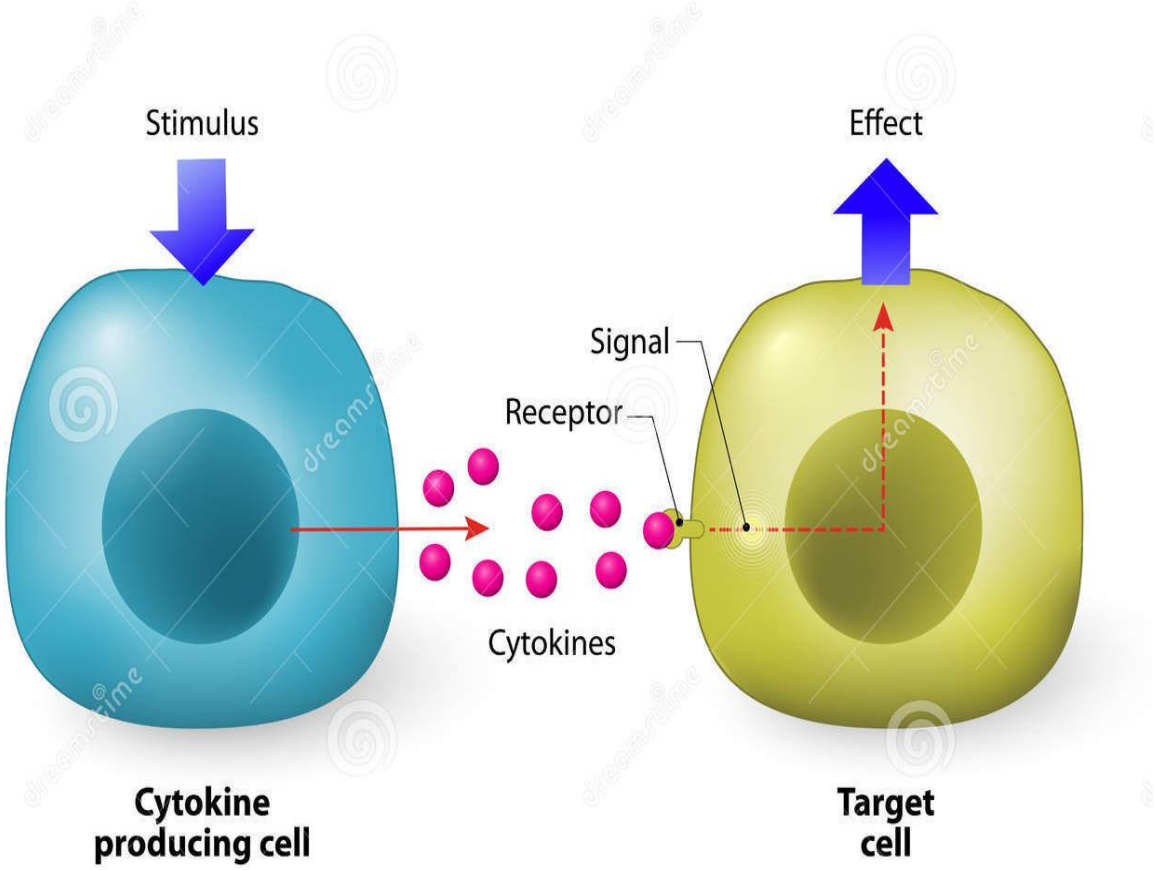
The term “cytokine” is derived from a combination of two Greek words which are “Cyto” meaning cell and “Kinos” meaning movement. Cytokines are a broad and loose catergory of small proteins (5-20 KDa) that are important in cell signaling. They can also be seen as cell signaling molecules that aid cell to cell combination in immune response and stimulate the movement of cells towards sites of inflammation, infection and trauma. They are released by cells and they affect the behavior of other cells. They include – Chemokines, interferons, interleukins, lymphokines, Tumour Necrosis factor, but generally not hormones or growth factors. They are produced by broad range of cells, including immune cells like macrophages, B-lymphocytes, T-lymphocytes and mast cells as well as endothelial cells, fibroblasts and various stromal cells. A given cytokine may be produced by more than one type of cell (Stedman, 2006 and lackie, 2010).

Cytokines being involved in immune responses are of great importance to the immune system and act through receptors. The various functions of cytokine include acting as an immunomodulating agent by modulating the balance between humoral and cell – based immune responses. They also regulate the maturation, growth and responsiveness of particular cell populations. They are also important regulators of both the innate and adaptive immune response. Some cytokines enhance or inhibit the action of other cytokines in complex ways (Horst, 2013). They have a larger distribution of sources for their production which includes almost all cells that have nucleus capable of producing interleukin – 1, interleukin 6 and Tumor Necrosis factor alpha (TNF - α) particularly endothelial cells, epithelial cells, and resident macrophages.

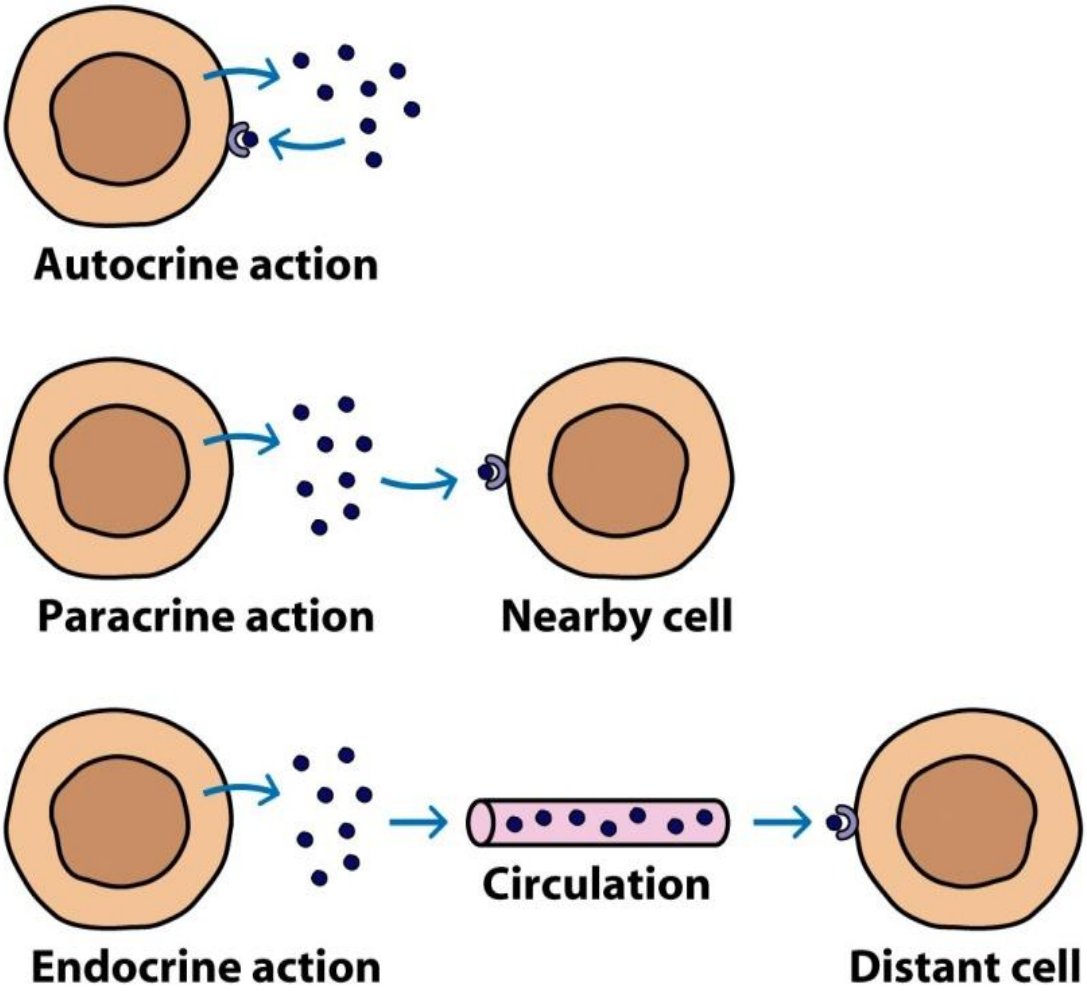
Cytokines can exert systemic as well as local effects in that its actions may affect the same cell it was secreted from, other cells nearby or may act in a more endocrine manner and produce effects across the whole body. Instances can be seen with cases of fever. Cytokines are indeed very important in host responses to infection, immune responses, inflammation, trauma, sepsis, cancer and resproduction (Stedman, 2006 and John, 2010). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action) (Freeman, 2007). They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors) proliferation and secretion of effector molecules (Freeman, 2007). It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types (pleiotropy). Cytokines are redundant in

their activity, meaning, similar functions can be stimulated by different cytokines. Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines.They can also act synergistically (two or more cytokines acting together) or antagonistically (cytokines causing opposing activities) (Freeman, 2007).

Figure 2.8 shows cytokines produced by a cell acting on the target cell, while figure 2.8b shows properties of cytokines.



(Figure 2.8, Cytokines) (Karki, 2018).



(Figure 2.8b, Properties of cytokines) (Freeman, 2007)

2.8.1 Nomenclature of cytokines

Based on presumed function, cell of secretion, or target of action, cytokines are therefore classed as lymphokines, interleukins, and chemokines. This is because cytokines are characterized by redundancy and pleiotroplsm. Interleukins - a term used for cytokines whose targets were assumed to be principally leucocytes were initially used by researchers, but currently, it is used widely to indicate newer cytokine molecules and the vast majority of these are produced by T-helper cells.

- Lymphokines produced by lymphocytes.
- Monokines produced by monocytes exclusively.
- Interferons involved in antiviral responses.
- Colony stimulating factors involved in the support of the growth of cells in semi-solid media.
- Chemokines involved in mediating chemo-attraction (that is Chemotaxis) between cells. (Chokkalingam *et al.,* 2013).

2.8.2 Classification of Cytokines

Cytokines are classified structurally and functionally:

2.8.2.1 Structural classification

Based on structural classification, structural homogeneity has been helpful to differentiate between cytokines that do demonstrate a considerable degree of redundancy. This led to its classification into four types.

1. The four α – helix bundle family: Here, the members are three dimensionally structured in which they are further divided into three sub-families:
   a. The IL – 2 sub-family (the largest contains immunological cytokines including Erythropoietin (EPO) and thrombopoietin (TPO).
   b. The interferon (IFN) sub-family.
   c. The IL-10 sub-family.
2. The IL-1 family which primarily includes IL-1 and IL – 18
3. The IL – 17 family, which helps promote proliferation of T-cells that cause cytotoxic effects.
4. The cysteine – knot cytokines. These include members of the transforming growth factor beta super family including TGF-β_1, TGF –β_2, TGF – β_3 (Cannon, 2000).

2.8.2.2 Functional classification

A classification that proves more useful in clinical and experimental practice outside of structural biology divides immunological cytokines into those that enhance cellular immune responses type I (IFN – Y, TNF – α etc) and Type 2 (TGF – β, IL – 4, IL – 10, IL – 13 etc) which favour antibody responses. A key focus of interest has been that cytokines in one of these two sub-sets tend to inhibit the effects of those in the other. Dysregulation of this tendency is under intensive study for its possible role in the pathogenesis of autoimmune disorders. (Tian *et al*., 2005).

Several inflammatory cytokines are induced by oxidative stress (David *et al.*, 2007). The fact that cytokines themselves trigger the release of other cytokines (Chokkalingam *et al.*, 2013) and also lead to increased oxidation stress makes them important in chronic inflammation, as well as other immune responses such as fever and acute phase proteins of the liver (IL – 1, IL – 6, IL – 12, IFN – α).

2.8.3 Types of Inflammatory Cytokines

Types include proinflammatory and anti-inflammatory cytokines.

2.8.3.1 Pro-inflammatory cytokines

A proinflammatory or inflammatory cytokine is a type of cytokine (signaling molecule) that is excreted from the immune cells and certain other cell types that promote inflammation. They are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. IL – β, IL – 2, IL – 6 and TNF – α are examples of proinflammatory cytokines (Xie *et al*., 2006).

Proinflammatory cytokines stimulate cell – mediated humoral and / or allergic immunity.

2.8.3.2 Anti - inflammatory cytokines

The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble receptors to regulate the human immune response. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized (Opal and DePalo, 2000). Major anti-inflammatory cytokines include – interleukin (IL-1) receptor antagonist, IL-4, IL-6, IL-10, IL-11 and IL-13. Specific receptors for IL-1, Tumor

Necrosis factor-α and IL-18 also function as pro-inflammatory cytokine inhibitors (Zhang, 2007).

The functional definition of an anti - inflammatory cytokine is the ability of the cytokine to inhibit the synthesis of IL-1, TNF-α and other major pro-inflammatory cytokines. For example, IL-4 promotes Th2 lymphocyte development, inhibition of lipopolysacharide (LPS) – induced pro-inflammatory cytokine synthesis while IL-10 inhibits monocyte, macrophage and neutrophils. Anti-inflammatory cytokines perform a multitude of functions during normal pregnancy by promoting placental formation, modulating trophoblast invasion and differentiation, inducing placental proliferation and angiogenesis, and inhibiting pro-inflammatory cytokines (Opal and DePalo, 2000). The immunological features of normal pregnancy are unique as the maternal Immune system has to accept a semi-allogeneic fetus, a product of two histo-incompatible individuals (Chatterjee *et al.,* 2014).

2.8.4 Cytokines and Cell – mediated Immunity (CMI)

Cell-mediated immunity is an immune response that does not involve antibodies, but rather involves the activation of phagocytes, antigen-specific cytotoxic T - lymphocytes , and the release of various cytokines in response to the antigen.

Historically, the immune system was separated into two branches: humoral immunity, for which the protective function of immunization could be found in the humor (cell-free bodily fluid or serum), and cellular immunity, for which the protective function of immunization was associated with cells. CD4 cells or helper T cells provide protection against different pathogens. Naïve T cells, mature T cells that have yet to encounter an antigen, are converted to activated effector T cells or helper T cells after encountering antigen presenting cells (APCs). These APCs such as macrophages, dendritic cells and B cells in some circumstances, load antigenic peptides onto the major histocompatibility (MHC) of the cell, in turn presenting the peptide to receptors on T-cells. The most important of these APCs are highly specialized dendritic cells, conceivably operating solely to ingest and present antigens. (Charles, 200l).

Activated Effector T cells can be placed into three functioning classes, detecting peptide antigens originating from various types of pathogens. The first being cytotoxic T cells which kill infected target cells by apoptosis without using cytokines, the second class being TH1 cells, which primarily function to activate macrophages, and the third class being TH2 cells, which primarily function to stimulate B cells into producing antibodies. Cellular immunity protects the body by:

- T-cell mediated immunity: activating antigen-specific cytotoxic T cells that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens.

- activating macrophages and natural killer cells, enabling them to destroy pathogens, and

- stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses(Charles, 2001).

The cytokines mediating cell-mediating immunity are: IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL-11, IL-12, IL-15, IL-16, IL-17, IL-18, IL-21, IL-23, TNF-α, IFN-α, IFN-β, IFN-y and the major cytokine mediating CMI is interferon y (IFN-y). Type 1 cytokines are those that primarily enhance cell –mediated immunity (*Xie et al.,* 2006).

2.8.5 Cytokines and Antibody - mediated Immunity (humoral immunity)

Humoral immunity is the aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. It is so named because it involves substance found in the humors, or body fluids. It contrasts with cell - mediated immunity. Its aspect involving antibody-mediated immunity.

The study of the molecular and cellular components that form the immune system, including their function and interaction, is the central science of immunology. The immune system is divided into a more primitive innate immune system, and acquired or adaptive immune system of vertebrates, humoral and cellular components, antibody production and the accessory processes that accompany it, including TH2 cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation. It also refers to the effector functions of antibodies, which include pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination (Charles, 200l).

The cytokines mediating humoral immunity include: IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-21, IL-25 and TGFβ. Humoral immunity is mediated by B cells and production of antibodies. IL-4, IL-10, IL-13 and transforming growth factor β trigger isotype switching of antibodies. Type 2 Cytokines are those that mainly augment humoral immunity (*Xie et al.,* 2006).

2.8.6 Interleukin – 2 (IL-2) and Pregnancy

1L - 2, a cytokine primarily produced by $CD4^+$ T lymphocytes, is considered a T-cell differentiation factor, because it promotes proliferation of T and B lymphocytes, as well as thymocytes (Sutton *et al.,* 2004). 1L-2 also enhances natural killer cell activity and immune response and induces the secretion of other cytokines such as gamma interferon(IFN-y), IL-4 and tumour necrosis factor alpha (TNF –α). 1L – 2 production is considered part of the pattern of cytokine secretion associated with a T-helper 1 (Th1) immune response (Sutton et al., 2004). Over-expression of 1L-2 inhibits pregnancy viability (Wegmann *et al.,* 1993). Women whose conceptions end in abortion have significantly high 1L-2 Serum levels. Elevated 1L-2 Serum concentrations have been found during the first trimester in women who later developed pre-eclampsia. 1L-2 production decreases in physiologic human pregnancy and also decreased in all trimester of pregnancy compared with non-pregnancy controls. It is increased in pathologic conditions (Marzi *et al*., 1996). Significant lower levels of Th1- type cytokines e.g 1L-2 were observed during pregnancy compared with non pregnant females (Kruse *et al.,* 2008). Equally, Kruse *et al.,* 2000 showed significant lower mRNA levels of the Th1-Type cytokines (1L – 2 and IFN-y) during pregnancy compared with non-pregnant female controls. Th1 cells have an essential role in the implantation and placental development. As a result, there exist a balance between Th1 and Th2, and this Th1/Th2 dichotomy aids in the explanation about the environment of cytokines underlying a successful pregnancy (Mellor and Munn, 2000).

2.8.7 Interleukin – 4 (IL – 4) and Pregnancy

1L-4 is a Th2-type cytokine and is beneficial for pregnancy, by promoting proliferation and differentiation of the trophoblastic cells and placentation. It also plays a protective role on the fetus' placental unit, inhibiting the production of Th1 cytokines (Jones *et al.,* 2000). Several studies implicate a role for 1L-4 in regulatory T cell (Treg) development. 1L-4 induces the formation of inducible Tregs from naïve CD4+ cells. Thus, 1L-4 not only mediates Th2 cell functions but also plays a part in the regulation of Tregs which play an important role in successful pregnancies (Pillermer *et al.,* 2009). IL-4 is a highly pleiotropic cytokine that is able to influence Th cell differentiation. It is an anti-inflamatory cytokines that function mainly by suppressing the pro-inflammatory milieu. IL-4 are activated T cells, mast cells, basophils, Eosinophils and NKT cells. Early secretion of IL-4 leads to polarization of Th cell differentiation toward Th2 – like cells. Th2 type cells secrete their own IL-4, and subsequent autocrine production of IL-4 supports cell proliferation. IL-4 is able to affect a variety of structural cells. It

can potentiate proliferation of vascular endothelium and skin fibroblasts yet decrease proliferation of adult human astrocytes and vascular smooth muscle cells. It induces a potent cytotoxic response against tumors (Wieseler – Frank *et al*., 2004). IL-4 is a Type 2 cytokine, hence augments humoral immunity (Clerici and Shearer, 1994). IL-4 is detectable at the feto-maternal interface during all phases of pregnancy (Lin *et al.,* 1993). It is produced not only by immune cells of the placenta but also by the maternal decidual amniochorionic membranes, cytotrophoblasts, and both maternal and fetal endothelial cells (Chaouat *et al*., 2004). IL-4 production is increased in the gravid state and levels of IL-4 increase throughout normal pregnancy (Marzi *et al.,* 1996). Progesterone is a known inducer of IL-4 and together they act to inhibit Th1 responses during pregnancy.

2.8.8 Interleukin – 10 (IL – 10) and Pregnancy

IL – 10 is the most important anti-inflammatory cytokine found within the human immune response. It was first determined in Th2 cells and was initially thought to be only produced by immune cells, but later studies demonstrated that IL-10 is also produced by non-immune cells (Saraiva and O' Garra, 2010). Immune cells that produce IL-10 include subsets of T cells such as Th1, Th2 and Th17, as well as monocytes, macrophages, dendritic cells, human B cells. Non-immune cells that produce IL -10 include keratinocytes, epithelia cells and tumor cells. IL-10 primarily exerts it's anti-inflammatory effect by inhibiting pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNF-α as well as Chemokines (Saraiva and O' Garra, 2010). In addition to its activity as a Th2 lymphocyte cytokine, IL-10 is also a potent de-activator of monocyte / macrophage pro-inflammatory cytokine synthesis. IL-10 up – regulates its own production by signaling between immune cells and also regulates recruitment, activation and suppression of both immune and non-immune cells.

In mice, IL-10 is expressed throughout pregnancy and peaks at gestation day 12 (Lin *et al.,* 1993). However, IL-10 plays a role in placental growth and remodeling IL-10 is not essential for the growth and development of the fetus in mice, but rather it plays an important role to inhibit excessive inflammation. These results suggest that IL-10 acts as a protective agent during infection and deficiency of IL-10 exacerbates inflammation in mice (Jiang *et al*., 2000). Normal pregnant women were determined to have increased IL-10 production during the first and second trimesters but not in the third trimester (Chatterjee *et al., 2014).* Moreover, IL-10 production decreases prior to labour and delivery of the fetus and placenta and increases post labour. Marzi *et al.,* (1996), reported that type 2 cytokines e.g. IL-4 and IL-10 increase in successful pregnancy

with a type 1 to 2 shift characterizing the third trimester. IL-10 and IL-4 show a constant presence at the first two trimesters with IL-10 showing a peak of production during labour (Ekerfelt *et al*., 1997). IL-4 and IL-10 play crucial roles in the success of pregnancy and there is strong evidence that a deficiency in IL-4 and/or IL-10 contributes to infertility, spontaneous abortion, Pre-term birth (PTB), Fetal Growth Rejection (FGR) and hypertensive disorders in pregnancy (Chatterjee *et al., 2014).*

2.8.9 Tumor Necrosis Factor – α (Tnf – α) and Pregnancy

TNF – α, also known as cachectin,is a pro-inflammatory cytokine that plays a well established, key role in some pain models. It has a major role in the cytokine network with a widest spectrum of biological activities. Normally, it is produced by the placental trophoblast cells and feto – placental macrophages, thus up-regulating the endothelial expression of platelet derived growth factor, endothelial – 1 and the plasminogen activator inhibitor. It induces structural and functional alterations in endothelial cells, enhances the formation of endothelial – 1 and reduces acetyl – choline induced vasodilation (Tavakkol *et al.,* 2005).

TNF – α is a type 1 cytokine and is implicated for pregnancy failure when the concentration is raised during pregnancy (Shaarawy and Nagui, 1997). TNF - α presents a stable production profile in all stages of pregnancy (Vassalidis *et al.,* 1998) and was increased throughout normal pregnancy (Jones *et al.,* 2000). TNF –α receptors have been shown to play important roles in both inflammatory and neuropathic hyperalgesia. It has been found that TNF - α injected into nerves induces Wallerian degeneration and generates the transient display of behaviours and endonevial pathologies found in experimentally painful nerve injury (Schafers *et al*., 2003). TNF – alpha is essential in the 0rchestration of the cytokine cascade, and it is a therapeutic target in many inflammatory diseases. The increased production of TNF - α has been related to the pathogenesis of various diseases, including rheumatoid arthritis, Crohns disease, atheriosclerosis psoriasis, sepsis, diabetes mellitus, and obesity (Patial and Parameswaran, 2010). Pregnancy success appears to rely on a discrete balance between the cytokines Th1 and Th2, which are involved in fetal growth and development. TNF – alpha, IL – 1beta, and IL – 6 are some of the fundamental cytokines in early pregnancy. It participates in blastocyst implantation and, adversely, in first trimester losses. As pregnancy develops, high TNF – alpha concentrations have been related to the development of preeclampsia and gestational diabetes mellitus (GDM) (Peracoli *et al*., 2007).

In the beginning of pregnancy, intense vascularization and cell proliferation helps the development of the embryo and the placentation, thus the presence of pro-inflammatory cytokines such as TNF – α and IFN – y is important at this early stage. The TNF – α modulates trophoblastic growth and the trophoblastic invasion of the spiral arterioles, although the overreaction can restrict the invasion and contribute to the pathophysiology of pre-eclampsia, (Peracoli *et al.*, 2007). Thus, it is essential to control this inflammatory response in the later stages of pregnancy, and this is probably why levels of IL-10 observed remain high throughout pregnancy linking to TNF-∝ level to regulate them.

2.9 The Role of the Immune System in Pregnancy

The immune system plays an important role in pregnancy, both in normal and pathologic states. The maternal immune response must be modulated to allow establishment and maintenance of a viable pregnancy, comprising allogenic tissues, without rejection (Chaouat *et al.*, 2004). TNF – α, IL-1β, and 1L-6 are just a few of the cytokines shown to play a key role in implantation and first trimester miscarriage. Later in pregnancy, elevated TNF-α levels have been associated with pre-eclampsia while decreased 1L- 10 levels have been associated with pre-term birth (Raghupathy, 2001).

Given the association between immune parameters and obstetric outcomes, early identification of alterations in the immune system may help predict and / or impact pregnancy outcomes. The longitudinal immunologic profile over the course of human pregnancy, however, has yet to be well defined, thus, complicating efforts to characterize the normal modulation of the immune system and to indentify systemic immune parameters associated with specific reproductive pathologies and outcomes (Denney *et al.*, 2011).

2.10 Cytokines and the Functional Immune Response

Cytokines are a diverse family of soluble small proteins, expressed by various cells and tissue types that act as mediators. Their expression profile has been used to categorize immune responses and the functional status of the immune system. Although cytokines are secreted by a number of immune cell types, T cells have often been characterized as playing a key role in determining the nature of an immune response (Fitzgerald, 2001). If there is a predominance of T Helper, (Th1) cells, the immune system will generate a cell mediated or cytotoxic response targeting intercellular pathogens or cancernogenic (Wilczynski, 2006). In contrast, a

predominance of T helper 2 (Th 2) cells would favour an antibody-mediated or humoral response to target extra-cellular pathogens like bacteria (Kidd, 2003). Th1 and Th2 responses were among the first classes of immune responses to be characterized. They generally function in opposition to one another and have been extensively studied (O Garra and Arai, 2000). The number of

human Cytokines described continues to increase with at least 33 interleukins (1Ls) identified to date (Chen *et al.,* 2006). In humans, Th1 associated cytokines include interferon gamma (IFN-y) and 1L -12 while Th2- associated cytokines include 1L-4, -5 and -13 (Chaouat *et al*., 2004). Another cytokine category, which includes TNF – α, 1L- 1β and 1L-6, is considered to exhibit pro-inflammatory function (Fitzgerald, 2001).

2.10.1 Cytokine Production during Normal Pregnancy

The definition of pregnancy as a "Th 2" or anti-inflammatory state was enthusiastically embraced and numerous studies attempted to prove and support this hypothesis . This theory postulates that pregnancy is an anti-inflammatory condition (Szekeres – Bartho and Wegmann, 1996), and a shift in the type of cytokines produced would lead to abortion or pregnancy complications. While many studies confirmed this hypothesis, a similar number of studies argued against this notion (Saito *et al*., 2006 and Saito *et al.,* 2010). The reason for these contradictory results may be due to oversimplification of disparate observations made during pregnancy. In the aforementioned studies, pregnancy was evaluated as a single event, when in reality, it has three distinct immunological phases that are characterized by distinct biological processes and can be symbolized by how the pregnant woman feels (Mor and Koga, 2008).

Implantation, placentation, and the first and early second trimester of pregnancy resemble "an open wound" that requires a strong inflammatory response. The first stage of pregnancy which involves a blastocyst implanting into the uterus is a predominantly pro-inflammatory phase. During this first stage, the blastocyst has to break through the epithelial linning of the uterus in order to implant, damage the endometrial tissue to invade, followed by the trophoblast replacement of the endomethelium and vascular smooth muscle of the maternal blood vessels in order to secure adequate placental-fetal blood supply (Dekel *et al.,* 2010). All these activities create a veritable "battle ground" of invading cells, dying cells and repairing cells. An inflammatory environment is required in order to secure the adequate repair of the uterine epithelium and the removal of cellular debris (Koga and Mor, 2010). Meanwhile the mother's well being is clinically affected. She feels sick because her whole body is struggling to adapt to

the presence of the fetus (in addition to hormone changes and other factors, this inflammatory response is responsible for "morning sickness"). The second phase of pregnancy is a predominantly anti-inflammatory phase. The cytokine skewing during the second phase of pregnancy can be systemic or local at the feto- maternal interface. This phase of pregnancy is, in

many ways, the optimal time for the mother. It is a period of rapid fetal growth and development. The mother, placenta and fetus are symbiotic, and the predominant immunological feature is induction of an anti-inflammatory state. The woman no longer suffers from nausea and fever as she did in the first stage, in part because the immune response is no longer the predominant endocrine feature (Mor *et al.*, 2011).

Finally, during the last immunological phase of pregnancy, the fetus has completed its development. All the organs are functional and ready to deal with the external world. Now the mother needs to deliver the baby, and this can only be achieved through renewed inflammation. Parturition is characterized by an influx of immune cells into myometrium in order to promote recrudescence of an inflammatory process (Romero *et al.,* 2006). This pro- inflammatory environment promotes the contraction of the uterus, expulsion of the baby and rejection of the placenta. Inflammation is tightly controlled during all stages of pregnancy however, excessive and persistent maternal inflammatory responses are associated with adverse pregnancy outcomes (Svensson *et al*., 2001). Preterm birth is associated with increased production of pro-inflammatory cytokines and chemokines, such as 1L - 1β, 1L – 6, TNF – α and CXCL8 (Gulleria and Pollard, 2010). These cytokines induce prostaglandin synthesis in the placenta tissues that triggers preterm labour and Fetal Graft rejection (FGR) (Kramer *et al.,* 2009). In conclusion, pregnancy is a pro-inflammatory and anti-inflammatory condition, depending upon the stage of gestation (Romero, 2005 and Mor, 2008).

2.11 T- regulatory Cells and Immune Suppression

Most recently, regulatory T cells (T reg) or T helper types 3 (Th 3) responses have been identified (Sakaguchu, 2000 and Curotto *et al*., 2005), and have been found to play an immune suppressive role including in the setting of pregnancy (Zenclussen, 2005). Although the exact mechanism by which regulatory T cells function has yet to be firmly established, the cytokine 1L - 10 appears to play a critical role (Akdis and Blaser, 2001 and Taylor *et al*., 2006). 1L-10 is a particularly intriguing cytokine. In humans, IL-10 is a pleiotropic cytokine with both immune stimulatory and counter- regulatory (immunosuppressive) functions that place it outside of the

Th1 – Th2 paradigm although it was originally described as a Th2 – associated cytokine in rodents (Wakkach, 2000 and Conti *et al*., 2003).

2.12 Innate versus Adaptive Arms of Immune System

Cytokines can be secreted predominantly by cellular elements of the innate arm of the imune system such as monocytes (monokines), predominantly by components of the adaptive arm of the immune system such as lymphocytes (lymphokines), or by both (Kidd, 2003). For instance monocytes are the predominant source of 1L -1β while lymphocyte are the predominant source of 1L-4 and 1FN – y. Monocytes and lymphocytes express TNF – α, 1L – 6 and IL – 10 (Wilczynoski, 2006). Monocytes and Lymphocytes respond to different stimuli and thus the expressed cytokines along with the limitations inherent in the collection of human biological specimen necessitate critical attention to the conditions under which specific samples are examined. For example, selection of a stimuli targeting one cell type (e.g, LPS stimulation of Monocytes) will not provide information regarding primary stimulation of Lymphocytes, thus limiting the capacity to evaluate their contribution to the physiologic or pathologic process in question (Kidd, 2003 and O' Garra and Arai, 2000).

2.13 Current Paradigms

Various paradigms have been proposed for the modification of the immune system that maintains a viable pregnancy (Ragupathy, 2001). These paradigms have focused on phenomena such as "missing self" (Makhsheed *et al.,* 1999). "This immunologic bias" (Denney *et al.*, 2011), and more recently "immunosubversion" (Block and Markovic, 2009). Current data support a significant role for placental mechanisms that may dampen immune responses rather than a shift in the immune system during pregnancy from a Th1 – Th 2 balance to a Th 2 bias (Wegmann *et al.*, 1993). Many of the studies supporting these various paradigms were conducted in murine models with problems inherent in generalizing rodent systems to humans. Existing human data are mostly limited to individuals with a history of reproductive pathology or is limited by cross-sectional study design (Raghupathy *et al*., 2001, Makhseed *et al*., 2003 and Azizieh *et al*., 2005).

2.14 Inflammation and Immune Cells during Implantation

As previously discussed, a high level of the proinflammatory T helper (Th) – 1 and cytokines (1L – 6, 1L 8, TNF – α) characterizes early implantation (Mor and Koga, 2008, Yoshinaga, 2008 and Jasper *et al*., 2010). These cytokine can be secreted by the endometrial cells as well as by

cells of the immune system that are recruited to the site of implantation 65 – 70% are uterine – specific natural killer (NK) cells (Manaster and Mandelboim, 2010), and 10 – 20% are

macrophages (Mos) and 2-4% are dendritic cells (DCs) (Manaster and Mandelboim, 2010 and Nagamatsu and Schust, 2010). Nk cells in human decidua have a role in regulating trophoblast invasion by the production of 1L-8 and interferon-inducible protein - 10 chemokines. Furthermore, decidual Nk cells are potent secretors of an array of angiogenic factors that is essential for the establishment of an adequate deciduas (Burke *et al.,* 2010). DCs are a heterogenous population of cells that initiate and co-ordinate the innate and adaptive immune response. These cells accumulate in the pregnant uterus prior to implantation and stay in the decidua throughout pregnancy (Laskarin *et al.,* 2007 and Scholz *et al.,* 2008). Several lines of evidence point to a pivotal role of macrophages and DCs in shaping the cytokine profile at the maternal fetal interface (Laskarin *et al.,* 2007 and Jasper *et al.,* 2010).

Furthermore, in recent studies, Mor *et al., (2011)* showed that depletion of uterine DCs (uDCs) cells resulted in a severe impairment of implantation and led to embryo resorption (Plaks *et al.,* 2008). The immune infiltrate, that plays a central role in the process of tissue renewal and differentiation, may also participate in the development of a receptive endometrium in the biopsy treated patients (Dekel *et al.,* 2010). In addition to their immediate influence, recruitment of cells of the immune system to the site of injury may create some "tissue memory" facilitating implantation in the following cycle of treatment. In fact, monocyte precursors of macrophages and DCs are known to be recruited to injured sites and provide essential beneficial effects during wound healing. These cells are long lived, and reside in some tissues for months, during which time they can differentiate into tissue resident macrophages or DCs (Luster and Von Andrian, 2005).

Wegmann *et al.,* (1993) presented the first theory to explain the involvement of cytokines in gestation. According to this proposition, the Th1 cytokines are deleterious, leading to an inflammatory response and placental necrosis, thus it can compromise fetal and placental development. Moreover, Th2 cytokines are beneficial for pregnancy, promoting proliferation and differentiation of the trophoblastic cells and placentation. In addition, have a protective role on the fetus, placental unit, inhibiting the production of Th1 cytokines. According to this theory, successful pregnancy is associated with preferential development of the Th2 profile. But with the advent of other researches that proposition is under review. According to that, one of the main aspects is the inclusion of many other cytokines and mediators on the maternal-fetal interaction.

Another important issue concerns the characterization of the immunological profile of the maternal-fetal relationship. Several studies showed that the pattern of cytokines is variable

throughout pregnancy, and the observed effects depend on the concentration and the period (Daher *et al.*, 1999). The Th 2 cytokines (1L – 4) can decrease Th1 responses (1L – 2 and IFN – y), creating an essential microenvironment for a good evolution of the pregnancy (Raghupathy, 1997 and Michie, 1998). Th 1 cells have an essential role in the implantation and placental development. Thus, the balance is fundamental between Th 1 and Th 2 and this Th 1 / Th2 dichotomy can aid in the explanation about the environment of cytokines underlying a successful pregnancy (Mellor and Munn, 2000). The balance of maternal immune response controlling the inflammatory mechanisms is dependent on 1L – 10. Regulatory features of 1L – 10 (Pleomorphic cytokine) in the immune-stimulatory and immune suppressive activity might be associated with the regulation of the Th1 – Th2 activities (Denney *et al.*, 2011) and may reduce Th1 (1L – 2 and IFN-Y)

In normal pregnancy, the secretion of 1L-10 assists in the maintenance of a less pro-inflammatory environment, favouring a more regulated immune microenvironment that is opposite to the presence of a fetus. The 1L – 10 influences the activity of placental trophoblastic, has suppressive effect on KC- like cells on the autocrine production of TNF – α and regulated fetal immuno protection (Rein *et al.*, 2003 and Piccinni, 2007). In the beginning of pregnancy, intense vascularization and cell proliferation helps the development of the embryo and the placentation. Thus, the presence of pro-inflammatory cytokines such as TNF – α and IFN-Y is important at this early stage. The TNF –α modulates trophoblastic growth and the trophoblastic invasion of the spiral artetioles, although the overreaction can restrict the invasion and contribute to the pathophysiology of preeclampsia (Peracoli *et al.*, 2007). Thus, it is essential to control this inflammatory response in the later stages of pregnancy and this probably why levels of 1L-10 observed in this study remain high throughout pregnancy, linking to TNF- α level to regulate them (Peracoli *et al*., 2007). The decrease observed in Th1 cytokines is associated with the presence of factors that inhibit the production of Th1 cytokines (1L-2) and these factors are important in the proliferation and differentiation of the trophoblastic cells and placentation and play a protective role on the fetal-placental unit as an attempt by the organism to maintain the pregnancy process (Feliciano *et al.*, 2012).

Normal pregnancy is accompanied by decreased production of type – 1 pro-inflammatory cytokines and increased production of Type – 2 anti-inflammatory cytokines, hence; an

important role in the maintenance of pregnancy is played by cytokines (Jones *et al.,* 2000). It has been proposed that successful pregnancy is a T-helper 2-type phenomenon. Th2 cells are associated primarily with the provision of help for B-cell antibody production via the characteristic Th2 – type cytokines – IL – 4, Il-5, IL-6 and IL – 10 (Cannon, 2000).

Anti-inflammatory cytokines perform a multitude of functions during normal pregnancy by promoting placental formation, modulating trophoblast invasion and differentiation, inducing placental proliferation and angiogenesis, and inhibiting pro-inflammatory cytokines. The immunological features of normal pregnancy are unique as the maternal Immune system has to accept a semi-allogeneic fetus, a product of two histo-incompatible individuals (Guerin *et al*., 2009). Medawar proposed that in order to accept a half foreign fetus, the mother needs to be in an immune-suppressed state (Chatterjee *et al.,* 2014).

Recent progress in our understanding suggests that the maternal immune system not only needs to be suppressed but at the same time also needs to protect the mother and the growing fetus from infection during pregnancy. Thus a successful pregnancy depends on the ability of the mother's immune system to become tolerant to paternal antigens as well as the ability to reject the fetus in case of pathogen infection. The maternal immune response is regulated by a complex array of cytokines to protect the conceptus and promote proper growth and development of the placenta. Wegmann and colleagues suggested that during pregnancy, there is a T – helper (Th)2 bias to promote tolerance to the half foreign fetus and Th1 cytokines are detrimental to the tolerance of the conceptus, similar to allografts in transplant recipients (Chatterjee *et al.,* 2014). It had been found that during tolerance induction to an allograft, there is a decrease in Th1 cytokines such as interleukin IL - 2 and IFN Y and an increase in Th2 cytokines including IL -4 and IL – 10. Conversely, high levels of IL-2 and INF-y were detected in rejecting allografts (Erdmann *et al*., 2004).

It has previously been demonstrated that upon mitogen stimulation, peripheral blood mononuclear cells (PBMC) obtained at the time of normal delivery from women with a history of successful pregnancy produced significantly higher concentrations of Th-2 type cytokines and significantly lower concentrations of Th1 – type cytokines as compared to women undergoing recurrent spontaneous abortion (Makhseed *et al.,* 2000). In a study carried out by Raghupathy *et al.,* 2000), highly significantly increased concentrations of three Th2 type cytokines tested (IL-4, IL-6 and IL-10) were found at the 12 week point in normal pregnancy.Denney *et al*., (2011) showed an overall decrease in pro-inflammatory cytokine trajectories in the innate and adaptive

arms of the immune system and an increase in counter-regulatory cytokines as pregnancy progresses and these changes support the role of immune modulation to permit maintenance of a viable pregnancy.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out at the Antenatal Clinic of Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi, Nigeria, a government tertiary health - care institution serving Nnewi Local Government Area and its environs. NAUTH is both a referral and non – referral centre and also manages uncomplicated obstetrics and gynaecology cases. Geographically, Nnewi falls within the tropical rain forest region of Nigeria, and the climate is of equatorial type with temperature ranging from 25 – 33^{o}C annually. There are two main seasons – the rainy season which starts from March to September and the dry season which starts from October to February. The population of Nnewi is about 1,047,309 as at 2019.

3.2 Study Design

This research was a longitudinal study carried out at the antenatal care clinic of NAUTH, Nnewi, Anambra State during the period of January to December, 2016. All pregnant women who presented for booking for antenatal care in their first trimester (1st - 3rd month) visit at NAUTH, Nnewi were recruited for the study. They were enrolled after providing their informed consent. Questionnaires were administered to obtain their medical and obstetrics history (age, parity, gestational age etc). Pregnancy and its duration were confirmed by ultrasound scan, and pregnant women with multiple pregnancy were excluded.

During the first trimester visit (3rd month, in particular), the blood pressure was measured using a sphygmomanometer, and at subsequent trimesters. The weight and height measured were used to calculate the body mass index (BMI), which was expressed as weight (kg)/Height (m^{2}). These pregnant women were screened, and at the end of the screening exercise, pregnant women who were not eligible were excluded. One hundred and sixty (160) apparently healthy pregnant women were enrolled in the research as the study group. The age range of these women was 20 – 40 years. Similarly, 160 age - matched non- pregnant women consisting of health science

students and the staff of NAUTH, who served as controls were enrolled for the study based on the inclusion criteria. Pregnancy test was conducted on the non-pregnant females to confirm they were not pregnant.

These pregnant women were on iron supplements and were followed up till the last trimester – second trimester (5^{th} month) and third (8^{th} month). The same tests were conducted on the non-pregnant control samples at NAUTH Laboratories.

At the second trimester, 156 pregnant women were followed up. During the third trimester, only 140 pregnant women completed the study. Those who could not complete the survey were inelligible because they had still birth, miscarried, changed address or felt the survey was a disturbance to them. Results from pregnant subjects were compared with that of controls, and comparisons were also made across the trimesters.

3.3 Specimen Collection

Nine mililiters (9mls) of blood was collected from each subject with a minimal stasis from the antecubital vein by means of hypodermic syringe and needle. 3mls was aliquoted into Potassium EDTA anticoagulated tubes, for malaria parasite screening using the Giemsa staining technique/ microscopic method, thin blood film for blood cell morphology, complete haemogram using an automated haematology analyzer, and the CD4+ T- cell absolute counts were done using the **Partec Cyflow counter**. The 4mls blood sample aliquoted into gel tubes were used to screen for HIV 1 & 11 using Alere Determine HIV 1/2 (Abbot co Ltd, Japan) and Uni – Gold (Trinity Biotech U.S.A). HBSAg was performed using one step rapid test (ABON Biopharm (Hanzhou) co Ltd. China), HCV and VDRL tests were done using rapid test (Nantong Egens Biotechnology Co. Ltd, China) and pregnancy test was also performed using rapid test (Hangzhou Biotest Biotech Co. Ltd , China). All are immunochromatographic tests for the qualitative detection of HBSAg, HCV and VDRL antibody in human serum. Serum ferritin and serum iron were done quantitatively by enzyme immunoassay (Biocheck, INC, Canada) and (TECO Diagnostics, USA) respectively. Cytokines levels– IL- 2, IL– 4, IL– 10 and TNF - α were performed using ELISA Kits (Perfect Ease Biotech (Beijing Co. Ltd, China). The remaining 2mls of blood was placed into sodium citrated anticoagulant bottles and used to perform PT and APTT tests using Dia – PT and APTT kits (Diagon Ltd, Hungary) .

3.4 Determination of Sample Size

The sample size was obtained using the formula by Taro Yamane, 1973.

$$n = \frac{N}{1 + N (e)^2}$$

Where:

N = Population size

e = Sampling error (0.05)

n = Sample size

Using this formula, the minimum number of sample size will be:

N = 216

e = 0.05

n = ?

$$\frac{216}{1 + 216 (0.05)^2}$$

$$\frac{216}{1 + 0.54} = \frac{216}{1.54} = 140.3$$

For this study, a minimum number of 140 subjects will be investigated.

3.4.1 Ethical Consideration

This research study was approved by the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, and Ethics Committee, Faculty of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State. An informed consent was obtained in writing before recruiting each subject into the study.

3.5 Inclusion Criteria

- Pregnant women in their first trimesters.
- Pregnant women from ages 20 – 40 years.
- Pregnant women with singleton pregnancy.

- Pregnant women with normotensive blood pressure < 140/90mmHg.
- Pregnant women with no known infection.
- Age-matched non – pregnant females (without high blood pressure or any known infection) served as controls.
- Pregnant women who gave their informed consent.

3.6 Exclusion Criteria

- Pregnant women with any of the following conditions – bleeding disorders, connective tissue disease, malaria parasitaemia, HIV 1 & II, HBsAg and HCV, gestational diabetes etc were excluded.
- Women with multiple pregnancies.
- Pregnant women who were unable to give informed consent.
- Women on non – steroidal anti-inflammatory drugs such as aspirin.
- Women with high blood pressure greater than 140/90mmHg.

3.7 Laboratory Methods

3.7.1 Retroviral disease screening (RVD screening)

3.7.1.1 Alere determine screening

The qualitative immunoassay determination of HIV 1/2 was performed using Alere determine (7D2342). (Alere Medical Co. Ltd Japan, 2015)

Principle: Alere Determine HIV 1/2 is an immunochromatographic test for the qualitative detection of antibodies to HIV – 1 and HIV – 2. Sample is added to the sample pad. As the sample migrates through the longitude pad, it reconstitutes and mixes with selenium colloid antigen conjugate. This mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site. If antibodies to HIV – 1 and / or HIV – 2 are present in the sample, the antibodies bind the antigen selenium colloid and the antigen at the patient window, 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 past the patient window, and no red line is formed at the patient window site. To ensure validity, a procedural control bar is incorporated in the assay device.

Procedure: The protective foil cover was removed from each test. Fifty microliter (50µl) of serum was applied to the sample pad using a precision pipette, and left for 15 minutes. The result was read.

Interpretation of results

Positive (Two Bars): Red bars appearing in both control window (Labelled "Control and the patient window labeled P "Patient") of the strip. Any visible red bar in the patient window was interpreted as positive.

Negative (one Bar): One red bar appearing in the control window of the strip (Labeled "control") and no red bar appearing in the patient window of the strip (Labelled "Patient")

Invalid: If there was no red bar in the control window of the strip, and even if a red bar appears in the patient window of the strip, the result was invalid and was repeated.

3.7.1.2 Uni -Gold^{tm} HIV screening

The Uni-Gold HIV 1 and 2 screening test was performed qualitatively by the rapid immunoassay technique. (1206502) (Trinity Biotech PLC, Ireland, 2014).

Principle: Uni-Gold^{TM} HIV is a rapid immunoassay based on the immunochromatographic sandwich principle. Recombinant proteins representing the immunodominant regions of the envelope proteins of HIV – I and HIV – 2, glycoprotein gp4, gp 120 (HIV) and glycoprotein gp35 (HIV – 2) respectively, are immobilized at the least region of the nitro cellulose strip. These proteins are also linked to colloidal gold and impregnated below the test region of the device. A narrow band of the nitro cellulose membrane is also sensitized as a control region.

During testing, two drops of serum, plasma or whole blood is applied to the sample port, followed by two drops of wash solution and allowed to react. Antibodies of any immunoglobulin class specific to the recombinant HIV – 1 or HIV – 2 proteins will react with the colloidad gold linked antigens.

The antibody protein colloidal gold complex moves chromatographically along the membrane to the test control regions of the test device excess conjugate forms a second pink / red band in the control region of the device. The appearance of this band indicates proper performance of the reagents in the kit.

Procedure: The required number of UNI-Gold HIV devices were removed from their pouches and used within 20 minutes of opening the foil pouch. Not more than 10 tests were performed at one time. The devices were laid on a clean flat surface and each labeled with the appropriate patient information / ID. The disposable pipette included in the kit was filled with sample and ensured that there were no air bubbles. The pipette was held vertically over the sample port,and the bulb squeezed and two (2) drops of plasma were discharged onto the sample pad. The sample was allowed to fully adsorb and no air bubbles introduced into the sample port. The disposable pipette was not allowed to touch the sample pad.

The dropper bottle of wash solution was held in a vertical position above the sample port, and two (2) drops of wash solution added to the sample port. The assay was timed from this point. The test results were read after 10 minutes but not later than 12 minutes incubation time.

Interpretation of results

Reactive Test Result: Two pink / red lines of any intensity in the device window, the first adjacent to letter "T" (test) and the second adjacent to "c" (control). This indicates a reactive result that is interpreted as preliminary positive for antibodies to HIV.

Non reactive test result: A pink / red line of any intensity adjacent to the letter "c" (control), but no pink / red line adjacent to "T" (test). This indicates a non reactive result that is interpreted as negative for antibodies to HIV.

Invalid result: No pink / red line appears in the device window adjacent to the letter "c" (control) irrespective of whether or not a pink / red line appears in the device window adjacent to "T" (test). This is an invalid result that cannot be interpreted.

3.7.2 Malaria parasite test

The malaria Parasite test was performed by microscopic method (Bain *et al*., 2012).

Principle: Thick blood films are extremely useful when parasites are scanty and these should be prepared and examined as a routine where malaria is suspected. Identification of the species is less easy than in thin films, and mixed infections may be missed, but if 5 minutes are spent examining a thick film, this is equivalent to about an hour spent in transversing a thin film. Once the presence of parasites has been confirmed, a thin film should be used for determining the species, and in the case of plasmodium falciparum, for assessing the severity of the infection by counting the percentage of positive cells.

Procedure: A thick film was made by placing a small drop of blood in the centre of a slide and spreading it out with a corner of another slide to cover an area about four times its original area, making sure the film was not too thick. The film was allowed to dry thoroughly for at least 30 min at 37^0 C. The slide was stained with Giemsa stain diluted 1 in 20 volumes of buffered water for 30 minutes, after which it was washed in buffered water pH 7.2 for 3 min. The back of the slide was cleaned with cotton wool, and the slide was then stood upright to air dry.

When the thick film was completely dry, a drop of immersion oil was applied on the film and the area that is well stained and not too thick was selected and examined with 100x objective.

3.7.2.1 Counting of malaria parasite number (Amah, 2016).

The counting of malaria parasite number per µl (microlitre) was done by counting parasite against WBC using thick blood film.The part of the thick film where the WBC were evenly distributed and the parasites were well- stained was selected. Using the oil immersion objective, 100 WBCs were systematically counted at the same time with the number of parasites in each field covered, and at least 100 high power microscope fields were examined before reporting "No parasite found".

In the calculation, an assumed 8,000 WBCs per µl of blood (Standard WHO WBC count) was used.The number of parasites per µl of blood was calculated as follows:

$$\text{Calculation : } \frac{\text{No of parasites counted}}{100} \times \text{WBC} = \text{No of parasite / µl}$$

$$= \frac{\text{No of parasites counted}}{100} \times 8{,}000 = \text{No of parasite / µl}$$

Results:

Chromatin of parasite	-	Dark Red
Cytoplasm of parasite	-	Blue
Schuffners dots	-	Red
Maurer's dots (clefts)	-	Red – mauve
Red cells	-	Grey to pale mauve

Reticulocytes	-	Grey Blue
Nuclei of neutrophils	-	Red
Granules of eosinophils	-	Red
Cytoplasm of mononuclear cell	-	Blue grey

3.8.1 Determination of blood cell morphology

This was done using the thin blood film (Potters *et al.*, 2009).

A drop of blood was placed on the end of a clean grease - free slide. Another clean glass slide with smooth edge was used as the spreader. The edge of the spread was placed in front of the drop of blood. The Spread was drawn back to touch the drop of blood and blood allowed to extend along the edge of the spreader. The spreader was pushed firmly along the slide at an angle of 45^{o} in a smooth movement. The end of the spreader was wiped clean. Then the film was air-dried immediately by waving the slide back and forth. When the blood film was completely dried within a few minutes, it was placed on a staining rack, and flooded with leishman stain for 2 minutes. Then, this was double-diluted with buffered distilled water (pH 6.8) using a pastuer pipette and left to stain for 8 minutes. The stain was washed with tap water, and the back of the slide cleaned and stood in a draining track for the smear to dry.When the stained film was completely dried, a drop of immersion oil was placed on the lower third of the blood film and examined microscopically using the 100x objective to check the staining and distribution of the cells. The objective was brought into place at the part of the film where the red cells were beginning to overlap. The morphology of the blood cells was reported.

Staining Results

Red cells - Pink red

Nucleus of WBC cells - Purple-violet.

3.8.2 Hepatitis B Surface Antigen (HBsAg) Screening Test

The qualitative detection of HBsAg in serum was performed using Immunochromatographic method (BSG5020027) (Abon Biopharm Hangzhou Co., Ltd, China, 2014).

Principle: The HBsAg one step hepatitis B surface antigen test strip (serum/plasma) is a qualitative lateral flow immunoassay for the detection of HBsAg in serum or plasma. The membrane is pre-coated with anti-HBsAg antibodies on the test line region of the strip during testing,the serum or plasma specimen reacts with the particle coated with anti-HBsAg antibody. The mixture migrates upward on the membrane and generates a coloured line. The presence of this coloured line in the test region indicates a positive result, while the absence indicates a negative result. To serve as a procedural control, a coloured line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

Procedure: All test strips, serum or plasma specimen and/or controls were allowed to equilibrate to room temperature (30^0c) prior to testing.

The pouch was brought to room temperature before being opened and the test strip was removed from the sealed pouch and used as soon as possible. The assay was performed within one hour for best results.

The test strip was vertically immersed in the serum or plasma with the arrows pointing toward the serum or plasma specimen for at least 10-15secs. The maximum (MAX) on the test strip was passed when immersing the strip. The test strip was then placed on a non-absorbent flat surface, the timer started and waited for red line(s) to appear i.e within 15 minutes.

Interpretation:

P**ositive**: Two distinct red lines appear one line should be in the control regions(c) and another line, in the test region (t)

Note: The intensity of the red colour in the test line region (t) will vary depending on the concentration of HBsAg present in the specimen. Therefore, any shade of red in the test region (t) was considered positive.

Negative: One red line appeared in the control region (c).no apparent red or pink line appeared in the test region (t).

Invalid: Control line failed to appear. Insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure.

3.8.3 Hepatitis C Virus (HCV) Screening Test

The detection and differentiation of Hepatitis C virus using lateral flow immunoassay HCV test Kit (20160518) (Nantong Egens Biotechnology Co. Ltd, China, 2015).

Principle: The Hepatitis C virus Antibody test (Serum/Plasma) is a lateral flow chromatographic immunoassay based on the principle of the double antigen sandwich technique. The membrane is pre – coated with HCV antigen on the test line region of the test. While detecting a positive sample, anti – HCV in the specimen (serum or plasma) reacts with the particle coated with HCV antigen. The mixture migrates upward on the membrane chromatographically by capillary action to react with HCV antigen on the membrane and generate a colored line. The presence of this colored line in the test region indicates a positive result, while its absence indicates a negative result.

To serve as a procedural control, a colored line will always appear in the control line region indicating that the proper volume of specimen has been added and membrane wicking has occurred.

Procedure

The pitch was opened at the notch and the device removed. The device was labeled with specimen ID number. The strip was immersed into the serum sample with the ….. end pointing towards the sample. The strip was taken out after 8 – 10 seconds and laid on a clean, dry, flat surface. The timer was set and the result was read in 15 minutes.

Interpretation

Positive: In addition to a pink colored control (c) band, a distinct pink colored band will also appear in the test (T) region.

Negative: Only one colored band appears on the control (c) region. No apparent band on the test (t) region.

Invalid: If a color band is not visible in the control region or a color band is only visible in the test region, the test is invalid.

3.8.4 Veneral Disease Research Laboratory (VDRL) Rapid Test

The syphilis antibody test was performed using plasma by the rapid test strip method (1705195) (Larson *et al.*, 1990).

Principle: The syphilis antibody rapid test strip (serum/plasma) is a lateral flow chromatographic immunoassay based on the principle of the double antigen – sandwich technique. In this test, syphilis recombinant antigen is immobilized in the test line region of the

strip in test device. After specimen is added to the specimen well of the device, It reacts with syphilis recombinant antigen coated particles in the test. This mixture migrates chromatographically along the length of the test strip and interacts with the immobilized syphilis antigens. If the specimen contains syphilis antibodies a colored line will appear in the test line region indicating a positive result. If the specimen does not contain syphilis antibodies, a colored line will not appear in this region, indicating a negative result. To serve as a procedural control, a colored line will always appear in the control line region, indicating that proper volume of specimen has been added and membrane wicking has occurred.

Procedure: The test strip specimen and / or controls were allowed to equilibrate to room temperature (30^0C) prior to testing. The test strip was removed from the foil pouch and used as soon as possible, and the assay performed within one hour for best results. The strip was dipped into the specimen for at least 10 seconds until thoroughly wet. The specimen was not allowed to reach above the level indicated by the arrows on the strip and time was set up. The strip was removed from the specimen and placed on a flat, dry surface. The result was read within 15 minutes when red line(s) appeared. The result was not interpreted after 15 minutes.

Interpretation of results

Positive: Two distinct red lines appear. One line should be in the control © and another line should be in the test region (T).

Negative: One red line appears in the control region (c) . No apparent red or pink line appears in the test region (T)

Invalid: Control line fails to appear, insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure.

3.8.5 Pregnancy Test

The qualitative detection of human chorionic gonadotropin (HCG) was performed by the rapid test strip method. (HCG 1500013) (Biotest Biotech Co. Ltd, China, 2014).

Principle: The HCG pregnancy rapid test strip is a rapid chromatographic immunoassay for the qualitative detection of human chorionic gonadotropin in urine/ blood to aid in the early detection of pregnancy. The test uses two lines to indicate results. The test utilizes a combination of antibodies including a monoclonal HCG antibody to selectively detect elevated levels of HCG. The control line is composed of goat polyclonal antibodies and colloidal gold particles.

The assay is conducted by immersing the test strip in a urine / blood specimens and observing the formation of colored lines. The specimen migrates via capillary action along the membrane to react with the colored conjugate. Positive specimens react with specific antibody HCG colored conjugate to form a colored line at the test line region of the membrane. Absence of this colored line suggests a negative result. To serve as a procedural control, a colored line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

Procedure: The pouch was brought to room temperature before opening it. The test sterile was removed from the sealed pouch and used immediately. The test strip was vertically immersed in the serum specimen, with arrow pointing toward the serum specimen for at least 15 seconds. The strip was placed on a non – absorbent flat surface, and the timer started. The result was read at 3 minutes when the colored line (s) appeared.

Interpretation:

Positive: Two distinct colored lines appear one in the control (c) region and the other in the test (T) region.

Negative: One colored line appears in the control line region (C). No line appears in the test line region (T).

Invalid: The result was invalid if no colored line appears in the control line region (C) even if a line appears in the test region (T).

3.8.6 Haematological Parameters

3.8.6.1 Full blood count

This was done using EDTA blood with sysmex KN – 21N, (Manufactured by sysmex corporation, Japan), a three – part auto analyzer able to run 19 parameters. Standardization, calibration of the instrument and processing of the samples were done according to the manufacturer's instructions (Beckman, 1995).

Principle : The Beckman counter method of sizing and counting particles uses measurable changes in electrical resistance produced by non – conductive particles suspended in an electrolyte. A suspension of blood cells passes through a small orifice simultaneously with an electric current. A small opening (aperture) between electrodes is the sensing zone through

which suspended particles pass. In the sensing zone, each particle displaces its volume of electrolyte. Beckman coulter measures the displaced volume as a voltage of the particle. The quantity of suspension drawn through the aperture is for an exact reproducible volume.

Procedure: Each blood Sample was mixed well and then approximately 20µl was aspirated by allowing the analyzer's sampling probe into the blood sample and depressing the start button. Results of the analysis were displayed after about 30 seconds, after which the analyzer generated a paper copy of the results on thermal printing paper.

3.8.7 Determination of Plasma Prothrombin Time (PT) Test

The PT test was performed using citrated plasma by the Dia – PT Prothrombin Time reagent kit(lot: 950126) (DIAGON Ltd, Hungary, 2014).

Principle: Dia – PT as calcium thromboplastin when added to patient's plasma, induces the formation of fibrin clot, which is measurable manually, or with optical or mechanical coagulation analyzers.

Procedure: The Dia – PT thromboplastin was brought to 37^0C. Fifty microliter (50µl) plasma was added to the test tube and incubated for 2 minutes at 37^0C. Then 100 µl Dia – PT thromboplastin was added and the timer started simultaneously. The coagulation time was determined when there was formation of clot.

3.8.8 Determination of Plasma Activated Partial Thromboplastin Time (APTT)

The APTT was done using citrated plasma by the Dia – PT reagent kit method (Lot no – 950201) (DIAGON Ltd Hungary, 2014).

Principle: The APTT involves the recalcification of the plasma in the presence of standardized amount of micronized silica.

The APTT is screening test for the qualitative and quantitative deficiencies of the intrinsic factors, causing bleeding tendency.

Procedure: The Dia – $Cacl_2$ was brought to 37^0C. Fifty microliter (50µl) plasma was added to the test tube and 50µl Dia – PTT reagent was added to the plasma. This was gently mixed and the mixture incubated for 3 minutes at 37^0C. fifty microliter (50µl) Dia – $Cacl_2$ was added and the timer simultaneously started. The coagulation time was then determined.

3.8.9 Determination of Serum Ferritin Concentration

The quantitative determination of serum ferritin concentration was performed by the Enzyme linked immunoabsorbent assay (ELISA) techniques using Human Ferritin Enzyme immunoassay Test Kit (BC – 1025) (BioCheck, Inc. Canada, 2014).

Principle: The Bio Check ferritin Quantitative Test is based on the principle of a solid phase enzyme linked immunosorbent assay. The assay system utilizes rabbit antiferritin for the solid phase (microliter wells) immobilization and mouse monoclonal anti -ferritin in the antibody enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the ferritin molecules being sandwiched between the solid phase and enzyme linked antibodies. After 45 minute incubation at room temperature, the wells were washed with water to remove unbound labeled antibodies. A solution of $3,3^{1},5,5^{1}$ – tertramethylbezidine (TMB) is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCL, and the resulting yellow color is measured spectrophotometrically at 450nm. The concentration of ferritin is directly proportional to the color intensity of the test sample.

Procedure: The desired number of coated wells were secured in the holder. About 20µl of standards, samples and controls were dispensed into appropriate wells. About 100µl of enzyme conjugate reagent was dispensed into each well, gently mixed completely for 30 seconds. This was incubated at room temperature (25^{0}C) for 45 minutes. The incubation mixture was removed by flicking well contents into a suitable waste container. The wells were rinsed 5 times with distilled or deionized water and struck sharply on absorbent paper to remove residual water droplets. About 100µl TMB reagent was dispensed into each well and gently mixed for 5 seconds. This was incubated at room temperature, in the dark, for 20 minutes. The reaction was stopped by adding 100µl of stop solution (IN HCL) into each well. This was gently mixed for 5 seconds and the optical density (OD) was read with a microtiter well reader within 15 minutes.

Calculation of Results

A standard calibration absorbance was obtained from each reference standard against its concentrations on the horizontal or x – axis. Then using the mean absorbance value for each sample, the corresponding concentration of ferritin in ng/ml was determined from the standard calibration curve (manufacturers standard operating procedures was strictly followed).

3.8.10 Determination of Serum Iron and Total Iron – binding Capacity

The quantitative determination of serum iron (SI) and total iron binding capacity (TIBC) were carried out spectrophotometrically with Teco diagnostics iron / TIBC reagent set, CA-92807(TECO Diagnostics U.S.A, 2013).

Principle: The iron in serum is dissociated from its fe (111) transferrin complex by the addition of an acidic buffer containing hydroxylamine. This addition reduces the Fe (111) to Fe(11). The chromogenic agent, ferene forms a highly colored Fe (ii) complex that is measured photometrically at 560nm.

The unsaturated iron binding capacity (UIBC) is determined by adding Fe(11) ion to serum so that they bind to unsaturated iron binding sites on transferrin. The excess Fe (11) ions are reacted with ferrozine to form the color complex, which is measured photometrically. The difference between n the amount of Fe (11) added and the amount of Fe (11) measured represents the unsaturated iron binding. The total iron binding capacity (TIBC) is determined by adding the serum iron value to the UIBC value.

Procedure:

Serum Iron: Test tubes were labeled "BLANK", "Standard" "Control" and "Sample". 2.5ml iron buffer reagents were added to all tubes. 0.5ml (500µl) sample was added to the different tubes and mixed, and 0.5ml iron free water to blank. The spectrophotometer was zeroed with the reagent blank at 560nm. The absorbance of all tubes were read and recorded (A1 reading). 0.05ml (50 µl) Iron color reagent was added to all the tubes and mixed. All tubes were placed in the water bath at 37^0C for 10 minutes. The instrument was zeroed with the reagent blank at 560nm, and the absorbances of all the tubes read and recorded (A_2 reading).

Calculations

A = Absorbance

Std = Standard

$$\frac{A_2\text{Test} - A_1\text{Test}}{A_2\text{Std} - A_1\text{Std}} \text{ X conc of Std} = \text{Total Iron (µg/ dl)}$$

Unsaturated Iron – Binding Capacity (UIBC)

This was carried out spectrophotometrically using Teco diagnostics reagent (TECO Diagnostics U.S.A, 2013).

Test tubes / cuvettes were labeled "Blank" "Standard", "Control" "Test". 2.0ml UIBC buffer reagent was added to all tubes. 1.0ml iron free water was added to "BLANK" and mixed. 0.5ml (500mm) Iron free water plus 0.5ml to "Standard" and mixed. The spectrophotometer was zeroed with reagent blank at 560nm. The absorbance of all tubes were read and recorded (A1 reading). 0.05ml (50 µl) of Iron color reagent was added to all tubes and mixed. Then, all tubes were placed in a heating bath at 37^{o} C for 10 minutes. The spectrophotometer was zeroed with blank at 560nm. The absorbance of all tubes were read and recorded (A_2 reading).

UIBC calculations

Conc of Std = $\frac{A_2 Test - A_1 Test}{A_2 Std - A_1 Std}$ x Conc of Std = UIBC (µg/dl)

Calculations

TIBC = Iron level + UIBC = TIBC (µg/dl)

SI unit conversion: µg/dl x 0.179 = µmol/L.

3.8.11 Determination of Cluster of Differentiation 4 (CD4+) Count.

The Partec cyflow counter for CD4 + cell count was used for the assay (Sysmex Partec, Germany, 2014).

Principle:

The basic principle of flow cytometry is the passage of cells in single file in front of a laser so they can be detected, counted and sorted.

Cell components are fluorescently labeled and then excited by the laser to emit light at varying wavelengths. The fluorescence can then be measured to determine the amount and type of cells present in a sample.

Procedure:

About 20 µl of well mixed blood was added into partec test tube (Rohren tube) containing 20 µl CD4 antibody. The solution was mixed gently and incubated in the dark for 15 minutes at room temperature. A total of 800 µl of $CD4^+$ buffer was added and mixed gently. Tube was connected to the counter and allowed to run ensuring CD4 cells were well gated. Results were automatically displayed in the screen in counts/ µl.

3.8.12 Interleukin– 2 (IL – 2) Level Determination

The determination of IL – 2 was performed by the Enzyme linked immunoassay technique (ELISA) using human Enzyme immunoassay test kit. (Perfemed, 2015).

Principle:

This ELISA kit uses sandwich ELISA as the method. The microelisa stripplate provided in this kit has been pre – coated with an antibody specific to IL – 2 standards or samples are added to the appropriate microelisa stripplate wells and combined to the specific antibody. Then a Horseradish peroxidase (HRP) conjugated antibody specific for IL -2 is added to each microelisa stripplate well and incubated.

Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain IL – 2 and HRP conjugated IL – 2 antibodies will appear blue in color and then turn yellow after the optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional of IL -2. The concentration of IL – 2 in the samples can be calculated by comparing the OD of the samples to the standard curve.

Procedure:

- **Dilution of Standards**: Ten wells were set for standards in a micro elisa striplate.100µl standard solution and 50µl standard dilution buffer were added in wells 1 and 2 respectively and mixed well. In well 3 and well 4 100µl solution from well 1 and well 2 were added respectively. Then 50 µl standard dilution buffer were added and mixed well. 50µl solution was discarded from well 3 and well 4. In well 5 and well 6, 50µl solution from well 3 and well 4 were added respectively. Then 50µl standard dilution buffer were added and mixed well. In well 7 and well 8, 50 µl solution from well 5 and well 6 added respectively. Then 50µl standard dilution buffer were added and mixed well. In well 9 and well 10 50µl from well 7 and well 8 were added respectively. Then 50µl standard dilution buffer were added

and mixed well, 50µl solution was discarded from well 9 and well 10. After dilution, the total value in all the wells were 50 µl and the concentrations were 180 pg/ml, 120 pg/ml, 60 pg/ml, 30 pg/ml and 15pg/ ml respectively.

- In the microelisa striplate, a well was left empty as blank control. In sample wells, 40 µl sample dilution buffer and 10 µl sample were added (dilution factor is 5). This was well mixed with gentle shaking.
- **Incubation**: It was incubated for 30mins at 37^0 C.
- **Dilution**: The Concentrated washing buffer was diluted with distilled water (30 times for 96T).
- **Washing**: The closure plate membrane was carefully peeled off, aspirated and refilled with the wash solution. The wash solution was discarded after allowing to stand for 30 seconds. The washing procedure was repeated 5 times. 50 µl / HRP – conjugate reagent was added to each well except the blank control well, and incubated for 30 minutes at 37^0C. This was washed as described above.
- **Coloring**: Fifty microliter (50µl) chromogen solution A and 50µl chromogen solution B were added to each well, mixed with gentle shaking and incubated at 37^0C for 15 minutes. Light was avoided during coloring.

Termination: Fifty microliter (50 µl) stop solution was added to each well to terminate the reaction. The color in the well changed from blue to yellow. The absorbance OD was read at 450nm using a microtiter plate reader. The OD value of the blank control well was set at zero. Assay was carried out within 15 minutes after reading stop solution.

Calculation of Results

Known concentrations of Human IL – 2 standard and its corresponding reading OD was plotted on the log Scale (x – axis) and the log scale (y – axis) respectively. The concentration of Human IL – 2 in sample was determined by plotting the samples OD on the Y – axis. The original concentration was calculated by multiplying the dilution factor.

3.8.13 Interleukin 4 (IL – 4) Level Determination

IL – 4 determination was done by the ELISA technique using human enzyme immunoassay test kit. (Perfemed, 2015).

Principle: IL – 4 ELISA Kit uses sandwhich – ELISA as the method. The micro elisa stripplate provided in this kit has been pre – coated with antibody specific to IL – 4. Standards or samples are added to the appropriate microelisa stripplate wells and combined to the specific antibody. Then a horse radish peroxidase (HRP) – conjugated antibody specific for IL – 4 is added to each micro elisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well, Only those wells that contain IL – 4 and HRP conjugated IL – 4 antibody will appear blue in color then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of IL – 4. The concentration of IL – 4 in the samples can be calculated by comparing the OD of the samples to the standard curve.

Procedure

- **Dilution of Standards**: Ten wells were set for standards in a micro elisa striplate. About 100µl standard solution and 50µl standard dilution buffer were added in wells 1 and 2 respectively and mixed well. In well 3 and well 4 100µl solution from well 1 and well 2 were added respectively. Then 50 µl standard dilution buffer were added and mixed well. 50µl solution was discarded from well 3 and well 4. In well 5 and well 6, 50µl solution from well 3 and well 4 were added respectively. Then 50µl standard dilution buffer was added and mixed well. In well 7 and well 8, 50 µl solution from well 5 and well 6 were added respectively. Then 50µl standard dilution buffer were added and mixed well. In well 9 and well 10 50µl from well 7 and well 8 were added respectively. Then 50µl standard dilution buffer was added and mixed well, 50µl solution was discarded from well 9 and well 10. After dilution, the total value in all the wells were 50 µl and the concentrations were 180 pg/ml, 120 pg/ml, 60 pg/ml, 30 pg/ml and 15pg/ ml respectively.
- In the microelisa striplate, a well was left empty as blank control. In sample wells, 40 µl sample dilution buffer and 10 µl sample were added (dilution factor is 5).This was well mixed with gentle shaking.
- **Incubation**: It was incubated for 30 mins at 37^0 C.
- **Dilution**: The Concentrated washing buffer was diluted with distilled water (30 times for 96T).
- **Washing**: The closure plate membrane was carefully peeled off, aspirated and refilled with the wash solution. The wash solution was discarded after resting for 30 seconds. The washing procedure was repeated 5 times. 50 µl / HRP – conjugate reagent was added to each

well except the blank control well, and incubated for 30 minutes at 37^0C. This was washed as described above.

- **Coloring**: Fifty microliter (50µl) chromogen solution A and 50µl chromogen solution B were added to each and well, mixed with gentle shaking and incubated at 37^0C for 15 minutes. Light was avoided during coloring.

Termination: Fity microliter (50µl) stop solution was added to each well to terminate the reaction. The color in the well changed from blue to yellow. The absorbance OD was read at 450nm using a microtiter plate reader. The OD value of the blank control well was set at zero. Assay was carried out within 15 minutes after reading stop solution.

Calculation of Results

Known concentrations of Human IL – 4 standard and its corresponding reading OD was plotted on the log Scale (x-axis) and the log scale (y – axis) respectively. The concentration of Human IL – 4 in sample was determined by plotting the samples OD on the Y – axis. The original concentration was calculated by multiplying the dilution factor.

3.8.14 Determination of Interleukin 10 (IL – 10) Level.

The determination of IL – 10 was performed by the ELISA method using human enzyme immunoassay test kit. (Perfemed, 2015).

Principle:

IL-10 ELISA Kit uses sandwhich – Elisa as the method. The micro elisa stripplate provided in this kit has been pre – coated with antibody specific to IL-10 Standards or samples are added to the appropriate microelisa stripplate wells and combined to the specific antibody. Then a horse radish peroxidase (HRP) – conjugated antibody specific for IL-10 is added to each micro elisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well, Only those wells that contain IL-10 and HRP conjugated IL-10 antibody will appear blue in color even turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of IL-10. The concentration of IL-10 can be calculated in the samples by comparing the OD of the samples to the standard curve.

Procedure

- **Dilution of Standards**: Ten wells were set for standards in a micro ELISA striplate. About 100µl standard solution and 50µl standard dilution buffer were added in wells 1 and 2 respectively and mixed well. In well 3 and well 4 100µl solution from well 1 and well 2 were added respectively. Then 50 µl standard dilution buffer were added and mixed well. 50µl solution was discarded from well 3 and well 4. In well 5 and well 6, 50µl solution from well 3 and well 4 were added respectively. Then 50µl standard dilution buffer were added and mixed well. In well 7 and well 8, 50 µl solution from well 5 and well 6 added respectively. Then 50µl standard dilution buffer were added and mixed well. In well 9 and well 10 50µl from well 7 and well 8 were added respectively. Then 50µl standard dilution buffer were added and mixed well, 50µl solution was discarded from well 9 and well 10. After dilution, the total value in all the wells were 50 µl and the concentrations were 90 pg/ml, 60 pg/ml, 30 pg/ml, 15 pg/ml and 7.5pg/ ml respectively.
- In the microelisa striplate, a well was left empty as blank control. In sample wells, 40 µl sample dilution buffer and 10 µl sample were added (dilution factor is 5).This was well mixed with gentle shaking.
- **Incubation**: It was incubated for 30mins at 37^0 C.
- **Dilution**: The Concentrated washing buffer was diluted with distilled water (30 times for 96T).
- **Washing**: The closure plate membrane was carefully peeled off, aspirated and refilled with the wash solution. The wash solution was discarded after allowing to stand for 30 seconds. The washing procedure was repeated 5 times. About 50 µl / HRP – conjugate reagent was added to each well except the blank control well, and incubated for 30 minutes at 37^0C. This was washed as described above.
- **Coloring**: About 50µl chromogen solution A and 50µl chromogen solution B were added to each and well, mixed with gentle shaking and incubated at 37^0C for 15 minutes. Light was avoided during coloring.

  Termination: About 50 µl stop solution was added to each well to terminate the reaction. The color in the well changed from blue to yellow. The absorbance OD was read at 450nm using a microtiter plate reader. The OD value of the blank control well was set at zero. Assay was carried out within 15 minutes after reading stop solution.

Calculation of Results

Known concentrations of Human IL – 10 standard and its corresponding reading OD was plotted on the log Scale (x – axis) and the log scale (y – axis) respectively. The concentration of Human IL – 10 in sample was determined by plotting the samples OD on the Y – axis. The original concentration was calculated by multiplying the dilution factor.

3.8.15 Determination of Tumor necrosis factor (TNF – α) Level

TNF – α was determined by the ELISA technique using human enzyme immunoassay test kit. (Perfemed, 2015).

Principe: TNF – α ELISA Kit uses sandwhich – ELISA as the method. The micro ELISA stripplate provided in this kit has been pre – coated with antibody specific to TNF – α Standards or samples are added to the appropriate microelisa stripplate wells and combined to the specific antibody. Then a horse -radish peroxidase (HRP) – conjugated antibody specific for TNF - α is added to each micro elisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well, only those wells that contain TNF – α and HRP conjugated TNF – α antibody will appear blue in color then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD value is proportional to the concentration of TNF - α. The concentration of TNF - α can be calculated in the samples by comparing the OD of the samples to the standard curve.

Procedure

- **Dilution of Standards**: Ten wells were set for standards in a micro elisa striplate. About 100µl standard solution and 50µl standard dilution buffer were added in wells 1 and 2 respectively and mixed well. In well 3 and well 4 100µl solution from well 1 and well 2 were added respectively. Then 50 µl standard dilution buffer was added and mixed well. About 50µl solution was discarded from well 3 and well 4. In well 5 and well 6, 50µl solution from well 3 and well 4 were added respectively. Then 50µl standard dilution buffer was added and mixed well. In well 7 and well 8, 50 µl solution from well 5 and well 6 added respectively. Then 50µl standard dilution buffer was added and mixed well. In well 9 and well 10 50µl from well 7 and well 8 were added respectively. Then 50µl standard dilution buffer was added and mixed well, 50µl solution was discarded from well 9 and well 10. After dilution, the total value in all the wells were 50 µl and the concentrations were 300 pg/ml, 200 pg/ml, 100 pg/ml, 50 pg/ml and 25pg/ ml respectively.

- In the microelisa striplate, a well was left empty as blank control. In sample wells, 40 µl sample dilution buffer and 10 µl sample were added (dilution factor is 5). Samples were loaded onto the bottom without touching the well wall. This was well mixed with gentle shaking.
- **Incubation**: Having been sealed with closure plate, it was incubated for 30mins at 37^0 C.
- **Dilution**: The Concentrated washing buffer was diluted with distilled water (30 times for 96T).
- **Washing**: The closure plate membrane was carefully peeled off, aspirated and refilled with the wash solution. The wash solution was discarded after resting for 30 seconds. The washing procedure was repeated 5 times. About 50 µl / HRP – conjugate reagent was added to each well except the blank control well, and incubated for 30 minutes at 37^0C. This was washed as described above.
- **Coloring**: About 50µl chromogen solution A and 50µl chromogen solution B were added to each and well, mixed with gentle shaking and incubated at 37^0C for 15 minutes. Light was avoided during coloring.
- **Termination**: About 50 µl stop solution was added to each well to terminate the reaction. The color in the well changed from blue to yellow. The absorbance OD was read at 450nm using a microtiter plate reader. The OD value of the blank control well was set at zero. Assay was carried out within 15 minutes after reading stop solution.

Calculation:

Known concentrations of Human TNF - $\boldsymbol{\alpha}$ standard and its corresponding reading OD was plotted on the log Scale (x – axis) and the log scale (y – axis) respectively. The concentration of Human TNF- $\boldsymbol{\alpha}$ in sample was determined by plotting the samples OD on the Y – axis. The original concentration was calculated by multiplying the dilution factor.

3.9 Statistical Analysis

Statistical analysis was done using computer software statistical package for social sciences (SPSS), version 20.0. One way analysis of variance (ANOVA) was used to compare between pregnant women at all trimesters and non – pregnant women (independent ANOVA), and for comparisons across the trimesters (the repeated measure ANOVA). Pearson's correlation analysis was undertaken to access the statistical relationships or associations among the variables according to trimesters and statistical significance was calculated using post hoc test to analyse the results of the experimental data. Differences were considered to be significant at $p < 0.05$.

3.10 Limitations

i) Some pregnant women were apprehensive about being involved in research study. It took time to convince them before they gave their informed consent, and during the follow up, some of them did not turn up for antenatal clinic at the appropriate time, so had to reach them elsewhere to collect their samples, making the study more tedious.

ii) Almost all the pregnant women started antenatal care at three months, so they were not assessed at baseline.

iii) It was a difficult task collecting blood samples from pregnant women considering the sample size.

iv) The research was capital intensive. It was solely funded by the researcher without assistance from the government.

(v) The Research work was long-term since it was follow-up study and was vulnerable to selection bias, because only willing participants who gave their informed consent agreed to be part of the long-term study.

(vi) This study gave room for attrition because some of the pregnant women could not complete the study because they had abortion, still birth, miscarriage, changed contact details and intentionally refused to continue in the research.

(vii) It was difficult to access the pregnant women at exactly the same week in each trimester.

CHAPTER FOUR

RESULTS

4.1: Demographic Data Analysis of the Pregnant Women Studied.

Table 4.1, shows the characteristics of the pregnant women studied. The mean age of the pregnant women was 28.13 $\pm$ 4.23 and body mass index (BMI), 28.17 $\pm$ 4.15. 61.25% of the pregnant women were primigravidas, while 38.75% were multigravidas. 45.00% were civil servants/private employees, 30.00% Traders, 18.13% House wives and 6.87% students. 65.00% attained tertiary education, 33.75%, secondary and 1.25%, primary

Table 4.1: **Demographic Data Analysis of the Pregnant Women Studied.**

(N=160)	**M (SD)** **N (%)**
Age (Years)	28.13 ± 4.23
BMI (kg/m^2)	28.17 ± 4.15
Gravidity	
Primigravida	98 (61.25)
Multigravida	62 (38.75)
Occupation	
Civil Servants/Private employees	72 (45.00)
Traders	48 (30.00)
Housewives	29 (18.13)
Students	11 (6.87)
Education	
Tertiary	104 (65.00)
Secondary	54 (33.75)
Primary	2 (1.25)

Key:

BMI – Body Mass Index

N – No of subjects

M – Mean

SD – Standard Deviation

4.2: Levels of Immunological Parameters of Pregnant Women based on Trimesters compared to Non – Pregnant Women (Mean +SD).

The mean levels of $CD4^+$ cell count in first (660.12 ± 484.92), second (625.45 ± 160.17) and third (621.92 ± 159.40) trimesters were significantly decreased compared to the control subjects (764.27 ± 182.58) ($F = 11.3$, $p < 0.001$). The mean levels of IL – 2 in first (51.18 ± 31.70), second (48.58 ± 31.01) and third (46.82 ± 31.13) trimesters were decreased significantly compared to controls (56.17 ± 31.45) ($F = 3.6$, $p = 0.014$). On the other hand, TNF – α mean values in first (168.97 ± 126.33), second (166.69 ± 67.43) and third (165.94 ± 68.97) trimesters showed no significant decrease compared to controls (169.27 ± 56.63) ($F = 0.1$, $p = 0.972$). IL – 4 with mean levels in first (28.12 ± 17.38), second (31.33 ± 17.51), and third (33.81 ± 17.78) were significantly increased compared to controls (27.73 ± 21.68) ($F = 4.8$, $p = 0.002$).
There was a significant increase when the mean values of IL – 10 in the first (30.54 ± 13.10), second (34.54 ± 16.41), and third (38.66 ± 22.89) trimesters were compared to controls (26.62 ± 17.61) ($F = 17.2$, $p < 0.001$).

Post Hoc Analysis

The mean levels of $CD4^+$ cell count in non-pregnant controls (764.27 ± 182.58) was significantly higher compared to the first trimester (660.12 ± 484.92) ($p = 0.001$). The mean level of $CD4^+$ cell count in second trimester (625.45 ± 160.17) was statistically decreased significantly when compared to that of the controls (764.27 ± 182.58) ($p < 0.001$). Similarly, that of third trimester (621.92 ± 159.40) was significantly decreased when compared to controls (764.27 ± 182.58) ($p < 0.001$). The third trimester (46.82 ± 31.13) showed a significant statistical decrease when compared to the controls (56.17 ± 31.45) ($p = 0.014$).

IL – 4 showed a significant increase when the third trimester (33.81 ± 17.78) was compared to the controls (27.73 ± 21.68) ($p = 0.006$). Also, a significant increase was observed when the third trimester (33.81 ± 17.78) was compared to the first (28.12 ± 17.38) ($p = 0.012$).

The mean level of IL – 10 showed a significant increase when the second (34.54 ± 16.41) and third (38.66 ± 22.89) trimesters were compared to the controls (26.62 ± 17.61) ($p < 0.001$). A significant increase was observed when the third trimester (38.66 ± 22.89) was compared to the first (30.54 ± 13.10) ($p < 0.001$) (Table 4.2).

Table 4.2: Levels of Immunological Parameters of Pregnant Women based on Trimesters compared to Non – Pregnant Women (Mean +SD

Control/ Trimester	$CD4^+$ (cells / ul)	IL – 2 (pg/ml)	TNF – α (ng/l)	IL – 4 (pg/ml)	IL – 10 (pg/ml)
Control[c] N = 160	764.27 ± 182.58	56.17 ± 31.45	169.27 ± 56.63	27.73 ± 21.68	26.62 ± 17.61
First Trim N = 160	660.12 ± 484.92	51.18 ± 31.70	168.97 ± 126.33	28.12 ± 17.38	30.54 ± 13.10
Second Trim N = 156	625.45 ± 160.17	48.58 ± 31.01	166.69 ± 67.43	31.33 ± 17.51	34.54 ± 16.41
Third Trim N = 140	621.92 ± 159.40	46.82 ± 31.13	165.94 ± 68.97	33.81 ± 17.78	38.66 ± 22.89
F (P – value)	11.3 (<0.001)*	3.6 (0.014)*	0.1 (0.972)	4.8 (0.002)*	17.2 (<0.001)*
	Post Hoc				
C vs 1st	0.001*	0.573	1.000	0.997	0.119
C vs 2nd	<0.001*	0.070	0.990	0.209	<0.001*
C vs 3rd	<0.001*	0.014*	0.979	0.006*	<0.001*
1st vs 2nd	0.602	0.652	0.993	0.306	0.107
2nd vs 3rd	0.999	0.941	1.000	0.538	0.093
1st vs 3rd	0.522	0.308	0.984	0.012*	<0.001*

Key:

Mean difference is significant at $p < 0.05$*

C = Control. SD = Standard Deviation.Trim = Trimester

4.3: Levels of Immunological Parameters of Pregnant Women at Different Trimesters (Mean ± SD).

The mean levels of $CD4^+$ cell count compared across the first (660.12 ± 484.92), second (625.45 ± 160.17) and third (621.92 ± 159.40) trimesters were not significantly decreased statistically (F = 1.0; p = 0.386). That of IL – 2 in the first (52.18 ± 31.70), second (48.58 ± 31.01), and third (46.82 ± 31.13) trimesters when compared were not significantly reduced (F = 1.6, p = 0.211). There was also no statistically significant decrease in the mean values of TNF - α when the first (168.97 ± 126.33), second (166.69 ± 67.43) and third (165.94 ± 68.97) trimesters were compared (F = 0.1, p= 0.941). When the first (28.12 ± 17.38), second (31.33 ± 17.51), and third (33.81 ± 17.78) trimesters were compared IL – 4 showed a significant increase (F = 5.4, p = 0.005). A significant increase was also observed in IL – 10 among the first (30.54 ± 13.10), second (34.54 ± 16.41), and third (38.66 ± 22.89) trimesters (F = 10.5, p < 0.001).

Post Hoc Analysis

There was no significant statistical decrease in the $CD4^+$ cell count when the second (625.45 ± 160.17) and the third (621.92 ± 159.40) trimesters were compared with the first trimester (660.12 ± 484.92) (p = 0.494) and (p = 0.425), and when the third trimester (621.92 ± 159.40) was compared to the second (625.45 ± 160.17) (p = 0.993).

There was no significant decrease in IL-2 when the second (48.58 ± 31.70) and third (46.82 ± 31.13) trimesters were compared to the first (52.18 ± 31.70) (P=0.476) and (p = 0.194), and when the third trimester (46.82 ± 31.13) was compared to the second (52.18 ± 31.70) (p = 0.836).

TNF – α showed no decrease when the second (166.69 ± 67.43) and third (165.94 ± 68.97) trimesters were compared with the first trimester (168.97 ± 126.33) (p = 0.966 and 0.941). Similarly, no statistically significant decrease was seen when the third trimester (165.94 ± 68.97) was compared with the second (166.69 ± 67.43) (p = 0.996).

IL-4 showed no significant increase when the second (31.33 ± 17.51) trimester was compared with first trimester (28.12 ± 17.38) (0.155), and the third (33.81 ± 17.78) compared to the second (31.33 ± 17.51) (p = 0.329). However, a significant increase was observed when the third (33.81 ± 17.78) trimester was compared to the first (28.12 ± 17.38) (p = 0.003). A significant increase was seen when the third (38.66 ± 22.89) was compared to the first (30.54 ± 13.10) (p < 0.001).

Table 4.3: Levels of Immunological Parameters of Pregnant Women at Different Trimesters (Mean ± SD).

Trimester	$CD4^+$ (cells / ul)	IL – 2 (pg/ml)	TNF – α (ng/l)	IL – 4 (pg/ml)	IL – 10 (pg/ml)
First Trim N = 160	660.12 ± 484.92	52.18 ± 31.70	168.97 ± 126.33	28.12 ± 17.38	30.54 ± 13.10
Second Trim N = 156	625.45 ± 160.17	48.58 ± 31.01	166.69 ± 67.43	31.33 ± 17.51	34.54 ± 16.41
Third Trim N = 140	621.92 ± 159.40	46.82 ± 31.13	165.94 ± 68.97	33.81 ± 17.78	38.66 ± 22.89
F (P – value)	1.0 (0.386)	1.6 (0.211)	0.1 (0.941)	5.4 (0.005) *	10.5 (<0.001) *
	Post Hoc				
1st vs 2nd	0.494	0.476	0.966	0.155	0.063
2nd vs 3rd	0.993	0.836	0.996	0.329	0.054
1st vs 3rd	0.425	0.194	0.941	0.003*	<0.001*

Key:

* Significant at $P < 0.05$

Trim = Trimester

4.4: Mean Levels of Haematological Parameters of Non – Pregnant Women and Pregnant Women based on Trimesters.

The mean levels of PCV in the first (34.03 ± 20.52), second (30.49 ± 2.37), and third (29.98 ± 4.23) trimesters, were significantly decreased statistically compared to the controls (35.25 ± 2.64) ($F = 12.2$, $p < 0.001$).

There was a significant decrease in the mean levels of Hb in the first (10.83 ± 0.85), second (9.66 ± 1.04), and third (8.91 ± 1.23) trimesters compared to the controls (11.64 ± 1.14) ($F = 260.4$, $p < 0.001$). WBC showed a significant increase in the first (6.50 ± 1.58), second (8.99 ± 1.53) and third (11.10 ± 1.63) trimesters, compared to the controls (6.14 ± 1.44) ($F = 459.8$, $p < 0.001$). A significant statistical decrease was observed in RBC count in the first (3.84 ± 0.44), second (3.44 ± 0.42) and third trimesters (3.18 ± 9.45) compared to the controls (4.43 ± 0.47) ($F = 304.4$, $p < 0.001$). Platelet count also showed a significant decrease when the first trimester (224.41 ± 54.61), second (220.13 ± 51.58, and third (217.69 ± 51.20) were compared to the controls ($F = 5.9$, $p = 0.001$).

Post Hoc Analysis

PCV in the second (30.49 ± 2.37) and third (29.98 ± 4.23) trimesters were significantly decreased compared to the controls (35.25 ± 2.64) ($p < 0.001$). Also, that in the second (30.49 ± 2.37) and third (29.98 ± 4.23) trimesters were significantly reduced statistically compared to the first (34.03 ± 20.52) ($p = 0.005$ and 0.001).

The Hb in the first (10.83 ± 0.85), second (9.66 ± 1.04) and third (8.91 ± 1.23) trimesters were significantly lowered statistically when compared to the controls (11.64±1.14) ($p <0.001$). In the second (9.66 ± 1.04) and third (8.91 ± 1.23) trimesters, the Hb was also significantly reduced statistically compared to the first (10.83 ± 0.85) ($p < 0.001$) and equally reduced when the third trimester (8.91 ± 1.23) was compared to the second (9.66 ± 1.04) ($p<0.001$).

A statistically significant increase was seen when the WBC in the second (8.99 ± 1.53) and third (11.10 ± 1.63) trimesters were compared to the controls (6.14 ± 1.44) ($p < 0.001$), and also when the second (8.99 ± 1.53) and third (11.10 ± 1.63) trimesters were compared to the first (6.50 ± 1.58), and the third trimester (11.10 ± 1.63) compared to the second (8.99 ± 1.53) ($p <0.001$).

RBC showed a statistically significant decrease when the first (3.84 ± 0.44), second (3.44 ± 0.42), and third (3.18 ± 0.44) trimesters were compared to the controls (4.43 ± 0.47) ($p < 0.001$).

A statistically significant reduction was also observed when the first (3.84 ± 0.44), second (3.44 ± 0.42) and third (3.18 ± 0.45) trimesters were compared to the controls (4.43 ± 0.47) ($p < 0.001$). When the second (3.44 ± 0.42) and third (3.18 ± 0.45) trimesters were compared to the first (3.84 ± 0.44), a statistically significant decrease was also observed ($p < 0.001$). When the third (3.18 ± 0.45) trimester was compared to the second (3.44±0.42), a statistically significant reduction was seen ($p < 0.001$).

Platelets in the second (220.13 ± 51.58) and third (217.69 ± 51.20) trimesters, showed a statistically significant decrease compared to the controls (237.45 ± 49.24) ($p = 0.004$ and 0.001).

Table 4.4: Mean Levels of Haematological Parameters of Non - Pregnant Women and Pregnant Women based on Trimesters.

Control/ Trimester	PCV (%)	Hb (g/dl)	WBC ($x10^9$/L)	RBC ($x10^{12}$/L)	Platelet ($x10^9$/L)
Control[c] N = 160	35.25 ± 2.64	11.64 ± 1.14	6.14 ± 1.44	4.43 ± 0.47	237.45 ± 49.24
FirstTrim N = 160	34.03 ± 20.52	10.83 ± 0.85	6.50 ± 1.58	3.84 ± 0.44	224.41 ± 54.61
Second Trim N = 156	30.49 ± 2.37	9.66 ± 1.04	8.99 ± 1.53	3.44 ± 0.42	220.13 ± 51.58
Third Trim N = 140	29.98 ± 4.23	8.91 ± 1.23	11.10 ± 1.63	3.18 ± 0.45	217.69 ± 51.20
F (P – value)	12.2 (<0.001) *	260.4 (<0.001) *	459.8 (<0.001) *	304.4 (<0.001) *	5.9 (0.001) *
	Post Hoc				
C vs 1st	0.649	<0.001*	0.085	<0.001*	0.054
C vs 2nd	<0.001*	<0.001*	<0.001*	<0.000*	0.004*
C vs 3rd	<0.001*	<0.001*	<0.001*	<0.001*	0.001*
1st vs 2nd	0.005*	<0.001*	<0.001*	<0.001*	0.837
2nd vs 3rd	0.962	<0.001*	<0.001*	<0.001*	0.964
1st vs 3rd	0.001*	<0.001*	<0.001*	<0.001*	0.554

Key: C = Control

* Significant at $p < 0.05$

Trim = Trimester

4 .5: Levels of Haematological Parameters of Pregnant Women at Different Trimesters (Mean ± SD).

The mean levels of PCV was significantly reduced statistically from the first trimester (34.03 ± 20.52), second (30.49 ± 2.37), to the third (29.98 ± 4.25) (F = 12.2, p = 0.001). That of Hb was equally reduced from the first trimester (10.83 ± 0.85) to the third (8.91±1.25) (F=2.60.4, p < 0.001). WBC was also significantly increased statistically from the first (6.50±1.58) to the third (11.10 ± 1.63) trimesters (F = 432.3, p < 0.001). A statistically significant reduction was observed in RBC from the first (3.84 ± 0.44) to the third trimester (3.18 ± 0.45) (F =119.8, p < 0.001). Platelets was insignificantly decreased statistically from the first (224.41 ± 54.61) to the third (217.69 ± 51.20) trimesters (F = 0.9, p = 0.424).

Post Hoc Analysis

The mean level of PCV in the second trimester (30.49 ± 2.37), showed a statistically non - significant decrease compared to the first (34.03 ± 20.52) (p = 0.100). That in the third trimester (29.98 ± 4.25), also showed an insignificant reduction when compared to the second (30.49 ± 2.37) (p = 0.905). However, the third trimester (29.98 ± 4.25) was significantly decreased statistically compared to the first (34.03 ± 20.52) (p = 0.002).

The Hb in the second (9.66 ± 1.04) and third (8.91 ± 1.25) were significantly decreased statistically compared to the first (10.83 ± 0.85) (p < 0.001). That, in the third trimester (8.91 ± 1.25) was also significantly reduced compared to the second (9.66 ± 1.04) (p < 0.001).

WBC showed a statistically significant increase when the second (8.99 ± 1.53) and third (11.10 ± 1.63) trimesters were compared to the first (6.50 ± 1.58), and when the third trimester (11.10 ± 1.63) was compared to the second (8.99 ± 1.53) (p <0.001).

A statistically significant decrease was seen in RBC when the second (3.44 ± 0.42) and third (3.18 ± 0.45) trimesters were compared to the first (3.84 ± 0.44) (p < 0.001), and when the third trimester (3.18 ± 0.45) was compared to the second (3.44 ± 0.42) (p < 0.001).

The Platelets showed a statistically significant decrease when the second trimester (22.13 ± 51.58) was compared to the first (224.41 ± 54.61) (p = 0.688), third trimester (217.69 ± 51.20) compared to the second (220.13 ± 51.58) (p = 0.886) and the first (224.41 ± 54.61) (p = 0.399).

Table 4.5: Levels of Haematological Parameters of Pregnant Women at different Trimesters (Mean ± SD).

Trimester	PCV (%)	Hb (g/dl)	WBC ($x10^9$/L)	RBC ($x10^{12}$/L)	Platelet ($x10^9$/L)
First Trim N = 160	34.03 ± 20.52	10.83 ± 0.85	6.50 ± 1.58	3.84 ± 0.44	224.41 ± 54.61
Second Trim N = 156	30.49 ± 2.37	9.66 ± 1.04	8.99 ± 1.53	3.44 ± 0.42	220.13 ± 51.58
Third Trim N = 140	29.98 ± 4.25	8.91 ± 1.25	11.10 ± 1.63	3.18 ± 0.45	217.69 ± 51.20
F (P – value)	6.7 (0.001) *	172.9 (<0.001) *	432.3 (<0.001) *	119.8 (<0.001) *	0.9 (0.424)
	Post Hoc				
1st vs 2nd	0.100	<0.001*	<0.001*	<0.001*	0.688
2nd vs 3rd	0.905	<0.001*	<0.001*	<0.001*	0.886
1st vs 3rd	0.002*	<0.001*	<0.001*	<0.001*	0.399

Key:

* Significant at p< 0.05

Trim = Trimester

4.6: Mean Values of Differential White Cell Counts of Non-pregnant Women compared to Pregnant Women based on Trimesters.

The mean level of neutrophil in the first (64.95 ± 66.17), second (67.52 ± 68.17) and third (69.41 ± 70.6) were significantly increased statistically compared to the controls (60.57 ± 61.74) (F=148.5, $p < 0.001$). Lymphocyte showed a significant reduction in first (33.83 ± 35.05), second (31.15 ± 32.48) and third (29.25 ± 30.52), compared to the controls (38.05 ± 39.40) (F = 0136.4, $p < 0.001$). Absolute neutrophil was significantly increased statistically in the first (5.77 ± 1.49), second (8.44 ± 1.66), and third (9.79 ± 1.89) trimesters compared to the controls (F = 781.4, $p < 0.001$). However, absolute lymphocyte was significantly reduced when the first (2.36 ± 0.47), second (2.09 ± 0.51) and third (1.85 ± 0.52) were compared to the controls (3.10 ± 0.76) (F =181.5, $p < 0.001$).

Post Hoc Analysis

A statistically significant increase was observed when neutrophil in the first (64.95 ± 66.17), second (67.52 ± 68.17), and third (69.41 ± 70.69) were compared to the controls ($p < 0.001$). Also, when the second (67.52 ± 68.17) and third (69.41 ± 70.69) trimesters were compared to the first (64.95 ± 66.17) and when the third (64.41 ± 70.69) was compared to the second (67.52 ± 68.17) ($p < 0.001$).

Lymphocyte showed a statistically significant decrease when the first (33.83 ± 35.05), second (31.15 ± 32.48) and third (29.25 ± 30.52) trimesters were compared to the controls (38.05 ± 39.40), the second (31.15 ± 32.48) compared to the first (33.83 ± 35.05), the third (29.25 ± 30.52) compared to the second (31.15 ± 32.48) and first (33.83 ± 35.05) ($p < 0.001$).

There was a statistically significant increase was observed when the absolute neutrophil in the first (5.77 ± 1.49), second (8.44 ± 1.66), and third (9.79 ± 1.89) trimesters were compared to the controls (3.13 ± 0.75), second (8.44 ± 1.66) and third (9.79 ± 1.89) trimesters compared to the first (5.77 ± 1.49), and third (9.79±1.89) compared to the second (8.44 ± 1.66) trimester ($p < 0.001$).

A statistically significant decrease was observed in the lymphocyte values when the first (2.36 ± 0.47), second (2.09 ± 0.51) and third (1.85 ± 0.52) trimesters were compared to the controls (3.10 ± 0.76), the second (2.09 ± 0.51) and third (1.85 ± 0.52) trimesters compared to the first (2.36 ± 0.47), and when the third trimester (1.85 ± 0.52) was compared to the second (2.09 ± 0.51) ($p < 0.001$).

Table 4.6: Mean Values of Differential White Cell Counts of Non-pregnant Women compared to Pregnant Women based on Trimesters.

Control/ Trimester	Neutrophil (%)	Lymphocyte (%)	Absolute Neutrophil ($x10^3$/L)	Absolute Lymphocyte ($x10^3$/L)
Control[c] N = 160	60.57 ± 61.74	38.05 ± 39.40	3.13 ± 0.75	3.10 ± 0.76
First Trim N = 160	64.95 ± 66.17	33.83 ± 35.05	5.77 ± 1.49	2.36± 0.47
Second Trim N = 156	67.52 ± 68.17	31.15 ± 32.48	8.44 ± 1.66	2.09 ± 0.51
Third Trim N = 140	69.41 ± 70.69	29.25 ± 30.52	9.79 ± 1.89	1.85 ± 0.52
F (P – value)	148.5 (<0.001) *	136.4 (<0.001) *	781.4 (<0.001) *	181.5(<0.001)*
	Post Hoc			
C vs 1st	<0.001*	<0.001*	<0.001*	<0.001*
C vs 2nd	<0.001	<0.001*	<0.001*	<0.001*
C vs 3rd	<0.001*	<0.001*	<0.001*	<0.001*
1st vs 2nd	<0.001*	<0.001*	<0.001*	<0.001*
2nd vs 3rd	<0.001*	<0.001*	<0.001*	<0.001*
1st vs 3rd	<0.001*	<0.001*	<0.001*	<0.001*

Key: C = Control

* Mean difference is significant at $p < 0.05$.

Trim = Trimester

4.7: Levels of Differential White Cell Counts of Pregnant Women at Different Trimesters (Mean ± SD).

The mean level of neutrophil was significantly increased statistically from the first trimester (64.95 ± 66.17) to the third (69.41 ± 70.69) trimester ($F = 48.9$, $p < 0.001$). On the other hand, the lymplocyte was significantly reduced statistically from the first (33.83 ± 35.05) to the third (29.25 ± 30.52) trimester ($F = 50.4$, $p < 0.001$). Absolute neutrophil increased significantly from the first (5.77 ± 1.49) to the third (9.79 ± 1.89) ($F = 299.4$, $p < 0.001$), while absolute lymphocyte was significantly lowered statistically from the first (2.36 ± 0.47) to the third (1.85 ± 0.52) trimester ($F = 53.2$, $p < 0.001$).

Post Hoc Analysis

There was a statistically significant increase in neutrophil in the second (67.52 ± 6817) and third (69.41 ± 70.69) compared to the first (64.95 ± 66.17), and when the third trimester (69.41 ±70.69) compared to the second (67.52 ± 68.17) ($p < 0.001$). Lymphocyte was significantly lowered when the second (31.15 ± 32.48) and third (29.25 ± 30.52) trimesters were compared to the first (33.83 ± 35.05), and the third trimester (29.25 ± 30.52) compared to the second (31.15 ± 32.48) ($p < 0.001$)

Absolute neutrophil showed a statistically significant increase when the second (8.44 ± 1.66) and third (9.79 ± 1.89) trimesters were compared to the first (5.77 ± 1.49), and the third trimester (9.79 ± 1.89) compared to the second (8.44 ± 1.66) ($p < 0.001$). However, absolute lymphocyte was statistically reduced significantly when the second (2.09 ± 0.51) and third (1.85 ± 0.52) trimesters were compared to the first (2.36 ± 0.47) ($p <0.001$) (Table 4.7).

Table 4.7: Levels of Differential White Cell Counts of Pregnant Women at Different Trimesters (Mean ± SD).

Trimester	Neutrophil (%)	Lymphocyte (%)	Absolute Neutrophil ($x10^3$/L)	Absolute Lymphocyte ($x10^3$/L)
First Trim N = 160	64.95 ± 66.17	33.83 ± 35.05	5.77 ± 1.49	2.36± 0.47
Second Trim N = 156	67.52 ± 68.17	31.15 ± 32.48	8.44 ± 1.66	2.09 ± 0.51
Third Trim N = 140	69.41 ± 70.69	29.25 ± 30.52	9.79 ± 1.89	1.85 ± 0.52
F (p – value)	48.9 (<0.001) *	50.4 (<0.001) *	299.4 (<0.001) *	53.2 (<0.001) *
	Post Hoc			
1st vs 2nd	<0.001*	<0.001*	<0.001*	<0.001*
2nd vs 3rd	<0.001*	<0.001*	<0.001*	<0.001*
1st vs 3rd	<0.001*	<0.001*	<0.001*	<0.001*

Key:

* Significant at p< 0.05

Trim = Trimester

4.8: Mean values of Red Cell Indices of Non-pregnant Women compared to Pregnant Women based on Trimesters.

The mean levels of MCV in the first (79.66 ± 5.18), second (85.64 ± 5.18) and third (87.67 ± 54.33) showed no statistically significant difference compared to the controls (81.19 ± 4.56) (F=2.0, p = 0.120). That of MCH in the first (29.51 ± 17.01), second (28.69 ± 17.01), and third (28.43 ± 1.73) were not statistically decreased significantly compared to the controls (30.32 ± 1.65) (F=2.0, p = 0.110). However, a statistically significant decrease was observed when the MCHC in the first (34.56 ± 1.65), second (34.56 ± 1.65) and third (33.79 ± 1.65) trimesters were compared to the controls (35.92 ± 1.90) (F = 57.9, p < 0.001).

Post Hoc Analysis

There was no statistically significant difference, when the MCV in the first (79.66 ± 5.18), second (85.64 ± 5.18), and third (87.67 ± 54.33) trimesters were compared to the controls (81.19 ± 4.56) (p = 0.978, 0.644 and 0.319) respectively. No statistically significant increase was observed when the second trimester (85.64 ± 5.18) was compared to the first (79.66 ± 5.18) (p = 0.392), third (87.67 ± 54.33) compared to the second (85.64 ± 5.18) (p = 0.950), and third (87.67 ± 54.33) compared to the first trimester (79.66 ±5.18) (p = 0.149). MCH showed no statistically significant decrease when the first (29.51 ± 17.01), second (28.69 ± 1701) and third (28.43 ± 1.73) trimesters were compared to the controls (30.32 ± 1.65) (p = 0.777, 0.224 and 0.120). Similarly, no statistically significant decrease was observed when the second (28.69 ± 17.01) and third (28.43 ± 1.73) trimesters were compared to the first (29.51 ± 17.01) (p = 0.772 and 0.585), and when the third trimester (28.43±1.73) was compared to the second (28.69 ± 17.01) (p = 0.990). MCHC showed a statistically significant decrease when the first (34.56 ± 1.65), second (34.57 ± 1.39) and third (33.79 ± 1.65) trimesters were compared to the controls (35.92 ± 1.90) (p < 0.001). However, no statistically significant decrease was seen when the second trimester (34.57 ± 1.39) was compared to the first (34.56 ± 1.65) (p =1.000), but a significant decrease was observed when the third (33.79 ± 1.65) was compared to the second (34.57 ± 1.39) and first (34.56 ± 1.65) trimesters (p < 0.001).

Table 4.8: Mean values of Red Cell Indices of Non-pregnant Women compared to Pregnant Women based on Trimesters.

Control/Trimester	MCV (fl)	MCH (pg / cell)	MCHC (g/dl)
Control[c] N = 160	81.19 ± 4.56	30.32 ± 1.65	35.92 ± 1.90
First Trim N = 160	79.66 ± 5.18	29.51 ± 17.01	34.56 ± 1.65
Second Trim N = 156	85.64 ± 5.18	28.69 ± 17.01	34.57 ± 1.39
Third Trim N = 140	87.67 ± 54.33	28.43 ± 1.73	33.79 ± 1.65
F (P – value)	2.0 (0.120)	2.0 (0.110)	57.9 (<0.001) *
	Post Hoc		
C vs 1st	0.978	0.777	<0.001*
C vs 2nd	0.644	0.224	<0.001*
C vs 3rd	0.319	0.120	<0.001*
1st vs 2nd	0.392	0.772	1.000
2nd vs 3rd	0.950	0.990	<0.001*
1st vs 3rd	0.149	0.585	<0.001*

Key:

* Significant at p< 0.05

C = control

Trim = Trimester

4.9 : Levels of Red Cell Indices of Pregnant Women at Different Trimesters (Mean ± SD).

The mean values of MCV from the first (79.66 ± 5.18), second (85.64 ± 53.46), to the third (87.67 ± 54.33) trimester, showed no statistically significant increase (F=1.8, p = 0.163).
There was no statistically significant decrease in MCH from the first (29.51 ± 17.01), second (28.69 ± 1.80), to the third (28.43 ± 1.73) trimesters (F = 0.7, p = 0.517). However, MCHC showed a statistically significant decrease across the trimesters, first (34.56 ± 1.65) second (34.57 ± 1.39), and third (33.79 ± 1.65) (F=16.5, p < 0.001).

Post Hoc Analysis

The mean levels of MCV in the second (85.64 ± 53.46) and third (87.67 ± 54.33) trimesters showed no statistically significant increase compared to the first (79.66 ± 5.18) (p = 0.358 and 0.159), and when the third trimester (87.67 ± 54.33) was compared to the second (85.64 ± 53.46) (p = 0.887). MCH equally showed no statistically significant reduction when the second (28.69 ± 1.80), and third (28.43 ± 1.73) trimesters were compared to the first (29.51 ± 17.01) (p = 0.681 and 0.514). A non-statistically significant decrease was observed when the third trimester (28.43 ± 1.73) was compared to the second (28.69 ± 1.80) (p = 0.962) .There was no statistically significant decrease in MCHC in the second trimester (34.57 ± 1.39) compared to the first (34.56 ± 1.65) (p = 0.993). However, a statistically significant reduction was observed when the third trimester (33.79 ± 1.65) was compared to the first (34.56 ± 1.65) and second (34.57 ± 1.39) (p <0.001) (Table 4.9).

Table 4.9: Levels of Red Cell Indices of Pregnant Women at Different Trimesters (Mean ± SD).

Trimester	MCV (fl)	MCH (pg / cell)	MCHC (g/dl)
First Trim N = 160	79.66 ± 5.18	29.51 ± 17.01	34.56 ± 1.65
Second Trim N = 156	85.64 ± 53.46	28.69 ± 1.80	34.57 ± 1.39
Third Trim N = 140	87.67 ± 54.33	28.43 ± 1.73	33.79 ± 1.65
F (P – value)	1.8 (0.163)	0.7 (0.517)	16.5 (<0.001) *
	Post Hoc		
1st vs 2nd	0.358	0.681	0.993
2nd vs 3rd	0.887	0.962	<0.001*
1st vs 3rd	0.159	0.514	<0.001*

Key:

* Significant at p< 0.05

Trim = Trimester

4.10 : Mean Levels of Serum Ferritin and Serum Iron/TIBC of Non-Pregnant Women compared to Pregnant Women at different Trimesters.

The mean level of serum ferritin in the control subjects (39.67 ± 54.70) was significantly increased statistically when compared to the first (28.83 ± 19.39), second (23.76 ± 18.74) and third (20.45 ± 18.42) trimesters ($F = 14.2$, $p < 0.001$). Similarly, the serum iron mean levels in the controls (80.78 ± 23.19) was significantly increased compared to the first trimester (74.18 ± 23.92), second (67.77 ± 17.22), and third (61.23 ± 17.35) trimesters ($F = 33.7$, $p < 0.001$). However, the TIBC mean level in the controls (338.37 ± 57.69) was significantly increased statistically compared to the first (349.50 ± 52.69), second (364.67 ± 54.53), and third (374.40 ± 55.40) ($F = 17.1$, $p < 0.001$).

Post Hoc Analysis

Serum Ferritin in the first (28.83 ± 19.39), second (23.76 ± 18.74), and third (20.45 ± 18.42) trimesters showed a statistically significant decrease when compared to the control subjects (39.67 ± 54.70) ($p = 0.003$ and <0.001). A non-statistically significant decrease was seen when the second trimester (23.76 ± 18.74) was compared to the first (28.83±19.39) ($p = 0.375$) and the third trimester (20.45 ± 18.74) ($p = 0.720$), but a statistically significant decrease was observed when the third trimester (20.45 ± 18.42) was compared to the first (28.83 ± 19.39) ($p = 0.040$).

Serum iron showed a statistically significant decrease when the first (74.18 ± 23.92), second (67.77 ± 17.22), and third (61.23 ± 17.35) trimesters were compared to the control subjects, (80.78 ± 38.19) ($p = 0.07$ and <0.001). Similarly, a statistically significant reduction was observed when the second trimester (67.77 ± 17.22) was compared to the first (74.18 ± 23.92) ($p = 0.010$), and the third (61.23 ± 17.22) ($p = 0.008$), and when the third trimester (61.23 ±17.35) was compared to the first (74.18± 23.92) ($p <0.001$). However, TIBC in first trimester (349.50 ± 52.69) showed a statistically insignificant increase when compared to the controls (338.37 ± 57.68) ($p = 0.174$), but a statistically significant increase in the second (364.67 ± 54.53) and third (374.40 ± 55.40), compared to the controls (338.37 ± 57.68). A statistically significant increase was also observed when the second (364.67 ± 54.53) and third (374.40 ± 55.40) were compared to the first (349.50 ± 52.69) ($p = 0.028$ and <0.001). On the other hand, a non-statistically significant increase was seen when the third trimester (374.40 ± 55.40) was compared to the second (364.67 ± 54.53) ($p = 0.282$) (Table 4.10).

Table 4.10: Mean Levels of Serum Ferritin and Serum Iron/TIBC of Non-Pregnant Women compared to Pregnant Women at different Trimesters.

Control/Trimester	Serum Ferritin (ng/ml)	Serum Iron (ug/dl)	TIBC (ug/dl)
Control[c] N = 160	39.67 ± 54.70	80.78 ± 38.19	338.37 ± 57.68
First Trim N = 160	28.83 ± 19.39	74.18 ± 23.92	349.50 ± 52.69
Second Trim N = 156	23.76 ± 18.74	67.77 ± 17.22	364.67 ± 54.53
Third Trim N = 140	20.45 ± 18.42	61.23 ± 17.35	374.40 ± 55.40
F (p – value)	14.2 (<0.001) *	33.7(<0.001) *	17.1 (<0.001) *
	Post Hoc		
C vs 1st	0.003*	0.007*	0.174
C vs 2nd	<0.001*	<0.001*	<0.001*
C vs 3rd	<0.001*	<0.001*	<0.001*
1st vs 2nd	0.375	0.010*	0.028*
2nd vs 3rd	0.720	0.008*	0.282
1st vs 3rd	0.040*	<0.001*	<0.001*

Key: C = Control

* Significant at p< 0.05

Trim = Trimester

4.11: Levels of Serum Ferritin and Serum Iron/TIBC of Pregnant Women at Different Trimesters (Mean ± SD).

The mean value of serum ferritin was significantly decreased statistically from the first trimester (28.83 ± 19.39) to the third trimester (20.45 ± 18.42) ($F = 10.2$, $p < 0.001$). There was also a statisticallysignificant decrease in the mean levels of serum iron from the first trimester (74.18 ± 23.92) to the third (61.23 ± 17.35) ($F = 21.9$, $p < 0.001$). TIBC showed a significant increase from the first trimester (349.50 ± 52.69) to the third (374.40 ± 55.40) ($F = 10.0$, $p < 0.001$).

Post Hoc Analysis

Serum Ferritin in the second (23.76 ± 18.74) and third (20.45 ± 18.42) showed a statistically significant decrease when compared to the first (28.83 ± 19.39) ($p = 0.019$ and < 0.001)), while no statistically significant decrease was observed when the third (20.45 ± 18.42) trimester was compared to the second trimester (23.76 ± 18.74) ($p = 0.179$).

There was a statistically significant reduction in serum iron when the the second (67.77 ± 17.22) and third (61.23 ± 17.35) trimesters were compared to the first trimester (74.18 ± 23.92) ($p = 0.003$ and <0.001), and when the third (61.23 ± 17.35) was compared to the second (67.77 ± 17.22) ($p = 0.002$). TIBC showed a significant increase when the second (364.67 ± 54.53) and third (374.40 ± 55.40) trimesters were compared to the first (349.50 ± 52.69) ($p = 0.014$ and <0.001), and when the third (374.40 ± 55.40) trimester was compared to the second (364.67 ± 54.53) ($p = 0.166$) (Table 4.11).

Table 4.11: Mean Values of Serum Ferritin and Serum Iron/ TIBC of Pregnant Women at different Trimesters.

Trimester	Serum Ferritin (ng/ml)	Serum Iron (ug/dl)	TIBC (ug/dl)
First Trim N = 160	28.83 ± 19.39	74.18 ± 23.92	349.50 ± 52.69
Second Trim N = 156	23.76 ± 18.74	67.77 ± 17.22	364.67 ± 54.53
Third Trim N = 140	20.45 ± 18.42	61.23 ± 17.35	374.40 ± 55.40
F (p – value)	10.2 (<0.001) *	21.9(<0.001) *	10.9 (<0.001) *
	Post Hoc		
1st vs 2nd	0.019*	0.003*	0.014*
2nd vs 3rd	0.179	0.002*	0.166
1st vs 3rd	<0.001*	<0.001*	<0.001*

Key:

* Significant at P < 0.05.

4.12: Mean Levels of Coagulation Parameters of Non-pregnant and Pregnant Women based on Trimesters.

The mean levels of PT in the first (12.86 ± 1.29), second (11.74±1.41), and third (10.96 ± 1.50) were significantly shortened statistically compared to the control subjects (14.68 ± 1.07) ($F=300.9$, $p < 0.001$). Similarly, the mean values of APTT in the first (30.10 ± 4.49), second (29.33 ± 4.59), and third (28.33 ± 4.76) trimesters were significantly shortened statistically when compared to the controls (32.09 ± 4.72) ($F=28.33$, $p < 0.001$)

Post Hoc Analysis

PT in the first (12.86 ± 1.29), second (11.74 ± 1.41) and third (10.96 ± 1.50) trimesters were significantly shortened when compared to the controls (14.68 ± 1.07) ($p < 0.001$). There was a statistically significant shortening when the second (11.74 ± 1.41) and third (10.96 ± 1.50) trimesters were compared to the second (11.74 ± 1.41) ($p < 0.001$)

APTT also showed a statistically significant shortening when the first (30.10 ± 4.49) second (29.33 ± 4.54), and third (28.33 ± 4.76) trimesters were compared to the control subjects (32.09 ± 4.72) ($p < 0.001$). A non-statistically significant shortening was observed when the second trimester (29.33 ± 4.54) was compared to the first (30.10 ± 4.49) ($p = 0.338$), and when the third trimester (28.33 ± 4.76) was compared to the second (29.33 ± 4.76) ($p = 0.127$). However, the APTT was significantly shortened statistically when the third trimester (28.33 ± 4.76) was compared to the first (30.10 ± 4.49) ($p = 0.001$) (Table 4.12).

Table 4.12: Mean Levels of Coagulation Parameters of Non-pregnant and Pregnant Women based on Trimesters.

Control/Trimester	PT (secs)	APTT (secs)
Control[c] N = 160	14.68 ± 1.07	32.09 ± 4.72
First Trim N = 160	12.86 ± 1.29	30.10 ± 4.49
Second Trim N = 156	11.74 ± 1.41	29.33 ± 4.54
Third Trim N =140	10.96 ± 1.50	28.33 ± 4.76
F (p – value)	300.9 (<0.001) *	28.33(<0.001) *
	Post Hoc	
C vs 1st	<0.001*	<0.001*
C vs 2nd	<0.001*	<0.001*
C vs 3rd	<0.001*	<0.001*
1st vs 2nd	<0.001*	0.338
2nd vs 3rd	<0.001*	0.127
1st vs 3rd	<0.001*	0.001*

Key:

*Significant at p< 0.05

Trim = Trimester

4.13: Mean Levels of Coagulation Parameters of Pregnant Women at Different Trimesters.

The mean level of PT was significantly shortened statistically from the first trimester (12.86 ± 1.29) to the third trimester (10.96 ± 1.50) ($F = 93.4$, $p < 0.001$). There was also a statistically significant shortening in APTT from the first trimester (30.10 ± 4.49) to the third (28.33 ± 4.76) ($F = 7.6$, $p = 0.001$).

Post Hoc Analysis

The mean level of PT was significantly shortened statistically from the first (12.86 ±1.29) to the third trimester (10.96 ± 1.50) ($F = 93.4$, $p < 0.001$).There was also a statistically significant shortening in APTT from the first (30.10 ± 4.49) to the third (28.33 ± 4.76) trimester ($F=7.6$, $p = 0.001$).

PT in the second (11.74 ± 1.41) and third (10.96 ± 1.50) trimesters showed a statistically significant shortening when compared to the first (12.86 ± 1.29) ($p < 0.001$). Also that in the third trimester (10.96 ± 1.50) showed a statistically significant shortening when compared to the second (11.74 ± 1.41) ($p < 0.001$). There was a statistically significant shortening in APTT when the third trimester (28.33 ± 4.49) was compared to the first (30.10 ± 4.49) ($p < 0.001$). However, APTT showed an insignificant shortening when the second trimester (29.33 ± 4.54) was compared to the first (30.10 ± 4.49) ($p = 0.212$), and when the third (28.33 ± 4.76) was compared to the second trimester (29.33 ± 4.54 ($P=0.071$) (Table 4.13).

Table 4.13: Mean Levels of Coagulation Parameters of Pregnant Women at Different Trimesters.

Trimester	PT (secs)	APTT (secs)
First Trim N = 160	12.86 ± 1.29	30.10 ± 4.49
Second Trim N = 156	11.74 ± 1.41	29.33 ± 4.54
Third Trim N = 140	10.96 ±1.50	28.33 ± 4.76
F (p – value)	93.4(<0.001) *	7.6(0.001) *
	Post Hoc	
1st vs 2nd	<0.001*	0.212
2nd vs 3rd	<0.001*	0.071
1st vs 3rd	<0.001*	<0.001*

Key:

* Significant at $p < 0.05$

Trim = Trimester

4.14: Correlation of Immunological Parameters with Haematological Parameters at First Trimester of Pregnancy.

In the first trimester of normal pregnancy, the $CD4^+$ cell count showed a statistically significant negative correlation with Hb (r = -0.141; p = 0.045), a non-significant negative correlation with PCV (r = -0.028, p = 0.693), WBC (r = -0.013, p = 0.856), platelet (r = 0.121, p = 0.084), Neutrophil (r = -0.035, p = 0.621), absolute lymphocyte (r = 0.012, p = 0.865), MCV (r = - 0.004, p = 0.958), MCHC (r = -0.093, p = 0.187), serum ferritin (r = -0.016, p = 0.821, TIBC (r = - 0.079, p = 0.264), PT (r = - 0.87, p = 0.218) and APTT (r = - 0.017, p = 0.808), but a non-significant positive correlation with RBC (r = 0.101, p = 0.153), lymphocyte (r = 0.033, p = 0.635), absolute Neutrophil (r = 0.023, p = 0.742), MCH (r = 0.006, p = 0.928), and serum iron (r = 0.056, p= 0.430). (Table 4.14).

Table 4.14 : Correlation of Immunological Parameters with Haematological Parameters at First Trimester of Pregnancy.

Variables	CD4+ (Cells/ µl)	IL-2 (pg/l)	TNF-α (ng/l)	IL-4 (pg/ml)	IL-10 (pg/ml)
PCV (%) r(P-value)	- 0.028(0.693)	0.110(0.117)	-0.051(0.465)	0.042(0.549)	-0.018(0.081)
Hb(g/dl) r(p-value)	-0.141(0.045)*	0.108(0.123)	-0.073(0.300)	0.014(0.842)	0.001(0.991)
WBC ($\times 10^9$/L) r(p-value)	-0.013(0.856)	-0.082(0.2430)	- 0.113(0.107)	0.162(0.021)*	0.074(0.295)
RBC $\times 10^{12}$ /L) r(p-value)	0.101(0.084)	-0.092(0.091)	-0.117(0.096)	0.067(0.339)	0.074(0.290)
Platelets(x10^9/L) r(p-value)	-0.121(0.084)	0.030(0.670)	0.038(0.592)	-0.068(0.331)	-0.018(0.083)
Neutrophil(%) r(p-value)	-0.035(0.0621)	0.012(0.865)	-0.042(0.554)	- 0.059(0.405)	0.004(0.956)
Lymphocyte(%) r(p-value)	0.033(0.635)	0.005(0.944)	0.045(0.521)	0.060(0.395)	-0..001(0.987)
Abs Neutrophil (x10^3/L) r(p-value)	0.023(0.742)	0.018(0.797)	0.117(0.094)	-0.041(0.560)	0.009(0.987)
Abs Lymphocyte(x10^3/L) r(p-value)	-0.012 (0.865)	-0.068(0.334)	0.055(0.435)	0.066(0.345)	-0.004(0.950)

Key:

* = Correlation is significant at 0.05 level.

Abs = Absolute

4.15: Correlation of Immunological Parameters with Red Cell Indices, Serum Ferritin, Iron/TIBC and Coagulation Parameters at First Trimester of Pregnancy

CD4+ cell count showed an insignificant inverse relationship with MCV (r = - 0.004, P = 0.958), MCHC (r = - 0.093, p = 0.187), Feritin (r = - 0.016, p = 0.821), TIBC (r = - 0.079, p = 0.264), PT (r = - 0.087, p = 0.218) and APTT (r = - 0.017, p = 0.808), but an insignificant positive correlation with MCH (r = 0.006, p = 0.928) and Iron (r = 0.056, p = 0.430). IL – 2 showed a non-significant positive correlation with MCV (r = 0.073, p = 0.302), MCHC (r = 0.092, p = 0.188) and PT (r = 0.018, p = 0.803), but an insignificant negative relationship with MCH (r = - 0.047, p = 0.508), Ferritin (r = - 0.067, p = 0.338), Iron (r = - 0.083, p = 0.238), TIBC (r = - 0.068, p = 0.337) and a significant negative relationship with APTT (r = - 0.139, p = 0.047). There was a non-significant positive correlation between TNF – α and MCV(r = 0.000, p = 0.996), MCHC (r = 0.014, p = 0.838), Ferritin (r = 0.002, p = 0.974), TIBC (r = 0.010, p = 0.889) and APTT (r = 0.012, p = 0.863), but an insignificant negative correlation with MCH (r = - 0.046, p = 0.514) and, Iron (r = - 0.186, p = 0.008), and a significant inverse relationship with PT (r = - 0.152, p = 0.030).

IL – 4 showed a non-significant inverse relationship with MCV (r = - 0.052, p = 0.461) MCH (r = - 0.067, p = 0.339), MCHC (r = - 0.062, p = 0.382, Ferritin (r = - 0.093, p = 0.187) and PT (r = - 0.081, p = 0.248), an insignificant positive correlation with Iron (r = 0.078, p= 0.265) and APTT (r = 0.016, p = 0.815), but a significant negative correlation with TIBC (r = - 0.141, p = 0.044). IL – 10 showed an insignificant positive correlation with MCV (r = 0.021, p = 0.769, MCH (r = 0.011, p = 0.875), MCHC (r = 0.129, p = 0.065) and PT (r = 0.090, p = 0.201), a non significant negative relationship with ferritin (r = - 0.050, p = 0.480) and TIBC (r = - 0.027, p = 0.704), but a significant positive correlation with Iron (r = 0.178, p = 0.011) and a significant inverse relationship with APTT (r = - 0.146, p = 0.037).

Table 4:15: Correlation of Immunological Parameters with Red Cell Indices, Serum Ferritin, Iron/TIBC and Coagulation Parameters at First Trimester.

Variables	CD4+ (Cells/ul)	IL-2 (pg/ml)	TNF-α (ng/l)	IL-4 (pg/ml)	IL-10 (pg/ml)
MCV(fl) r(p-value	-0.004(0.958)	0.073(0.302)	0.000(0.996)	-0.052(0.461)	0.021(0.769)
MCH(pg) r(p-value)	0.006(0.928)	-0.047(0.508)	-0.046(0.514)	-0.067(0.339)	0.011(0.875)
MCHC (g/dl) r(p-value)	-0.093(0.187)	0.092(0.188)	0.014(0.838)	-0.062(0.382)	0.129(0.065)
Ferritin (ng/ml)r(p-value)	-0.016(0.821)	-0.067(0.338)	0.002(0.974)	-0.093(0.187)	-0.050(0.480)
Iron(µg/dl) r(p-value)	0.056(0.430)	-0.083(0.238)	-0.186(0.008)	0.078(0.265)	-0.178(0.011)*
TIBC (µg/dl) r(p-value)	-0.079(0.264)	-0.068(0.337)	0.010(0.889)	-0.141(0.044)*	-0.027(0.704)
PT(secs) r(p-value)	-0.087(0.218)	0.018(0.803)	-0.152(0.030)*	-0.081(0.248)	0.090(0.201)
APTT(secs) r(p-value)	-0.017(0.808)	-0.139(0.047)*	0.012(0.863)	0.016(0.815)	-0.146(0.037)*

Key : * = Correlation is significant at 0. 05 level.

4.16 : Correlation of Immunological Parameters with Hematological Parameters in Pregnancy at Second Trimester.

The CD4+ cell count showed a non-significant inverse relationship with PCV (r = - 0.062, p = 0.380), WBC (r = -0.103, p = 0.144) platelet (r = - 0.128, p = 0.068), Neutrophil (r = -.0102, p = 0.148) and an insignificant positive association with Hb (r = 0.015, p = 0.832), RBC (r = 0.037, p = 0.579), Lymphocyte (r = 0.102, p = 0.148), absolute Neutrophil (r = 0.073, p = 0.301) and absolute lymphocyte (r = 0.105, p = 0.134). There was an insignificant positive association between IL – 2 and PCV (r = 0.044, p = 0.530), Hb (r = 0.081, p = 0.247), platelet (r = 0.011, p = 0.878), lymphocyte (r = 0.041, p = 0.564), but an insignificant inverse correlation with WBC (r = - 0.046, p = 0.518), RBC (r = - 0.091, p = 0.198), Neutrophil (r = - 0.041, p = 0.564), absolute neutrophil (r = - 0.028, p = 0.695) and absolute lymphocyte (r = - 0.084, p = 0.232). TNF - α showed a non-significant positive relationship with PCV (r = 0.109, p = 0.120), Hb (r = 0.077, p = 0.276), lymphocyte (r = 0.028, p = 0.692), absolute Neutrophil (r = 0.038, p = 0.585) and absolute lymphocyte (r = 0.120, p = 0.088), but an insignificant negative correlation with WBC (r = - 0.089, p = 0.207), RBC (r = - 0.015, p = 0.828), platelet (r = - 0.026, p = 0.708) and Neutrophil (r = - 0.028, p = 0.629). There was a significant negative association between IL – 4 and PCV (r = - 0.183, p = 0.009), a non-significant positive correlation with Hb (r = 0.063, p = 0.367), WBC (r = 0.109, p = 0.122) and absolute lymphocyte (r = 0.065, p = 0.354), an insignificant negative correlation with platelet (r = - 0.076, p = 0.281), Neutrophil (r = - 0.067, p = 0.341), lymphocyte (r = - 0.014, p = 0.842) and absolute Neutrophil (r = - 0.057, p = 0.418), but a significant positive relationship with RBC (r = 0.173, p = 0.014). A non significant inverse correlation was observed between IL – 10 and PCV (r = - 0.035, p = 0.621), Hb (r = - 0.026, p = 0.708), Platelet (r = - 0.043, p = 0.542), lymphocyte (r = - 0.068, p = 0.330) and absolute lymphocyte (r = - 0.009, p = 0.900), but an insignificant positive correlation with WBC (r = 0.081, p = 0.248), RBC (r = 0.039, p = 0.578), Neutrophil (r = 0.068, p = 0.330) and absolute Neutrophil (r = 0.126, p = 0.073).

Table 416: Correlation of Immunological Parameters with Haematological Parameters in Pregnancy at Second Trimester.

Variables	CD4+ (Cells/ul)	IL-2 (pg/ml)	TNF-α (ng/l)	IL-4 (pg/ml)	IL-10 (pg/ml)
PCV(%) r(p-value)	-0.062(0.380)	0.044(0.350)	0.109(0.120)	-0.183(0.009)	-0.035(0.621)
Hb (g/dl) r(p-value)	0.015(0.832)	0.081(0.247)	0.077(0.276)	0.063(0. 367)	-0.026(0.708)
WBC ($x10^9$/ r(p-value)	-0.103(0.144)	-0.046(0.518)	-0.089(0.207)	-0.109(0.122)	0.081(0.248)
RBC($x10^{12}$/l) r(p-value)	0.039(0.579)	-0.091(0.198)	-0.015(0.828)	0.173(0.014)*	0.039(0.578)
Platelet ($x10^9$/L) r(p-value)	-0.128(0.068)	0.011(0.878)	-0.026(0.708)	-0.076(0. 281)	-0.043(0.542)
Neutrophil(% r(p-value)	-0.102(0.148)	-0.041(0.564)	-0.028(0.692)	-0.069(0. 341)	0.068(0.330)
Lymphocyte(%) r(p-value)	0.102(0.148)	0.041(0.564)	0.028(0.692)	-0.014(0.842)	-0.068(0.330)
Abs Neutrophil r(p –value)	0.073(0.301)	-0.028(0.695)	0.038(0.585)	-0.057(0.418)	0.126(0.073)
Abs Lymphocyte r(p-value)	0.105(0.134)	-0.084(0.232)	0.120(0.088)	0.065(0.354)	-0.009(0.900)

Key: * = Correlation is significant at 0.05 level.

Abs = Absolute

4.17 : Correlation of Immunological Parameters with Red Cell Indices, Serum Ferritin, Iron/TIBC and Coagulation Parameters at Second Trimester.

At the second trimester, CD4+ cell count showed a non-significant negative correlation with MCV (r = - 0.079, p = 0.261), MCHC (r = - 0.130, p = 0.065), an significant positive relationship with ferritin (r = 0.035, p = 0.616), Iron (r = 0.041, p = 0.556), TIBC (r = 0.028, p = 0.692), and APTT (r = 0.020, p = 0.776), but a significant negative association with MCH (r = - 0.149, p = 0.034) and PT (r = - 0.148, p = 0.034). There was a non-significant positive relationship between IL – 2 and MCV (r = 0.035, p = 0.619), MCH (r = 0.024, p = 0.732), MCHC (r = 0.115, p = 0.102), TIBC (r = 0.111, p = 0.113), PT (r = - 0.036, p = 0.607), but an insignificant inverse correlation with Ferritin (r = - 0.090, p = 0.200), Iron (r = - 0.018, p = 0.800) and APTT (r = - 0.115, p = 0.103), TNF - α showed a non-significant positive relationship with MCV (r = 0.041, p = 0.561), MCH (r = 0.070, p = 0.319), MCHC (r = 0.005, p = 0.941), Ferritin (r = 0.087, p = 0.214), TIBC (r = 0.044, p = 0.528) but an insignificant inverse association with Iron (r = - 0.131, p = 0.061),PT (r = -0.046, p = 0.511), and APTT (r = - 0.029, p = 0.683). IL-4 showed an insignificant positive correlation with MCV (r = 0.062, p = 0.379), Iron (r = 0.066, p = 0.351) and APTT (r = 0.005, p = 0.940), but a non-significant inverse association with MCH (r = - 0.085, p = 0.229), MCHC (r = - 0.021, p = 0.765), Ferritin (r = - 0.084, P = 0.234), TIBC (r = - 0.091, p = 0.195) and PT (r = - 0.96, p = 0.174). An insignificant positive association was seen between IL-10 and MCV (r = 0.000, p = 0.995), MCH (r = 0.060, p = 0.394), MCHC (r = 0.120, p = 0.087), TIBC (r = 0.000, p = 0.994), and PT (r = 0.100, P = 0.156), but a non-significant negative relationship with Ferritin (r = - 0.028, p = 0.696), a significant positive association with Iron (r = 0.148, p = 0.035), and a significant inverse correlation with APTT (r = -0.199, p = 0.004).

MCV (r = - 0.005, p = 0.946), Ferritin (r = -0.009, p = 0. 896), TIBC (r = -0.042, p = 0.549), a non significant positive relationship with MCH (r = 0.078, p = 267), PT (r = 0.079, p = 0.259 and a significant positive association with MCHC (r = 0.150, p = 0.032) and serum iron (r = 0. 168, p = 0.016), but a significant inverse correlation with APTT (r = - 0.190, p = 0.007).

Table 4.17 : Correlation of Immunological Parameters with Red Cell Indices, Serum Ferritin, Iron/TIBC and Coagulation Parameters at Second Trimester.

Variables	CD4+ (Cells/ul)	IL-2 (pg/ml)	TNF-α (ng/l)	IL-4 (pg/ml)	IL-10 (pg/ml)
MCV(gl) r(p-value)	-0.079(0.261)	0.035(0.619)	0.041(0.561)	0.062(0.379)	0.000(0.995)
MCH(pg) r(P-value)	-0.149(0.034)*	0.024(0.732)	0.070(0.319)	-0.085(0.229)	0.060(0.394)
MCHC (g/dl) r(P-value)	-0.130(0.065)	0.115(0.102)	0.005(0.941)	-0.021(0.765)	0.120(0.087)
Ferritin(ng/ml r(p-value)	0.035(0.616)	-0.090(0.200)	0.087(0.214)	-0.084(0.234)	-0.028(0.696)
Iron(µg/dl) r(p-value)	0.041(0.556)	-0.018(0.800)	-0.131(0.061)	0.066(0.351)	0.148(0.035)*
TIBC(µg/dl) r(P-value)	0.028(0.692)	0.111(0.113)	0.044(0.528)	-0.091(0.195)	-0.000(0.994)
PT(secs) r(p-value)	-0.148(0.034)*	0.036(0.607)	-0.046(0.511)	-0.096(0.174)	0.100(0.156)
APTT(secs) r(p-value)	0.020(0.776)	-0.115(0.103)	-0.029(0.683)	0.005(0.940)	-0.199(0.004)**

Key: * Correlation is significant at 0.05 level.
** Correlation is significant at 0.01 level.

4.18 : Correlation of Immunological Parameters with Haematological Parameters at Third Trimester of Pregnancy.

The CD4+ cell count in the third trimester was insignificantly correlated positively with the PCV (r = 0.023, p = 0.740), lymphocyte (r = 0.134, p = 0.057), absolute Neutrophil (r = 0.009, p = 0.897), but had a non-significant negative relationship with Hb (r = - 0.078, p = 0.270), WBC (r = - 0.028, p = 0.695), RBC (r = - 0.024, p = 0.731), Platelet (r = - 0.115, p = 0.101), Neutrophil (r = - 0.131, p = 0.062) and absolute lymphocyte (r = - 0.058, p = 0.411). IL – 2 showed a non-significant inverse relationship with PCV (r = - 0.044, p = 0.531), Hb (r = - 0.012, p = 0.868), WBC (r = - 0.035, p = 0.618), RBC (r = - 0.024, p = 0.731), Neutrophil (r = - 0.046, p = 0.514) and absolute lymphocyte (r = - 0.061, p = 0.386), but had a non-significant positive correlation with platelet (r = 0.067, p = 343), Lymphocyte (r = 0.039, p = 0.584), and absolute Neutrophil (r = 0.104, p = 0.140). There was an insignificant inverse correlation between TNF-α and PCV (r = - 0.028, p = 0.695), Neutrophil (r = - 0.019, P = 0.787), a non-significant positive correlation with Hb (r = 0.065, p = 0.356), Platelet (r = 0.035, p = 0.615), Lymphocyte (r = 0.020, p = 0.777), absolute Neutrophil (r = 0.055, p = 0.433) and absolute lymphocyte (r = 0.121, p = 0.084), but showed a significant negative association with WBC (r = -0.137, p = 0.050), and RBC (r = - 0.138, p = 0.049). IL – 4 showed an insignificant inverse relationship with PCV (r = - 0.092, p = 0.189), Hb (r = - 0.018, p = 0.797), RBC (r = - 0.012, p = 0.869), Platelet (r = -0.131, p = 0.062), Neutrophil (r = - 0.027, p = 0.703), absolute Neutrophil (r = - 0.030, p = 0.667), but a non-significant positive correlation with WBC (r = 0.111, p = 0.113), lymphocyte (r = 0.030, p = 0.675) and absolute lymphocyte (r = 0.059, p = 0.399). There was a non-significant positive correlation between IL – 10 and PCV (r = 0.028, p = 0.644), WBC (r = 0.030, p = 0.675), RBC (r = 0.037, p = 0.600), Platelet (r = 0.003, p = 0.968), Neutrophil (r = 0.65, p = 0.355), absolute Neutrophil (r = 0.079, p = 0.264), but an insignificant negative association with Hb (r = - 0.031, p = 0.656), lymphocyte (r = - 0.059, p = 0.404) and absolute lymphocyte (r = - 0.018, p = 0.803).

Table 4.18: Correlation of Immunological Parameters with Haematological Parameters at Third Trimester of pregnancy.

Variables (pg/ml)	CD4+ (Cells/ul)	IL-2 (pg/ml)	TNF-α (ng/l)	IL-4 (pg/ml)	IL-10 (pg/ml)
PCV(%) r(p-value)	0.023(0.740)	-0.044(0.351)	-0.028(0.695)	-0.092(0.189)	0.028(0.644)
Hb(g/dl) r(p-value)	-0.078(0.270)	-0.012(0.868)	0.065(0.356)	-0.018(0.797)	-0.031(0.656)
WBC ($x10^9$/L) r(p-value)	-0.028(0.695)	-0.035(0.618)	-0.137(0.050)*	0.111(0.113)	0.030(0.675)
RBC($x10^{12}$/l) r(p-value)	-0.024(0.731)	-0.024(0.731)	-0.138(0.049)*	-0.012(0.869)	0.037(0.600)
Platelet ($x10^9$/L) r(p-value)	-0.115(0.101)	0.067(0.343)	0.035(0.615)	-0.131(0.062)	0.003(0.968)
Neutrophil (%) r(p-value)	-0.131(0.062)	-0.046(0.514)	-0.019(0.787)	-0.027(0.703)	0.065(0.355)
Lymphocyte(%) r(p-value)	0.134(0.057)	0.039(0.584)	0.020(0.777)	0.030(0.675)	-0.059(0.404)
Abs Neutrophil r(p –value)	0.009(0.897)	0.104(0.140)	0.055(0.433)	-0.030(0.667)	0.079(0.264)
Abs Lymphocyte r(p-value)	-0.058(0.411)	-0.061(0.386)	0.121(0.084)	0.059(0.399)	-0.018(0.803)

Key : * = Correlation is significant at 0.05 level.

Abs = Absolute

4 .19: Correlation of Immunological Parameters with Red Cell Indices, Serum Ferritin, Iron /TIBC and Coagulation Parameters at Third Trimester.

At the third trimester, CD4+ cell count showed an insignificant positive correlation with MCV (r = 0.058, p = 0.408), MCHC (r =0.036, p = 0.612), Ferritin (r = 0 .030, p = 0.665), Iron (r = 0.029, p = 0.676) TIBC (r = 0.042, p = 0.554) and APTT (r = 0.020, p = 0.775), but a significant negative relationship with MCH (r = 0.157, p = 0.025) and PT (r = - 0.148, p = 0.034). IL -2 showed an insignificant negative correlation with MCV (r = - 0.008, p = 0.909), MCH (r = - 0.049, p = 0. 484), MCHC (r = -0.709, p = 0.263), Ferritin (r = - 0.105, p = 0.135), Iron (r = - 0.054, p = 0.441) and APTT (r = - 0. 135, p = 0. 056), but a non - significant positive relationship with TIBC (r =0.088, p = 0.208) and PT (r = 0.047, p = 0.508) There was an insignificant positive correlation between TNF-α and MCV (r = 0. 002, p = 0.978), MCH (r = 0. 016, p = 0.819), MCHC (r = 0.091, 0.194), Ferritin (r = 0.101, p = 0.150), TIBC (r = 0.021, p = 0.770), a non- significant inverse relationship with PT (r = - 0. 016, p = 0.821) and APTT (r = - 0. 028, p = 0. 686), but a significant negative correlation with Iron (r = - 0.181, p = 0. 009). IL – 4 showed an insignificant positive correlation with MCV (r = 0.063, p = 0.370), Iron (r = 0. 052, p = 0.459) and APTT (r = 0.012, p = 0 .870), but an insignificant inverse correlation with MCH (r = -0.056, p =0.426), MCHC (r = -0.105, p = 0.134), Ferritin (r = - 0.113, p =1.109), TIBC (r = - 0. 092, p = 0.193) and PT (r = - 0.073, p = 0.302). There was a non -significant inverse association between IL – 10 and MCV(r = -0.005, p = 0.946), Ferritin (r = -0.009, p = 0.896), TIBC (r = -0.042, p = 0.549), a non- significant positive relationship with MCH (r = 0.078, p = 0.267), PT (r = 0.079, p = 0.259) and a significant positive association with MCHC (r = 0.150, p = 0.032), and serum iron (r = 0.168, p = 0.016), but a significant inverse correlation with APTT (r = - 0.190, p = 0.007).

Table 4.19: Correlation of Immunological Parameters with Red Cell Indices, Serum Ferritin, Iron/TIBC and Coagulation Parameters at Third Trimester.

Variables	CD4+ (Cells/ul)	IL-2 (pg/ml)	TNF-α (ng/l)	IL-4 (pg/ml)	IL-10 (pg/ml)
MCV(fl) r(p-value)	0.058(0.408)	-0.008(0.909)	0.002(0.978)	0.063(0.370)	-0.005(0.946)
MCH(pg) r(p-value)	-0.157(0.025)*	0.049(0.484)	0.016(0.819)	-0.056(0.426)	0.078(0.267)
MCHC (g/dl) r(p-value)	-0.036(0.612)	-0.079(0.263)	0.091(0.194)	-0.105(0.134)	0.150(0.032)*
Ferritin (ng/ml) r(p-value)	0.030(0.665)	-0.105(0.135)	0.101(0.150)	-0.113(0.109)	-0.009(0.896)
Iron(µg/dl) r(p-value)	0.029(0.676)	-0.054(0.441)	-0.181(0.009)**	0.052(0.459)	0.168(0.016)
TIBC(µg/dl) r(p-value)	0.042(0.554)	0.088(0.208)	0.021(0.770)	-0.092(0.193)	-0.042(0.549)
PT(secs) r(p-value)	-0.148(0.034)*	0.047(0.508)	-0.016(0.821)	-0.073(0.302)	0.079(0.259)
APTT(secs) r(p-value)	0.020(0.775)	-0.134(0.056)	-0.028(0.686)	0.012(0.870)	-0.190(0.007)**

Key: * Correlation is significant at 0.05 level.

** Correlation is significant at 0.01 level.

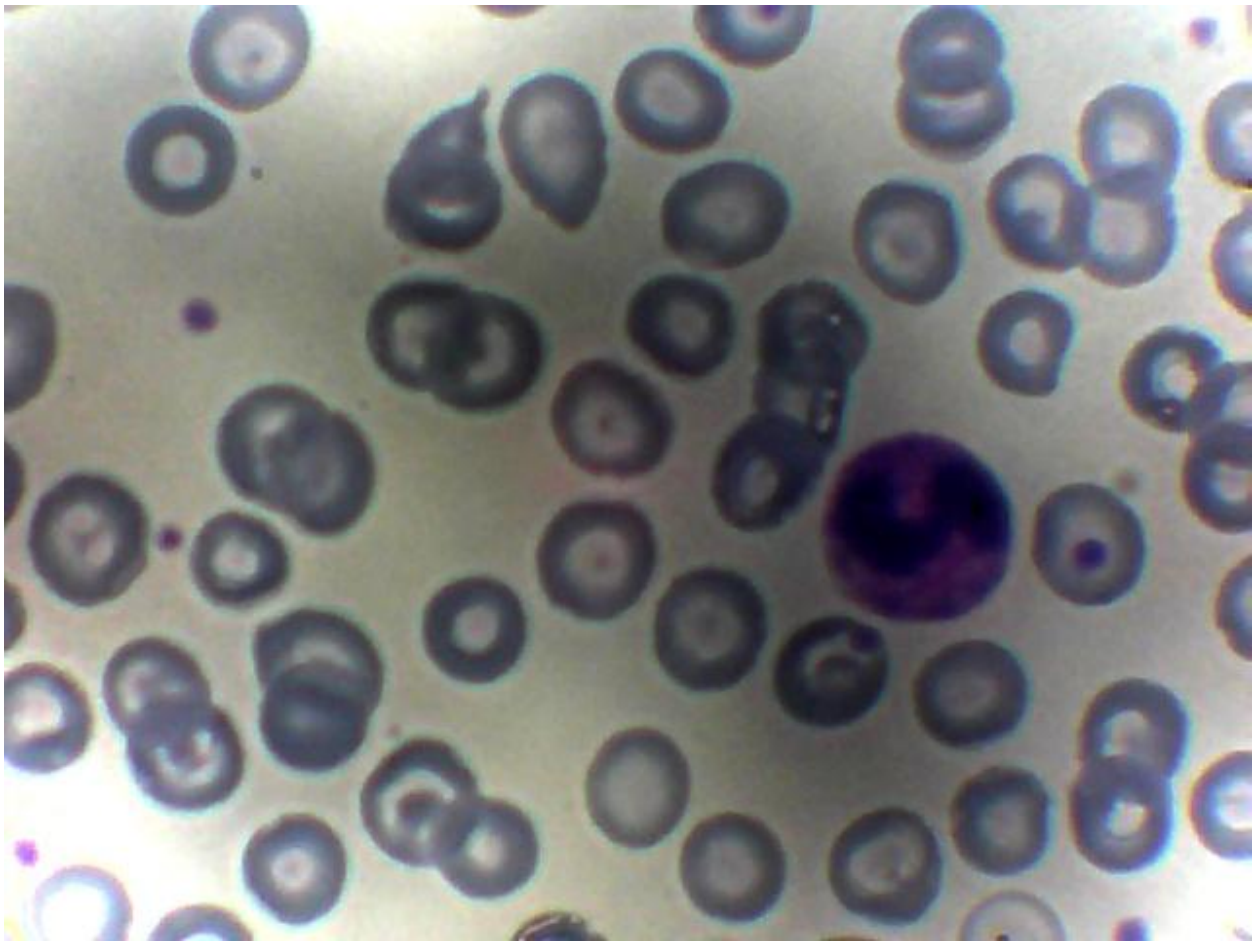


Fig 4: Photomicrograph of blood film of pregnant women with severe iron deficiency, showing anisocytosis, hypochromasia, target cells, tear drop cells and microcytes. X100 magnification.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

In this study, 61.25% of the pregnant women studied were primigravidas while 38.75% were multigravidas. Primigravidas were more in number probably because they were first timers, anxious and had not visited antenatal clinic before. Majority (45.00%) of the women were civil servants/private employees, followed by traders (30.00%). Most women of these days are hardworking and want to assist their husbands financially owing to the economic hardship in the country despite their pregnancy condition. 18.13% were housewives, may be because they were asked to stay at home and nurture the kids, while 6.87% were students, who still want to further their education to make their lives better. More pregnant women (65.00%) had tertiary education, showing that women now value education more than before, 33.75% had secondary education, while a minute fraction, 1.25.% attained primary education.

This study revealed that, the mean CD4+ cell count was significantly lower at all trimesters compared to the non – pregnant women. This could be probably due to the fact that during pregnancy, the immune system is compromised. Changes in the hormonal environment may play a role in the suppression of maternal immune response. It agrees with the study done by Aina *et al*., (2005) and Ufelle *et al*., (2017) who reported a lower mean CD4+ count in pregnancy compared with non – pregnant women.

The decrease in CD4+ cell count in pregnancy may be due to the fact that pregnancy is an immunocompromised state which alters T - Lymphocytes subsets (Tanjong *et al*., 2012). In pregnancy, immune function is suppressed and the state of pregnancy represents an extreme challenge for the immune system. The maternal immune system during pregnancy is altered in order to tolerate the semi – allogenic fetus (Chen *et al.,* 2012). This study also showed that the CD4+ cell count progressively declined from first to third trimesters insignificantly.This decrease might be because of the increasing physiological demand associated with pregnancy. It agrees with the work done by Ufelle *et al.,* 2017, who stated that CD4+ cell count was

significantly decreased, when compared to non – pregnant females and decreases as the gestational age increases, but disagrees with the work of Akinbami, (2014), who reported an insignificant association between CD4+count and gestational age. He stated that there was a slight variation in CD4+ cell count by trimester – the highest in first trimester, then third and lastly the second.The progressive decrease in $CD4^+$ cell count at different gestational ages could be attributed to increasing physiological demand during pregnancy and the changes in the hormonal environment of pregnancy contribute to local suppression of cell mediated immunity at the maternal fetal interface (Bakalor *et al.*, 2001).

Gomo *et al.*, 2004, also reported that no relationship exists between gestational age and CD4+ cell count in HIV negative women. There was a significant negative correlation between CD4+ count and Hb at the first trimester. CD4+ count showed a significant inverse relationship with MCH and PT at thesecond and third trimesters.

In this study, the levels of four different cytokines during physiologic pregnancy were determined. IL – 4 was insignificantly in the first and second trimesters, but significantly increased in third trimester compared to the non – pregnant controls. This coud be due to the fact that during pregnancy, levels of Th2 cytokire increase in order to enhance the proliferation and differentiation of the cells of the trophoblast and protect the fetal placental unit. Their presence might also inhibit the production of Th1 cytokines to prevent pregnancy complications.This agrees with the work done by Marzi *et al.*, (1996), who showed that IL – 4 production is increased in the gravid state and that a successful pregnancy is characterized by increased production of Th2 cytokines e.g. IL – 4. Jones *et al.*, (2000), also stated that normal pregnancy is accompanied by an increased production of type 2 anti - inflammatory cytokines. The insignificant increase in the first stage of pregnancy may be because the first stage is not predominantly an anti – inflammatory phase, while the third phase is anti – inflammatory, and a shift would lead to pregnancy complications. IL – 4 being a Th2 cytokine, is beneficial for pregnancy, promoting proliferation and differentiation of the trophoblastic cells and placentation. In addition, it has a protective role on the fetus placental unit, inhibiting the production of Th1 cytokine. Hence successful pregnancy is associated with preferential development of the Th2 profile. The increase in the production of hormones e.g. progesterone during pregnancy is also part of it. Progesterone is a known inducer of IL – 4. It enhances IL – 4 production by human T – cells and together they act to inhibit Th1 responses during pregnancy (Marzi *et al.*, 1996 and

Jones *et al.,* 2000). IL – 4 being a Th2 – type cytokine, is produced by the deciduas and are required for embryo implantation and development. Hence, the increase in pregnancy more than non - pregnancy state.

This study showed a progressive increase in IL – 4 from the first to the third trimester. This could be as a result of increased hormonal production especially progesterone as pregnancy progresses, and also in order to suppress the production of Th1 cytokines, of which their increased production leads to pregnancy failure.This is similar to the work done by Marzi *et al.,* 1996 and Ekerfelt *et al.,* 1997 who stated that IL – 4 showed a constant presence at the first two trimester and the highest quantities were observed in the third trimester, when the concentration of progesterone is at its highest. The second phase of pregnancy is an anti – inflammatory state, so IL – 4 is increased. This stage is a period of rapid fetal growth and development. The mother, placenta, and fetus are symbiotic, and the predominant immunological feature is induction of an anti – inflammatory state. There was a significant positive correlation between IL- 4 and WBC, and a significant negative correlation with TIBC in the first trimester. A significant negative correlation was also observed with PCV and a significant positive association with RBC in the second trimester.

IL – 10 being an anti –inflammatory cytokine or a Th2 – type cytokine was insignificantly high in the first trimester, but significantly increased in the second and third trimesters compared to their non – pregnancy counterpart. This may be probably because, IL – 10 being a pleomorphic cytokine, regulates the balance between Th1 and Th2 cytokines and works together with IL – 4 to maintain a successful pregnancy.This agrees with the study carried out by Chatterjee *et al.,* (2014), who showed that IL – 10 production increased in pregnancy than in non – pregnant state. Abdolreza *et al.,* (2011) also stated that during pregnancy IL – 10 levels were increased than in non pregnancy state. Increase in IL – 10 in pregnant women was also observed by Holmes *et al.,* 2003. There was increase in IL – 10 levels from first to the third trimester, and this also agrees with Abdoreza *et al.,* (2011), who stated that during pregnancy, IL-10 levels were increased with increase in gestational age and together with IL – 4 play crucial roles in the success of pregnancy (Chatterjee *et al.,* 2014). It agrees with the work done by Denney *et al.,* (2011), who showed an overall increase in counter – regulatory cytokines e.g. IL – 10 as pregnancy progresses. Chatterje *et al.,* (2014) stated that normal pregnancy was determined to have increased IL – 10 production during the first and second trimesters but, not in the third trimester and decreases prior to labour and delivery of the fetus and placenta, but increases post labour.

They play protective roles during pregnancy and being Th2 – type cytokines are increased during pregnancy in order to regulate pro – inflammatory cytokines, especially TNF – α. In normal pregnancy, the secretion of IL – 10 assists the maintenance of a less pro – inflammatory environment, favoring a more regulated immune micro environment that is opposite to the presence of a fetus. The balance of maternal immune response controlling the inflammatory mechanism is dependent on IL – 10. Regulatory features of IL – 10 (Pleomorphic cytokine) in the immune stimulatory and immunosuppressive activity might be associated with the regulation of the Th1 – Th2 activities (Denney *et al.*, 2011). Normal progesterone, the concentration of which increases in pregnancy favours the production of IL – 10. This accounts for the observation that the highest quantities of IL – 4 and IL – 10 were observed in the third trimester of pregnancy, when the concentration of progesterone is at its highest. IL – 10 showed a significant positive relationship with APTT in the first trimester. A significant positive correlation was observed with IL – 10 and serum iron and a negative correlation with APTT in the second and third trimesters, but a significant positive correlation in with MCHC in the third trimester.

In this study, IL – 2 was insignificantly decreased in the first and second trimesters, but significantly lower in the third compared to non pregnant controls. This decrease could be because, IL – 2 being a Th1 cytokine, its presence is inhibited by Th2 cytokines to maintain a balance and prevent pregnancy complications, such as pre- eclampsia, abortion, small for geststional age babies (SGA) and so on.. This agrees with the work done by Marzi *et al.*, 1996 who stated that IL – 2 production decreases in physiologic human pregnancy but increases in pathologic pregnancy compared to non – pregnant controls. It is consistent with the study done by Kruse *et al.*, (2008), who showed that significant lower levels of Th1 – type cytokines e.g IL-2 were observed during pregnancy compared to non- pregnant females. Kruse *et al.*, (2000) also observed that lower mRNA levels of the Th1 – type cytokines (IL – 2 and IFN – y) were observed during pregnancy compared to non pregnant female controls. Normal pregnancy is accompanied by decreased production of type 1 pro- inflammatory cytokines and increased production of type 2 anti- inflammatory cytokines (Holmes *et al.*, 2003, Abdolreza *et al.*, 2011 and Davila *et al.*, 2011). IL – 2 was decreased may be because it is a Th1 type / pro – inflammatory cytokine. The decrease observed in Th1 cytokines is associated with the presence of factors that inhibit the production of Th1 cytokines and these factors are important in the proliferation and differentiation of the trophoblastic cells and placentation and play a protective role on the fetal – placental unit as an attempt by the organism to maintain the pregnancy process

(Feliciano *et al.,* 2012). Despite this, Th1 cells have an essential role in the implantation and placental development. Hence, there exist a balance between Th1 and Th2, and this Th1 / Th2 dichtonomy aids in the explanation about the environment of cytokines underlying a successful pregnancy (Feliciano *et al*., 2012).

According to Robison and Klein, (2012), the depression of pro – inflammatory cytokines was associated with high HCG serum levels, of which the effect on cytokine production is not entirely clear. There may be other early pregnancy signals which may not only have a profound effect on immune regulation at the fetal maternal interphase but also influence the cytokine expression pattern within blood cells (Robison and Klein, 2012). IL -2 was decreased from first trimester to the third, decreased more from first to second trimester than from second to third trimester. This is consistent with Kruse *et al.,* (2000) who was able to detect reduced IL – 2, IL – 18 and IFN – Y mRNA expression levels during the first trimester of normal pregnancy. Marzi *et al.,* 1996 showed that IL – 2 decreased in all trimesters compared to non – pregnant controls and that elevated IL – 2 serum concentrations have been found during the first trimesters in women who later develop pre – eclampsia. Denney *et al.,* (2011) indicated an overall decrease in pro – inflammatory cytokine trajectories in the innate and adaptive arms of the immune system and increase in counter regulatory cytokines as pregnancy progresses. IL – 10 showed a significant negative correlation with APTT in the first trimester. As IL -10 increased in the first trimester, APTT decreased in order to maintain a successful pregnancy.

This study showed that although there was a decrease in TNF – α level at all trimesters compared to non – pregnant women, there was no significant difference between them. This .might be probably due to the fact that it is a pro – inflammatory cytokine of which its presence during pregnancy is important for trophoblastic invasion and growth, but where the concentration is high, leads to pregnancy failure. Hence, its presence is regulated by anti – inflammatory cytokines especially IL -10 to prevent over – reaction.This supports the study of Jones *et al.,* (2000.) Kruse *et al.,* (2000), showed that TNF - ∝ mRNA levels were slightly higher compared to age – matched non - pregnant women. TNF – α was stable in all the trimesters. In the beginning of pregnancy, intense vascularization and cell proliferation helps the development of the embryo and the placentation, thus the presence of pro – inflammatory cytokines such as TNF - ∝ is important at this early stage. It modulates trophoblastic growth and the trophoblastic invasion of the spiral arterioles. Although its presence is essential, overreaction can restrict the invasion and contribute to the pathophysiology of pre – eclampsia (Peracoli *et al.,* 2007).

Thus, it is essential to control this inflammatory response in the pregnancy. In other words, for TNF – ∝ not to lead to pregnancy failure, it has to maintain a stable production profile in all stages. Hence, this is why levels of IL – 10 remain high throughout pregnancy in order to regulate the level of TNF - ∝ . TNF – α showed a significant inverse relationship with PT at the first trimester and with WBC, RBC and serum iron at the third trimester.

In this study, the packed cell volume (PCV) was insignificantly lower in first trimester, but significantly decreased in the second and third trimesters compared to non-pregnant controls. This is consistent with the study of Osonuga *et al.,* (2011) who also showed the same pattern. Obeagu *et al., (*2014) also observed a decrease in pregnancy compared with non-pregnant controls. The hemoglobin (HB) level was significantly reduced in the first, second and third trimesters compared to non-pregnant females. This agrees with Namama, (2015) and Obeagu *et al.*, (2014), who showed the same pattern.

During pregnancy, the iron requirement is significantly greater than that in the non-pregnant state, despite the temporary respite from losses incurred during menstruation (Bothwell, 2000). The HB and PCV levels during pregnancy are naturally lower than when not pregnant.The plasma increases by about 50% during pregnancy and the increased plasma dilutes the red cells, making their level drop. In other words, more iron is required to meet the expansion of maternal red cell mass and the needs of the fetus. The additional progesterone and estrogen that are secreted by the placenta during pregnancy cause a release of rennin from the kidneys. Renin stimulates the aldosterone rennin-angiotension mechanism, leading to sodium retention and increased plasma volume. The increase in plasma volume is relatively greater than the increase in red cell mass, which results in a fall in maternal red cell, hence the physiological anemia that occurs in pregnancy.

Several factors can be responsible for a high rate of iron deficiency. Multiparity, poor socio-economic and educational status is the principal reasons for a high prevalence of iron deficiency anemia in our population (Ijlal *et al.*, 2000 and Namama, 2015).

According to Hoffbrand *et al.,* (2000), prevalence of iron deficiency (ID) and iron deficiency Anemia (IDA) is increased 2 fold or more for those women who are minorities, below the poverty level or with < 12y of education. Risk is also increased with parity –nearly 3-fold higher for women with 2-3 children and nearly 4-fold greater for women with 4 or more children, thus implicating pregnancy. The PCV was significantly reduced from the first to the third trimester. The reduction was more pronounced in the second trimester, but the lowest value was achieved in the third trimester. Duria *et al.,* (2017), showed a decrease in PCV from the first to the third

trimester with lowest values achieved in the second trimester. Akinbami *et al.*,(2013) and Akingbola *et al.,* (2006) found a progressive decline in PCV from the first to the third trimester, but a slight rise in the third trimester and a drop in the second trimester, while Osonuga *et al.*,(2011) observed that no statistical significant difference in the value of PCV throughout the whole process of pregnancy. The difference in PCV may be due to the physiological haemodilution associated with pregnancy and increased demand by foetus for growth and development. There was a significant difference in Hb from first to the third trimester. This is consistent with Akinbami *et al.,* (2013) and Akingbola *et al.,* (2006), who showed a reduction in Hb from the first to the third trimester. Duria *et al.,* (2017), also showed a decrease in Hb from the first to the third trimester. There was reduction in Hb similar to PCV due to haemodilution in pregnancy. Also, lack of quality health care available to pregnant women, inadequate management of their blood profiles with dietary supplementation may be the reasons for reduced levels. The Hb showed a significant negative relationship with CD4+ in the first trimester. There was a significant negative correlation between PCV and IL - 4 in the second trimester.

This work showed an insignificant increase in total WBC in the first trimester, but significantly increased in the second and third trimesters compared to non pregnant controls. There was a progressive increase from the first to the third trimester. The increase might be due to increase in neutrophils and leukocytosis associated with pregnancy as a result of anxiety, nausea, pain and vomiting.This is in line with the work done by Akingbola *et al.,* (2006), Osonuga *et al .,(*2011), Chandra *et al.,* (2012), Akinbani *et al.,(* 2013), Obeagu *et al.,* (2014), and Duria *et al.,(* 2017).
Pregnancy is associated with leukocytosis. This leukocytosis might be as a result of increases in neutrophils (Akinbami *et al.,* 2013, Chandra *et al.,* 2013 and Duria *et al.,* 2017).
The increase in neutrophils during pregnancy may be due to the physiologic stress induced by the pregnant state. Total WBC count during pregnancy is hardly less than 7000/mm^3. The exact cause of relative leukocytosis during gestation is yet to be explored by further researches, however, physical and emotional stress associated with pregnancy (Abramson and Melton (2000), and depressed neutrophil apoptosis (Von Dadelszen *et al.,* (1999), could be possible cause. According to Akinbami *et al.,* (2013), the increase in WBC is primarily due to an increase in neutrophils and may represent a response to stress due to redistribution of the WBC'S between the marginal and circulating pools. Pain, nausea, vomiting and anxiety have been reported to cause leukoytosis in the absence of infection. However, a rising WBC count in pregnancy is not a reliable indicator of infection in sub clinical chorionamnionitis; rather, clinical methods of detection such as maternal pyrexia, offensive vaginal discharge, and fetal tachycardia

are better indicators, especially of preterm labour and membrane rupture. The increase in WBC may also be as a result of the body building immunity of the foetus and it is achieved by the state of selective immune tolerance, immunosuppression and immune modulation in the presence of a strong antimicrobial immunity. A significant inverse relationship was observed between WBC and TNF – α at the third trimester, and a significant positive association with IL – 4 in the first.

The Red Blood cell (RBC) was significantly reduced at all trimesters compared to non-pregnancy. It was significantly decreased from the first to the third trimester.
Obeagu *et al.,* (2014) and Duria *et al.,* (2017) also showed a decrease in RBC in pregnancy compared with non-pregnant state. Similarly, Duria *et al.,*(2017) also showed that the maximum decrease in RBC count was in the third trimester. The reduction in RBC may be due to physiological anemia, which occurs as a result of haemodilution. In pregnancy, plasma volume increase by about 50% higher than RBC mass (30%), and this discrepancy leads to physiological anemia. That is to say that, during pregnancy, plasma rennin activity tends to increase and atrial natriuretic peptide levels tend to reduce, though slightly. This suggests that, in pregnancy the elevation in plasma volume is in response to an underfilled vascular system resulting from systemic vasodilatation and increase in vascular capacitance, rather, than actual blood volume expansion, which would produce the opposite hormonal profile instead (Crocker *et al.,* 2000).
RBC showed a significant negative association with TNF – α at the third trimester and a significant positive relationship with IL – 4 at the second trimester.

Platelet count was insignificantly reduced in the first trimester, but significantly lower in the second and third trimesters compared to non- pregnant controls. This reduction could be as a result of gestional thrombocytopenia. This agrees with the work done by Chandra *et al*., (2012), Akinbami *et al.,(2013)* and Obeagu *etcond and third trimesters al.,(* 2014), who also showed the same pattern.
Chandra *et al., (2012)* also observed lowest value in the third trimesters. Obeagu *et al.,* (2014), observed lowest value of Platelet in the third trimester followed by first, then highest in the second trimester. This gestational thrombocytopenia may have occurred partly due to physiological haemodilution and partly due to increased platelet activation and accelerated clearance (Chandra *et al.,* 2012). This gestational thrombocytopenia does not have complications related to thrombocytopenia.

Neutrophils were increased significantly in all the trimesters compared to non - pregnant controls. This might be due to leukocytosis that occur during pregnancy as a result of impairment of neutrophilic apoptosis.This agrees with the work done by Osonuga *et al.*, (2011), Akinbami *et al.,* (2013) and Obeagu *et al.,* (2014). Leukocytosis occurs during pregnancy due to physiologic stress induced by the pregnant state. Neutrophils are the major type of Leukocytes on differential counts. This Leukocytosis may likely be due to impaired neutrophilic apoptosis in pregnancy. The neutrophil cytoplasm shows toxic granulation. Neutrophil chemotaxis and phagocytic activity are depressed, especially due to inhibitory factors present in the serum of a pregnant female (Jessica *et al.,* 2007). There is also evidence of increased oxidative metabolism in neutrophils during pregnancy. Immature forms of neutrophils may be found in the peripheral blood film of healthy women during pregnancy and do not have any pathological significance (Karalis *et al.,* 2005), rather they simply indicate adequate bone marrow response to an increased drive for erythropoiesis during pregnancy. Hence, the increase in neutrophil counts.

The Lymphocytes were significantly decreased in pregnancy compared to non- pregnant controls. This could be as a result of immunosuppression associated with pregnancy.Osonuga *et al.,* (2011) and Obeagu *et al*.,(2013) showed the same pattern. When compared in all the trimesters, were also significantly reduced from the first to the third trimester. Osonuga *et al.,* (2011) and Obeagu *et al., (*2013) observed percentage lymphocytes to increase in second trimester with a decrease in the third trimester. Duria *et al.,* (2017) showed that lymphocyte counts were comparable during the first two trimesters, but dropped significantly, over the last one, while Chandra *et al.,* (2012) observed lymphocyte count to be decreased through the first and second trimesters and increases during the third trimester. The decrease in lymphocyte count can be attributed to change that occur during pregnancy due to the development and growth of the fetus and may be because pregnancy is an immunocompromised state which results in a weakened immunosystem.

In this study, although the MCV was reduced in the first trimester compared to the controls, there was no significant difference. This could be because the demand during pregnancy has not really increased at this early stage. It was insignificantly increased in the second and third trimesters compared to the non – pregnant females.This might be due to increased demand of the fetus for growth and development and for placental development. It was not significantly increased from the first to the third trimester. Obeagu *et al.,* (2014) observed a significant increase. Bain *et al.,* (2012) stated that there was a slight increase in MCV in the second

trimester. The highest value observed in the third trimester. Duria *et al.,* (2017), showed a steady increase in MCV throughout pregnancy and highest value at the second trimester while Bain *et al.,* 2012, observed a slight increase at the second trimester. Ramsay,(2010), stated that during pregnancy the total blood volume increased by about 1.5 liters. Chandra *et al.,* (2012), showed that there is a small increase in mean corpuscular volume (MCV), of an average of 4fl in an Iron-replete woman.This might be due to increased demand by the fetus for growth and development and for the development of the placenta.The increase in MCV may be due to increased production of RBCs to meet the demands of pregnancy (i.e due to the production of higher proportion of young RBCs which are larger in size (Crocker *et al.,* 2000). During pregnancy, the total blood volume increases by about 1.5 litres, mainly to supply the demands of the new vascular bed and to compensate for blood loss occurring at delivery (Ramsay, 2010). Of this around one litre of blood is contained within the uterus and maternal blood spaces of the placenta. Increase in blood volume is therefore, more marked in multiple pregnancies and in iron deficient states.

The MCH insignificantly increased in non-pregnant controls compared to pregnant females. Although there was a decrease across the trimesters, the MCH showed no significant difference. This could be due to changes occurring during pregnancy.This is in line with the work of Akinbami *et al.,* (2013), who stated that MCH remained relatively stable through all trimesters. The changes may be due to physiological alterations that occur during pregnancy. MCH showed a significant negative correlation with CD4+ count in the second and third trimesters.

The MCHC was significantly decreased at all trimesters compared to non- pregnancy, no significant difference was observed between the first trimester and the second but a significant difference was observed when the first and second trimesters were compared to the third. Similarly, Akinbami *et al.,* (2013), showed MCHC to be stable in the first and second trimester but dropped in the third. This observation could be a reflection of iron deficiency anaemia. MCHC showed a significant positive correlation with IL – 10 at the third trimester.

This study shows that serum ferritin decreased significantly in pregnancy as compared to the non – pregnant controls. This decrease may be because during pregnancy, there is increased need of iron and this triggers ferritin mobilization from its stores.This is in line with the work done by Namama, (2015), who showed that levels of serum ferritin were decreased in pregnancy compared to that in non – pregnancy. Okwara *et al.,* (2013), also showed that non- pregnant women had more iron stores, therefore had less need for iron than their pregnant counterparts.

The higher iron need in pregnancy triggered its mobilization from its stores. During pregnancy, there is an immense stress on iron metabolism and it frequently induces iron deficiency which is characterized by a reduced ferritin level.(Naghmi *et al.,* 2007). On the other hand, may be some of the pregnant women started pregnancy with low iron stores, hence, the reason for low ferritin in pregnancy compared with the controls. This research work focused on physiological pregnancy not pathological, and ferritin levels are reduced in physiological pregnancy, but increased in pathological state (Naghmi *et al.,* 2007 and Enaam *et al*., 2014). Ferritin is an acute phase reactant protein and is sometimes found elevated independent of the iron status during illness and inflammation. According to Bain *et al*., (2012), serum ferritin decreases in early pregnancy and usually remains low throughout pregnancy, even when supplementary iron is given. Pregnancy is commonly associated with urinary tract infections and some occult infection. In such individuals, high serum ferritin levels are likely to be seen despite iron deficiency (Hou *et al.,* 2000).

Ferritin progressively decreased from the first to the third trimester. This could be due to increased demand for fetal growth and development as pregnancy progresses. This disagrees with the work done by Naghmi *et al.*,(2007) and Namama,(2015) who showed that ferritin decreased from first to second trimester with a slight rise in the third trimester. It is in line with Okwara *et al.,* (2013), who stated that serum ferritin declined progressively from first trimester to the trimester. The immense stress on iron metabolism during pregnancy frequently induces iron deficiency hence the reduction in ferritin level. This also implies a progressive mineral transfer from the mother to the fetus.

Serum ferritin showed significantly lowest values in the third trimester compared to the first and second trimesters. This disagrees with that of Naghmi *et al.,* (2007), who showed that significantly lowest values of ferritin was seen in the second trimester with slight increase in the third.The demand of iron is variable during the three trimesters and the practice of iron supplementation is also not uniform. Also the decrease in serum ferritin level may be associated to plasma volume expansion and the higher need of iron in pregnancy caused its mobilization from the stores. Therefore serum ferritin levels can be variable during different trimesters of pregnancy.

In this study, serum iron was decreased significantly at all stages of pregnancy compared to the controls. The decrease mignt be because iron is needed during pregnancy to expand the red blood cell mass and for fetal and placental growth.This is in line with the work of Bothwell, (2000), who stated that iron was more reduced in pregnancy than in non – pregnant women

because iron requirements are significantly greater in pregnancy than in non- pregnant state, despite the temporary respite from iron losses incurred during menstruation. Similarly, Chaudhari *et al.,* (2013), also showed decreased level of serum iron in pregnancy, Ama-Mariah *et al.,* (2011), showed the same pattern. During pregnancy, hemodynamic changes lead to expansion of blood plasma volume up to 50% and increase in red cell mass up to 20% which results in haemodilition. The overall iron requirement during pregnancy is significantly greater than that in the non-pregnant state. Iron is needed for expansion of the red blood cell mass and for transfer to both the growing fetus and the placental structures. Also iron was reduced in pregnancy maybe because some of the pregnant women started pregnancy with low or no iron stores or because they consumed diets of low iron bioavailability. It was decreased from the first trimester to the third, when compared across the trimesters. This follows the same pattern with Chaudhari *et al*., (2013) and Okwara *et al.,* (2013). There is an unequal distribution of iron requirement during pregnancy, as iron is needed for fetal and placental development (Bothwell, 2000). There is a significant increase in the amount of iron required to increase the red cell mass, expand the plasma volume and allow for the growth of the fetal – placental unit (Yip, 2001). According to Anonymous (2002), during pregnancy, anaemia increases > 4 fold from the first to the third trimester in the low-income women. In the Camden study where the cohort is mostly minority, the data Suggests that the prevalence of anaemia increases > 6-fold from 6.7% (1^{st} trimester) to 27.3% (2^{nd} trimester) to 45.6% in the 3^{rd} trimester. Serum iron showed a significant inverse correlation with TNF – α at the third trimester, likewise TIBC at the third trimester. Serum iron showed a significant positive correlation with IL – 10 at the first and second trimesters.

TIBC was insignificantly increased in the first trimester, but increased significantly in the second and third compared to non-pregnant females. When compared across the trimesters, the values increased from the first to the third trimester. This is consistent with the work done by Namama, (2015) who showed increased levels of TIBC from the first to the third trimester.The study of Chaudhari,(2013), also followed the same pattern. Okwara *et al.,* (2013) showed that TIBC was lowest in non- pregnant controls compared with the three trimesters of pregnancy. Amah-Tariah *et al.,* (2011), also observed increases in TIBC from the first to the third trimester. Total iron binding capacity (TIBC) is known to be increased in pregnancy and during iron overload.

This study revealed that the PT was significantly shortened in all the trimesters compared to the non-pregnant female controls. This could be as a result of hormonal changes which help to maintain placental function and prevent maternal bleeding during delivery. This is in agreement

with the study of Abbasi - Ghanavati *et a*l., (2009), who showed that PT was shortened in pregnancy than in non- pregnant controls. It is also consistent with the work done by Hellgren in 2003. Durotoye *et al.,* (2012) also found reduced PT during pregnancy compared to non-pregnancy. Uchikova *et al.,* (2015), reported PT as being significantly shortened in pregnancy compared to controls. It disagrees with the work done by Okungbowa *et al*., (2015) who showed a significant increase in PT in pregnant women when compared to non-pregnant women. Pal *et al*., (2010), reported that PT remains unchanged in pregnancy. Singh *et al., (*2016), showed a high prevalence of shortened PT (22.5%) and APTT (37.6%), in pregnancy. There was a significant shortening in PT from the first to the third trimester. This might be in order to prevent haemorrhage at every stage of pregnancy.This is in line with the work done by Durotoye *et al.,* (2012), who stated that the mean prothrombin time in the subjects in the first, second and third trimesters of pregnancy, showed that production of the coagulation factors increases as pregnancy advanced as there was statistically significant reduction in prothrombin time from the first to the third trimesters.

During pregnancy, the coagulation system undergoes significant changes, for example, hormonal changes. These changes help in maintaining placental function during pregnancy, protects from fetal haemorrhage during delivery, but at the same time predisposes to thromboembolism (Bremme, 2003). Thrombophilia predisposes a woman to an increased risk of developing both early and late complications in pregnancy. This includes recurrent miscarriages and late placental vascular-mediated problems (Fetal loss, preeclampsia, placental abruption and intrauterine growth restriction) (Simcox *et al.,* 2015).

Different values of PT by various authors may be associated with the sensitivities of the reagents and techniques employed. However, variability in PT results from different researchers have been traced to the differing sensitivities of the thromboplastin reagents used, concentration of citrate anticoagulation and method of analysis (Kamal *et al.,* 2007).

APTT was also shortened in pregnancy as compared to the non- pregnant females, and it is in line with Durotoye *et al.,* (2012), Ibeh *et al.,* (2015) and Singh *et al.,* (2016). This shows that levels of factors (FV, FViii, Fix and Fxii) in the intrinsic pathway are also increased in normal pregnancy (Durotoye *et al.,* 2012 and Hammerova *et al.,* 2014). The study of Ibeh *et al.,* (2015) also showed that the APTT was significantly lower in the first, second and third trimester compared to controls. There was significant difference in the levels of APTT when compared across the trimesters. This also agrees with the study of Durotoye *et al.,* (2012) and Ibeh *et al.,*

(2015) who followed the same pattern. This might be due to increased synthesis or increased activation by coagulation factors.

Both PT and APTT were shortened to protect the mother from the hazard of bleeding imposed by placentation and delivery. This is similar to the work of Durotoye *et al.,* (2012) who showed shortened PT and APTT in all trimesters compared to controls. The reason might be in order to maintain placental function and prevent maternal bleeding in every stage of the pregnancy. A significant inverse association was observed between PT and CD4+ at the second and third trimesters, and with TNF – α at the first trimester. APTT showed a significant inverse relationship with IL – 2 at the first trimester and with IL – 10 at the first, second and third trimesters.

5.2 Conclusion

In Summary, this study has shown that by longitudinal analysis;

Pregnancy significantly decreased absolute CD4+ cell count. This could be due to immunosuppression associated with pregnancy. CD4+ had a significant negative relationship with Hb in the first trimester, and with MCH and PT in the second trimester.

Pregnancy decreased levels of IL - 2, and IL -2 showed a significant negative relationship with APTT at the first trimester.

Levels of TNF-α were insignificantly decreased at all trimesters of pregnancy. TNF- α showed a significant negative correlation with PT in the first trimester. TNF – α showed a significant negative correlation and with WBC, RBC and serum iron at the third trimester.

Pregnancy elevated levels of IL- 4. IL- 4 had a significant positive relationship with WBC and a significant negative correlation with TIBC at the first trimester, but a significant negative association with PCV, and a significant positive correlation with RBC at the second trimester.

IL-10 levels increased in pregnancy. A significant positive association was observed between IL-10 and serum iron, and a significant negative correlation with APTT at the first trimester. A significant positive correlation was observed between IL-10 and serum iron, and a significant negative relationship with APTT at the second trimester. There was a significant positive correlation with MCHC and serum iron, but a significant negative relationship with APTT at the third trimester.

Pregnancy decreased levels of PCV, Hb, RBC, platelet count, lymphocyte, absolute lymphocyte, serum ferritin, serum iron, MCH and MCHC but increased levels of WBC, neutrophil, absolute neutrophil (leukocytosis), and TIBC.

Pregnancy is a transient hypercoagulable state, of which the evidence wass seen in the significant shortening of PT and APTT compared to the non- pregnancy states.

Therefore, this study has shown that pregnancy alters some haematological and immunological parameters. Hence, there is need to monitor pregnancies at risk in order to prevent adverse outcomes.

5.3.1 Recommendation

(i) Further study should follow pregnancy till delivery and pueperuim to ascertain the outcome and even work on cord blood.

(ii) Cytokines, PT and APTT should be included in addition to routine antenatal tests and subsidized for pregnant women in ante-natal clinics.

(iii) Pregnant women should be encouraged to register for antenatal clinics from the first month for early gestational evaluation.

(iv) The cost of ante-natal care should be included in the national health insurance scheme.

5.4 Contributions to Knowledge

(i) No previous longitudinal study has been done in immunological and haematological in uncomplicated pregnancy in this locality. Hence, the research work has enlightened clinicians on the need to know the state of these parameters during the period of pregnancy in order to monitor and predict pregnancy outcome.

(ii) This research study has also enlightened pregnant women on the need for early antenatal booking and iron supplementation in order to prevent adverse pregnancy outcomes.

(iii) The study underscored the need for early evaluation of cytokines in pregnant women to forestall complications in pregnancy.

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APPENDIX I

ETHICAL APPROVAL

NNAMDI AZIKIWE UNIVERSITY TEACHING HOSPITAL

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

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

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Board of Management

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

Our Ref: NAUTH/CS/66/VOL.8/23

Your Ref: _____

Date: 22nd December, 2015

Aloy-Amadi Oluchi Chinwe
Department of Medical Laboratory Science,
Faculty of Health Sciences and Technology,
Nnewi Campus

ETHICS COMMITTEE APPROVAL

RE: CD4 COUNT, CYTOKINE LEVELS AND HAEMATOLOGICAL PARAMETERS IN PREGNANT WOMEN AT NNAMDI AZIKIWE UNIVERSITY TEACHING HOSPITAL (NAUTH) NNEWI

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

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

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

.....
Dr. (Mrs.) Ebenebe J.C
Chairman, NAUTH Ethics Committee

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

APPENDIX II

QUESTIONNAIRE

I am Aloy-Amadi Oluchi C. a Post Graduate Student of the Department of Medical Laboratory Science, Faculty of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, doing a research on $CD4^+$ cell count, and levels of some cytokines and haematological parameters of pregnant women attending Nnamdi Azikiwe University Teaching Hospital, Nnewi. Please this research is purely academic. Participation is voluntary and you will not be exposed to any risk. Privacy is assured. Your laboratory tests will be free and the results communicated to you. I therefore solicit your co-operation and assure you that your response shall be treated with strict confidentiality.

Thank you.

A. PERSONAL/DEMOGRAPHIC DATA

Instructions

Please fill or tick where appropriate:

Phone No: …… Hospital No: ……..…….

Address: ……..

Email: …….

State: …….....

Nationality: ……..……..

Tribe: ……. Religion: ……..……..

Age: …… Marital Status**:** Married [ ] Single [ ] Divorce [ ] Separated [ ] Widow [ ]

Stage of Pregnancy: ……..

Occupation: Civil servant [ ] Trader [ ] Housewife [ ] Artisan [ ] others: …….

…….....

Level of Education: Primary [ ] Secondary [ ] Tertiary [ ] None [ ]

Height: ……

Weight: ……. BP: ……...

B. PRESENT PREGNANCY HISTORY

1. Date of the first day of your last period: ……
2. Was it normal period? Yes [ ] No [ ]
3. Did you use birth control pill before your last period? Yes [ ] No [ ]

   If yes, which one did you use? ……..

4. When did you stop using it? ……
5. Did you run a Pregnancy test? Yes [ ] No [ ]

   Date of Pregnancy test: …….
6. Are you on any iron supplement? Yes [ ] No [ ] if yes, specify:

   ……..……
7. Pregnancy is a physiological state, not a pathological state Yes [ ] No [ ]

C. PREGNANCY RISK FACTORS

Since the inception of this pregnancy have you

1. Had any vaginal bleeding that required a visit to the clinic? Yes [ ] No [ ]
2. Sick/hospitalized? Yes [ ] No [ ] if yes, when? …….
3. Had high or low blood pressure? Yes [ ] No [ ]
4. Taken any medication or drugs? Yes [ ] No [ ]

   If yes, list them: …….

   Providers' comment: …….

D. PREVIOUS PREGNANCY HISTORY

How many

1. Pregnancies have you had? *(including current one)*
2. Deliveries have you had?
3. Miscarriages have you had?
4. Abortions have you had?
5. Living children have you had?

Providers' comment: …….

Has any of your pregnancies involved;

1. A baby weighing less than 2.5kg? Yes [ ] No [ ]
2. A baby weighing more than 4.5kg? Yes [ ] No [ ]
3. Premature labour? (Before 8 months)? Yes [ ] No [ ]
4. Cesarean section? …… Yes [ ] No [ ]

   If yes, what was the indication? …….

   Provider's comment …….

E. MEDICAL HISTORY

Do you have or have you ever had

S/N		YES	NO	PROVIDERS, COMMENT
1	Asthma			
2	Abnormal Pap test			
3	Diabetes			
4	Hepatitis – B			
5	Hepatitis – C			
6	HIV			
7	Syphilis			
8	High blood pressure			
9	Low blood pressure			
10	Mental illness			
11	Depression			

F. FAMILY HISTORY

Has anyone in your family ever had;

S/N		YES	NO	PROVIDERS, COMMENT
1	Asthma			
2	Hypertension			
3	Diabetes			
4	Twin			
5	Sickle cell/ Thalassemia			
6	Birth defects			

	Have your husband;	YES	NO	PROVIDERS, COMMENT
1	Had blood transfusion			
2	Tested positive for HIV, hepatitis – B, hepatitis – C.			
3	Had syphilis or Gonorrhea			

APPENDIX III

DETERMINATION OF SAMPLE SIZE

The sample size was obtained using the formula by Taro Yamane, 1973.

$$n = \frac{N}{1 + N (e)^2}$$

Where:

N= Population size

e = sampling error (0.05)

n = sample size

Using this formula, the minimum number of sample size will be:

N = 216

e = 0.05

n = ?

$$\frac{216}{1 + 216 (0.05)^2}$$

$$\frac{216}{1 + 0.54} = \frac{216}{1.54} = 140.3$$

For this study, a minimum number of 140 subjects will be investigated.

APPENDIX IV

CALCULATIONS OF THE PARAMETERS

- **INTER LEUKIN -2 (IL – 2)**

Known concentrations of Human IL-2 standard and its corresponding reading OD is plotted on the log scale (X – axis) and the blog scale (Y – axis) respectively.

The concentration of Human IL -2 in sample is determined by plotting the sample's O.D on the Y – axis. The original concentration is calculated by multiplying the dilution factor.

PRECISION

Intra-assay precision (Precision within an assay): 3 samples with low, middle and high level Human IL – 2 were tested 20 times on one plate, respectively.

Inter- assay Precision (Precision between assays): 3 samples with low. Middle and high level human IL -2 were tested on a 3 different plates, 8 replicates in each plate.

CV (%) = 8D/mean x 100

Intra – Assay: CV < 10%

Inter – Assay: CV > 12%

ASSAY RANGE: 3.3pg/ml – 200 pg/ml

SENSITIVITY: 1.2pg/ml

- **INTER LEUKIN -4 (IL -4)**

Intra-assay precision (Precision within an assay): 3 samples with low, middle and high level Human IL – 4 were tested 20 times on one plate, respectively.

Inter- assay Precision (Precision between assays): 3 samples with low. Middle and high level human IL -4 were tested on a 3 different plates, 8 replicates in each plate.

ASSAY RANGE: 3.8pg/ml -200pg/ml

Sensitivity: 0.8pg/ml

- **INTER LEUKIN -10 (IL – 10)**

Intra-assay precision (Precision within an assay): 3 samples with low, middle and high level Human IL – 10 were tested 20 times on one plate, respectively.

Inter- assay Precision (Precision between assays): 3 samples with low. Middle and high level human IL -10 were tested on a 3 different plates, 8 replicates in each plate.

ASSAY RANGE: 1.2 pg/ml – 100pg/ml

SENSITIVITY: 0.3pg/ml

- **TUMOR NECROSIS FACTOR ALPHA (TNF - ∝)**

Intra-assay precision (Precision within an assay): 3 samples with low, middle and high level Human TNF - ∝ were tested 20 times on one plate, respectively.

Inter- assay Precision (Precision between assays): 3 samples with low. Middle and high level human TNF - ∝ were tested on a 3 different plates, 8 replicates in each plate.

ASSAY RANGE: 20ng/l -400ng.L

SENSITIVITY – 6ng/L

- **SERUM FERRITIN**

1. Calculate the mean absorbance value (OD_{450}) for each set of reference standards, controls and samples.

2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on a graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.

3. using the mean absorbance value for each sample, determine the corresponding of ferritin in ng/mg from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

4. Any diluted samples must be further converted by the appropriate dilution factor. Example of standard curve.

1. Results of a typical standard run of the assay are shown below.

Ferritin (ng/ml)	Absorbance (450nm)
0	0.074
15	0.150
80	0.362
250	1.017
500	1.699
1000	2.729

EXPECTED VALUES

Male	20 -250ng/ml
Female	10 – 120ng/ml
Children (6 months – 15 years)	7 -140ng/ml
Infants (2 – 5 months)	50 – 200ng/ml
Infants (1 months)	200 – 600ng/ml
Newborn	25 – 200ng/ml

- **SERUM IRON/TIBC**

CALCULATIONS

A = Absorbance

Std = standard

$\frac{A_2 \text{ Test} - A_1 \text{ Test}}{A_1 \text{std} - A_1 \text{ std}}$ x Conc of std = Total Iron (μg/dl)

EXAMPLE:

A_1 Test = 0.08 A_2 Test = 0.15

A_1std = 0.00 A_2 std = 0.40

Then $\frac{0.15 - 1.08}{0.40 - 0.00} = \frac{0.07}{0.40} = \frac{0.175 \times 500}{}$

=87.5μg/dl

- **UNSATURATED IRON – BINDING CAPACITY (UIBC)**

$$\text{Conc of std} - \frac{(A_2 \text{ Test} - A_1 \text{ Test}) \times \text{Conc of std}}{(A_2 \text{std} - A_1 \text{ std})} = \text{UIBC } (\mu\text{g/dl})$$

E.g.

$$500 - \frac{0.2 - 0.10 \times 500}{0.4 - 0.00} = \text{UIBC } (\mu\text{g/dl})$$

Therefore:

$500 – (0.25 \times 500) = 375\ \mu$g/dl (UIBC)

TIBC (Total Iron Binding Capacity):

Iron Level + UIBC = TIBC (μg/dl)

S.I unit conversion: μg/dl x 0.179 = μmo/IL

CALIBRATION: The procedure is calibrated with iron standard (500μg/dl) included in each kit.

EXPECTED VALUES

Iron, Total = 60 – 150 μg/dl

TIBC = 250 – 400 μg/dl

Iron saturation = 20 – 55 %

APPENDIX V

PREPARATION AND STORAGE OF REAGENTS AND SPECIMEN COLLECTION

- INTER LEUKIN – 2 (IL – 2)

ASSAY MATERIALS PROVIDED WITH THE KIT (IL -2)

MATERIALS	96 DETERMINATIONS	STORAGE
Users manual	1	R.T
Closure Plate Membrane	2	R.T
Sealed Bags	1	R.T
Microelisa Stripplate	1	2 – 8^{o}c
Standard: 270pg/ml	0.5ml x 1 bottle	2 – 8^{o}c
Standard dilutent	1.5ml x 1 bottle	2 – 8^{o}c
HRP conjugate reagent	6ml x 1 bottle	2 – 8^{o}c
Sample diluent	6ml x 1 bottle	2 – 8^{o}c
Chromogen solution A	6ml x 1 bottle	2 -8^{o}c
Chromogen solution B	6ml x 1 bottle	2 – 8^{o}c
Stop solution	6ml x 1 bottle	2 – 8^{o}c
Wash solution	20ml (30x) x 1 bottle	2 – 8^{o}c

IL -2 Standard 270 pg/ml – 0.5ml x 1 bottle – 2 - 8 ^{0}C

Preparation and storage of reagents and specimen collection are as for IL – 4, IL – 10 and TNF – α.

IL – 4 standard 270 pg/ml – 0.5ml x 1 bottle – 2 - 8 ^{0}C
IL – 10 standard 135pg / ml – 0.5 ml x 1 bottle – 2 - 8 ^{0}C
TNF – α standard 450 pg/ml – 0.5 ml x 1 bottle – 2 - 8 ^{0} C

SAMPLE PREPARATION

1. SERUM PREPARATION: After collection of the whole blood, allow the blood to clot by learning it undisturbed at room temperature. This usually takes 10 -20 minutes undisturbed at remove the clot by centrifuging at 2,000 – 3,000 rpm for 20 minutes if precipitates appear during reservation, the sample should be centrifuged again.

2. PLASMA PREPARATION: Collect the whole blood into tubes with anticoagulant (EDTA or citrate) After incubation at room temperature for 10 -20 minutes, tubes are centrifuged for 20 minutes 2,000 – 3000 rpm. Collect the supernatant carefully as plasma samples. If precipitates appear during reservation, the sample should be centrifugated again.

PRECAUTIONS:

1. Store the kit at 4^{o}c upon receipt. The kit should be equilibrated to room temperature before the assay. Remove any unneeded strips from Human IL -2 Antibody coated plate, rascal them in Zip – lock foil and keep at 4^{o}c.
2. Precipitates may appear in concentrated washing buffer. Please heat the buffer to dissolve all the precipitates, which will not affect the results.
3. Accurate pipette should be used to avoid experimental error. Samples should be added to the micro plate in less than 5 minutes. If a large number of samples are included. Multiple channel pipette is recommended.
4. Standard curve should be included in every assay. Replicate wells are recommended. If the OD value of the sample is greater than the first well of standards, please dilute the sample (n times) before test. When calculating the original IL -2 concentration, please multiply the total dilution factor (x ∩ x s)
5. In order to avoid cross – contamination, micro plate sealers are for one time use only.
6. Please keep substrate away from light.
7. All the operation should be in accordance with the manufacturer's instructions strictly. The results determined by the microtiter plate reader.
8. All the samples, washing buffer and wastes should be treated as infectious agents.
9. Reagents from different lots should not be mixed.
10. Sample extraction and ELISA assay should be performed as soon as possible after sample collection. The samples should be extracted according to the relevant literature. If ELISA assay cannot be performed immediately, samples can be stored at – 20^{o}c. Repeated freeze – thaw cycles should be avoided.

- **SERUM FERRITIN ASSAY**

REAGENTS AND MATERIALS

1. Antibody – Coated wells (1 plate, 96 wells) – microtiter wells coated with rabbit anti – ferritin.
2. Enzyme conjugate Reagents (13ml) – contains mouse monoclonal anti – ferritin conjugated to horse radish peroxidase.
3. Reference standard set (0.5ml/vial) – contains 0, 15, 80, 250, 500 and 1000ng/ml human liver or spleen ferritin in bovine serum with preservatives. 0.5ml each, liquid, ready to use.
4. TMB Reagent (one step) (1 bottle, μ ml) – contains 3,3^1, 5, 5^{1}tetramethyl benzidine (TMB) stabilized in buffer solution.
5. Stop solution (IN HCL) (1 bottle, μ ml) – contains diluted hydrochloric acid.
6. Distilled or deionized water.
7. Precision pipettes: 0.02, 0.05, 0.1, 0.2 and 1ml.
8. Disposable pipette tips.
9. Micro titer well reader capable of reading absorbance at 450nm
10. Voltex mixer, or equivalent
11. Absorbent paper
12. Graph paper.
13. Quality control material e.g. Bio Rad Lypho check control sera).

SPECIMEN COLLECTION AND PREPARATION

1. Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques. This kit is for use with serum samples without additives only. Avoid grossly hemolytic (bright red), lipenic (milky), or turbid samples.
2. Specimens should be capped and may be stored for up to 48 hours at 2-8^{o}c. Specimens held for a longer time should be frozen only once at -20^{o}c prior to assay. Thawed samples should be inverted several times prior to testing.
3. Specimens with expected values greater than 1,000ng/ml leng dialysis patients) should be diluted with zero standard prior to assaying. A 1:10 initial dilution is recommended.

INSTRUMENTATION

A micro titer well reader with a band- with of 10nm or less and an optical density range of 0 to 2 OD or greater at 450nm wave length is acceptable for absorbance measurement.

REAGENT PREPARATION

1. All reagents should be allowed to reach room temperature ($18 – 25^{o}c$) before use.
2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3. Samples with expected values greater than 1000ng/ml should be diluted with zero standard prior to assaying. A 1:10 initial dilution is recommended.

PRECAUTIONS

1. The kit contains human material. The source material used for manufacture of the kit tested negative for HBS A_g, HIV ½ and HCV by FDA – approved methods.

   However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum samples should be considered potentially infectious, handling and disposal should be as defined by an appropriate national biohazard safety guideline or regulation, where it exists.

2. Do not use reagents after expiration date and do not mix or use components from kit with different lot numbers.

3. Do not use the reagent when it becomes cloudy or contamination is suspected

4. Do not use the reagent if the vial is damaged

5. Replace caps on reagents immediately. Do not switch caps

6. Each well can be used only once.

7. Do not pipette reagents by mouth

8. Solutions containing additives or preservatives, such as sodium azide, should not be used in the enzyme reaction.

9. Avoid contact with the IN HCL. It may cause skin irritation and seek medical attention if irritation persists.

10. For in Vitro diagnostic use

STORAGE CONDITIONS

1. Store the unopened kit at 2 -8^{o}c upon receipt and when it is not in use, until the expiration shown on the kit label.

   Refer to the package label for the expiration date.

2. The opened and used reagents are stable until the expiration date if stored properly at 2-8^{o}c

3. Keep micro titer plate in a sealed by with dessicant to minimize exposure to damp air.

- **SERUM IRON/ TIBC DETERMINATION**

REAGENTS AND MATERIALS

REAGENTS –

1. Iron buffer reagent: Acetate buffer containing 220m M Hydroxylamine hydrochloride, PH 4.5 with surfactant.
2. UIBC buffer reagent: Tris buffer 0.5m, PH 8.0 with surfactant, sodium azide as preservative.
3. Iron colour reagent: Ferrozine (16.6mM) in Hydroxylamine hydrochloride.
4. Iron standard (500μg/dl): 500 μg ferrous chloride in Hydroxylamine hydrochloride

MATERIALS -

1. Spectrophotometer capable of reading at 560nm
2. Iron – free deionized water
3. Pipetting devices
4. Test tubes/ rack
5. Heating bath/block

SPECIMEN COLLECTION AND STORAGE

1. Fresh, unhemolyzed serum is the specimen of choice
2. Serum should be separated as soon as clot has formed.
3. Heparimized plasma may be used but other anticoagulants should not be used to avoid possible iron contamination
4. Serum iron is reported to be stable for four days at room temperature (15-30^{o}c) and seven days at 2-8^{o}c

PRECAUTIONS

1. For in vitro diagnostic use
2. UIBC buffer contains sodium azide and may react with lead and copper plumbing to form highly explosive metal azides. Oh disposed, flush with a large volume of water to prevent azide accumulation.
3. Avoid ingestion of reagent, as toxicity has not yet been determined.
4. Specimens should be considered infectious and handled appropriately.

STORAGE AND STABILITY

All the reagents and standard should be stored at room temperature (15 – 30^{o}c)

REAGENTS DETERIORATION

1. Appearances of turbidity, possible mold growth, or crystal formation that will not readily dissolve are signs or reagents deterioration.
2. Failure to obtain accurate results in the assay of controls materials may indicate reagent deterioration

QUALITY CONTROL: Serum controls with known normal and abnormal values should be run routinely to monitor the validity of the reaction.

- **CD4 COUNT ASSAY**

RECOMMENDED AND REQUIRED MATERIALS

1. Partec flow cytometry instrument (e.g. cyFlow counter code No.CY – S- 3022 or cyFlow SL 3 code No. CY- S- 1023).
2. Partec test tubes (code No. 04-2000).

3. Micro pipettes and pipette tips (e.g. Eppendorf, Code No. 3112000.029 and 3111000.0165).
4. Powder – Free latex gloves (e.g. Sageskin, Code No.545 – 95006)
5. Venous blood collection system with EDTA as anticoagulatant (e.g. Greiner Bio-one: Vacuette EDTA Tubes, K3E/EDTA K3, 3ml, Code No.454217, Vacuette blood collection Needles 38 x 0.8mm, code No, 450076, Vacuette Tube holder's, Code No. 450201).

HANDLING AND STORAGE

CD4 mAB PE is supplied in 2ml of phosphate – buffered saline (PBS), PH 7.4, containing BSA and 0.09% sodium azide, sufficient for 100 tests. 20 μl of CD4 mAB PE are sufficient for labeling 1 x 10^6 cells. When stored at 2 – 8^oc in the dark, the CD4 easy count kit is stable until the expiration date printed on the kit label. Do not freeze or expose to elevated temperatures.

- **HEPATITIS- B SURFACE ANTIGEN (HBsAg)**

REAGENTS – The strip contains anti HBsAg particles and anti – HbsAg coated on the membrane.

SPECIMEN COLLECTION AND PREPARATION

- The HBsAg one step HBsAg test strip can be performed using either serum or plasma.
- Separate the serum or plasma from blood as soon as possible to avoid hemolysis. Only clear, non- hemolyzed specimens can be used.
- Testing should be performed immediately after the specimens have been collected. Do not leave the specimens at room temperature for prolonged periods. Specimens may be stored at 2-8^oc for up to 3 days. For long term storage, specimens should be kept below -20^oc.
- Bring specimens to room temperature prior to testing. Frozen specimens must be completely thawed and mixed well prior to testing. Specimens should not be frozen and thawed repeatedly.
- If specimens are to be slipped, they should be packed in compliance with Federal, State or Local regulations for the transportation of etiologic agents.

MATERIALS- Test strips, specimen collection container, centrifuge (for plasma only) Timer.

PRECAUTIONS

1. For professional in vitro diagnostic use only. Do not use after expiration date.
2. Do not eat, drink or smoke in the area where the specimens or kit are handled.
3. Handle all specimens as if they contain infectious agents. Observe established precautions against micro biological hazards throughout testing and follow the standard procedures for proper disposal of specimens.
4. Wear protective clothing such as Laboratory Coat, disposal gloves and eye protection when specimens are being tested.
5. Humidity and temperature cab adversely affect results.

STORAGE AND STABILITY

The kit can be stored at room temperature or refrigerated (2 -3^{o}c). The test strip is stable through the expiration date printed on the sealed pouch. The test strip must remain in the sealed pouch until use. Do not freeze. Do not use beyond the expiration date.

- **HEPATITIS – C (HCV) DETERMINATION**

REAGENTS AND MATERIALS

- So pouch: One HCV test strip and one Desiccant.
- Clock or Timer
- Specimen collection containers
- Centrifuge

SPECIMEN COLLECTION – Consider any materials of human origin and infectious and handle them using standard bio safety procedures.

A. PLASMA

1. Collect blood specimen into a Lavender, blue or green top collection tube (containing EDTA, citrate or heparin, respectively in vacutainer by vein puncture.

2. Separate the plasma by centrifugation

3. Carefully withdraw the plasma into new pre-labelled tube

B. SERUM

1. Collect blood specimen into a red top collection tube (containing no anti coagulatants in Vacutainer) by vein puncture.
2. Allow the blood to clot
3. Separate the serum by centrifugation
4. Carefully withdraw the serum into a new pre-labeled tubes.

Test specimens as soon as possible after collecting. Store specimens at 2^{o}c to 8^{o}c, if not tested immediately. The specimens could be stored at 2^{o}c to 8^{o}c up to 5 days. The specimens should be frozen at -20^{o}c for longer storage. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen specimens to room temperature showly and mix gently. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

STORAGE AND STABILITY- All reagents are ready to use as supplied. Store unsured test device unopened at 2^{o}c – 30. If stored at 2-8^{o}c, ensure that the test device is brought to room temperature before opening. The opening device is stable through the expiration date printed on the sealed pouch. Do not freeze the kit or expose the kit over $30^{oc.}$

- **HCG PREGNANCY RAPID TEST**

REAGENTS: The test contains anti-HCG particle and anti-hcG coated on the mwmbrane

MATERIAL: Test strips package insert specimen collection container, timer.

PRECAUTION:

1. For profession in vitro diagnostic use only. Do not use after the expiration date.
2. The test should remain in the sealed pouch or closed canister until ready to use.
3. All specimens should be considered potentially hazardous and handled in the same manner as an infection agent.
4. The used should be discarded according to local regulations.

STORAGE AND STABILITY

Store as package at room temperature or refrigerated (2-30^{o}).the test is suitable through the expiration date printed on the sealed pouch or lable of the closed canister until use. Do not use beyond the expiration date.

- **ALERE DETERMINE ™ HIV 1/2**

MATERIAL

- Alere Determine™ HIV 1/2 Test card, HIV 1/2 recombinant antigen and synthetic peptide coated.

ACCESSORIES (required but not provided): for testing whole blood samples

1. 1 bottle (2.5 ml) chase buffer (7d2243) prepared in phosphate buffer. Preservatives: Antimicrobial Agent.
   Whole blood (fingerstick assay)
2. EDTA Capillary tubes (7D2222)

SPECIMEN COLLECTION

Human serum, plasma and whole blood collection by venipuncture should be done aseptically in such a way as to avoid heamolysis NOTE: For whole blood and plasma specimen, EDTA collection tubes must be used.

Whole blood collection by finger stick

Before collecting a fingerstick specimen, place an EDTA capillary tube on a clean dry surface.

1. Choose the fingerstick tip of the middle, ring, or index finger (whichever is the least callused) for adult and children older than one year. Warm the hand as needed with a warm, moist towel or warm water to increase blood flow.
2. Clean fingertip with alcohol allow to air dry position the hand palm side up
3. Use a new lancet each, place the lancet off-center on the fingertip. Firmly press the lancet against the finger and puncture the skin. Dispose of the lancet in an appropriate bio hazard sharp container.
4. Wipe away the first drop of blood with a sterile gauze pad.
5. Hold the finger lower than the elbow and apply gentle, intermillent pressure to the base of the puncture finger several times. Touch the tip of the EDTA capillary tube to the drop of blood. Avoid air bubbles.

WARNING AND PRECAUTIONS

- For in vitro diagnostic use.

CAUTION:

Appropriate bio safety practices should be used when handling specimen and reagent these precautions include, but are not limited to the following:

- Wear gloves
- Do not pipette by mouth
- Do not eat, drink, smoke, apply cosmetic, or handle contact lenses in area where these material are handled.
- Clean and disinfect all spills of specimen or reagent using a suitable disinfectant, such as $0.5^{o/o}$ sodium hypochlorite.
- Decontaminate and dispose of all specimen, reagent and other potentially contaminant material in accordance with local regulations.

ALERE DETERMINE^{TM} HIV 1/2 MATERIALS

- Alere determine^{TM} HIV 1/2 Test cards, HIV 1/2 recombinant antigen and synthetic peptide coated.

ACCESSORIES (required but not provided):

1. 1 Bottle (2.5ml) chase Buffer (7D2243) prepared in phosphate buffer. Preservatives: Antimicrobial Agents

   Whole Blood (Finger Assay)

2. EDTA capillary tubes (7D2222)

SPECIMEN COLLECTION

Human serum, plasma, and whole blood collection by venipuncture should be done aseptically in such a way as to avoid haemolysis.

NOTE: For whole blood and plasma specimens, EDTA collection tubes must be used.

Whole blood collection by Fingerstick:

Before collecting a fingerstick specimen, place an EDTA capillary tube on a cleandry surface.

1. Choose the finger tip of the middle, ring, or index finger (whichever is the least callused) for adults and children older than one year. Warm the hand as needed with a warm, moist towel or warm water to increase blood flow.
2. Clean finger tip with alcohol; allow to air dry. Position the hand palm- side up.
3. Use a new lancet each, please the lancet off-center on the finger tip. Firmly press the lancet against the finger.

Storage: The Ablere Determine HIV/12 test cards and chase buffer must be stored at 2 – 30 0 c until expiration date. DO not use kit components beyond expiration date. Immediately reseal all unused tests in the foil pouch containing the disccant by pressing seal from end to end to close.

Specimen Storage

1. Serum and plasma specimens should be stored at 2 – 8 0 c if the test is to e run within 7 days of collection. If testing is delayed more than 7 days, the specimen should be frozen (- 20 0c or colder).
2. Whole blood collected by venipuncture should be stored at 2 – 8 0 c if the test is to be run within 7 days of collection. Do not freeze whole blood specimens.
3. Whole blood collected by fingerstick should be tested immediately.

- **PROTHROMBIN TIME TEST (PT)**

PROTHROMBIN TIME REAGENT

Catalogue No: 81100	10 X 10ml
Catalogue No: 81050	10 X 5ml
Catalogue No: 81025	5 X 5ml

INTENOID USE

For in Vitro Diagnostic use only.

Dia PT is a rabbit brain thromboplastin with own solvent for prothrombin time determinations.

ACTIVE INGREDIENTS

Dia PT is a freeze-dried, tissue thromboplastin from rabbit brain with stabilizes. Solvent is a buffer, which contains calcium ions and sodium oxide as preservative.

PRECAUTION

Dia-PT, due to its ingredients should be handled with care, observing the precautions recommended for bio-hazardous material.

- The solvent contains sodium oxide. It can form metallic oxides, which may be potentially explosive in metal plumbing. Execute proper precaution.
- Specimens, samples and all materials coming into contact with them should be handled as if capable of transmitting infection and disposed of with proper precaution.
- Do not use the reagent beyond the expiration date printed on the label.
- Avoid microbial contamination of the reagent results may occur.
- According to the present knowledge the reagent does not contain any particles, which can spread from animals to humans.
- All reagents, waste and ultilized disposable laboratory equipments should be considered as hazardous waste. Their handling and disposal should be done according to the valid hazardous material processing regulation.

PREPARATION – Dia-PT reagent is dissolved with the entire contents of one vial solvent of the same lot.

Keep the thromboplastin at 18 ^{o}c to 25 ^{o}c for 30 minutes, swirl the vial gently before use and do not shake. Avoid the contact of fluid with the stopper. Using of stirring bar is necessary.

SPECIMENS- Dia-PT requires freshly, decalcified plasma. To obtain it, mix mine part of freshly drawn various blood with one part trisodium citrate. Mix the blood carefully and centrifuge plasma before testing. Refer to national committee for chemical laboratory standard (NCCLS) guidelines H3-A3 and H21-A3.

STORAGE AND STABILITY

Dia-PT reagent in intact vial is stable until the expiration date given on the vial, when stored at 2 - 8 ^{o}c. stability after reconstitution in the original vial: 8 hours at 37 ^{o}c, 1 day at 22 ^{o}c, 2 days at 16 ^{o}c and 12 days at 2-8^{o}c. do not freeze.

EXPECTED RESULTS

Dia-PT test results can be reported in the following until

1. Seconds, which means the observed clotting time.

2. Ratio, which means the clotting time of the sample divided by the clotting time of the normal plasma pool.
3. Percentage, which means the proportional part of the normal PT activity, which is calculable from the calibration curve. We recommend to use the master curve enclosed in the box.
4. International Normalized Ratio (INR), which means the ratio raised to the power of international sensitivity index (ISI).

The lot and method dependent on ISI value is provided on the separated sheet.

The international committee on thrombosis haemostatis (ICTH) and the international committee for standardization in Haematology (ICSH) have recommended the INR as a comparable dimension of PT results.

The normal range that is as second of the prothrombin time at healthy factors (age, gender, hematocint).

In general, the INR is considered as normal between 0.9-1.3. the pathological range begins at 1.6 INR.

MATERIALS REQUIRED

- Normal and pathological controls for quality control (Dia-cont; 1-11; cat. No;91020).
- Optical or mechanical coagulation analyzer for measuring.

- **ACTIVATED PARTIAL THROMBOPLASTIN TIME** (APTT) REAGENT

Cat No:	71048	12 X 4ml
Cat No:	71024	12 X 2ml
Cat No:	71030	6 X 4ml

INTENDED USE

- For in vitro diagnostic use only.

Dia-PTT is a rabbit brain cephalin for Activated partial thromboplstin Time determination.

ACTIVE INGREDIENTS - Dia-PTT is a freeze-dried, rabbit brain cephalin and micromized silica in buffered medium with stabilizer.

PRECAUTION

- Dia-PTT, due to its ingredients should be handled with care, observing the precautions recommendations recommended for biohazardour material.
- Specimens, samples and all materials coming into contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions.
- Do not use the reagent beyond the expiration date printed on the label.
- Avoid microbial contamination of the reagents or erroneous results may occur.
- According to the present knowledge the reagent does not contain particles, which can spread from animal to human.
- All reagents, waste and utililized displosable laboratory equipments should be considered as hazardour waste. Their handling and disposal should be done according to the valid hazardour material processing regulation.

PREPARATION

Dia-PTT reagent is dissolved with distilled water. The amount is on the label. Keep the reagent after reconstitution at 18- 25 ^{o}c for 30 minutes. Sirvil the vial gently before use and do not shake. Avoid the contact of fluid with the stopper. Using of stirring bar is necessary.

SPECIMENS

Dia-PTT requires freshly, decalcified plasma. To obtain it, mix nine parts of freshly drawn various blood carefully and centrifuge plasma before testing.

STORAGE AND STABILITY

Dia-PTT reagent in intact vial is stable until the expiration date given on the bial, when stored at 2-8 ^{o}c, stability after reconstitution in the original vial: one day at 22 ^{o}c, two days at 16 ^{o}c and five days at 280 ^{o}c. do not freeze.

EXPECTED RESULTS

Dia-PTT test results can be reported in the following units:

1. Seconds, which means the clotting time of clotting time.
2. Ratio, which means the clotting time of the sample, divided by the clotting time of the normal plasma pool.

The normal range that is the second of the activated partial thromboplastin time of healthy persons is influenced by several factors (age, gender, hematorit). In general, the range is considered as normal, between 25-43 secs.

MATERIALS REQUIRED

- Cacl2 for measuring (Dia-cacl2; cat. No. 41192).
- Normal and pathological controls for quality control (Dia-cont 1- 11; cat No: 91020)
- Optical or mechanical coagulation analyzer for measuring.
- Replace this with the CD4 determination, you typed under method.

- **CD4 COUNT DETERMINATION**

The CD4 count was performed using the CD4 easy count kit (1504301)

SPECIFICITY AND ANTIGEN DISTRIBUTION: The mouse monoclonal antibody MEM 241 Recognizes the human CD4 antigen, a transmembrane glycoprotein (55KDa) of the immunoglobulin supergene family, present on, a subset of T-lymphocytes ("helper/inducer T-cells) and also expressed at a lower level on monocytes, tissue macrophages and granulate. Approximately 20-60% of human peripheral blood mononuclear cells as well as a sub population of monocytes but with a weaker signal are stained.

METHOD

20ml white blood (EDTA) was added to a partec test tube. 20ml of CD4mAb PE was added, mixed gently and incubated for 15 minutes at room temperature protected from light. Then 800ml of no lyse buffer was added and shaken gently. Blood samples were then analyzed on a partec device.

FLOW CYTOMETRIC ANALYSIS- CD4-PE fluorescence was analyzed on a partec flow cytometer with an excitation light source of 488mm or 532nm (blue or green solid state laser). To count CD4-T cells the test tube with 840ml of the ready prepared blood sample were transferred to the partec flow cytometerand a count analysis carried out.

- **VDRL(SYPHILIS AB) RAPID TEST STRIP**

Materials Supplied: Test strip, desiccant package insert, clock / timer, specimen containers, centrifuge (for plasma only).

SPECIMEN COLLECTION

1. Syphilis Ab Rapid test strip (serum / Plasma) can be performed using either serum or plasma.
2. Separate the serum or plasma from blood as soon as possible to avoid haemoly. Specimen can be used.

3. Testing should be performed immediately after the specimens have been at room temperature for prolonged periods. Specimens may be stored at room temperature for prolonged periods. Specimens may be stored at 2 – 8 0 c for up to 3 days for long term storage, specimens should be kept below – 20 0 c.
4. Bring specimens to room temperature prior to testing, frozen specimens must be completely thawed and mixed well prior to testing. Specimens should not be frozen and thawed repeatedly.
5. If specimens are to be shipped, they should be packed in compliance with usual regulations for transportation of actiological agents.

Warnings and precautions

1. For professional in vitro diagnostic use only. Do not use after expiration date.
2. Warning: The reagents in the kit contain sodium azide which may react with lead or copper plumbing to form proteins partially explosive metal azides. When disposing of such reagents, always flush with large volumes of water to prevent azide build – up.
3. Do not use it if the tube/ pouch is damaged or broken.
4. Test is for single use only. Do not re – use under any circumstances.
5. Handle all specimens as if they contain infections agents. Observe established precautions against microbiological hazards throughout testing and follow the disposal of specimens.
6. Wear Protective clothing such as laboratory coats, disposable gloves and eye protection when specimens are assayed.
7. Humidity and temperature can adversely affect result.
8. Do not perform the test in a room with strong air flow, i.e. an electric fan or strong air conditioning.

Storage and stability

All reagents are ready to use as supplied store unused test device unopened at 2 0 c – 30 0 c. If stored at 2 – 8 0 c, ensure that the test device is brought to room temperature before opening. The test device printed on the sealed pouch. DO not freeze the kit or expose the kit over 30 0 c.

QUALITY CONTROL

A procedural control is included in the test. A red line appearing in the control region (c) is the internal procedural control. It confirms sufficient specimen volume and correct procedural technique. Control standards are not supplied with this kit, however it is recommended hat positive and negative controls be tested as a good laboratory practice to confirm the test procedure and to verify proper test performance.