T LYMPHOCYTE SUB POPULATIONS, CYTOKINES AND PREGNANCY SPECIFIC BETA-1 GLYCOPROTEIN IN ECLAMPSIA IN KADUNA STATE, NIGERIA

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JANUARY, 2015

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BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN IMMUNOLOGY,
IMMUNOLOGY UNIT, DEPARTMENT OF MEDICINE,
FACULTY OF MEDICINE,
AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA.

JANUARY, 2015

DECLARATION

I hereby declare that this dissertation entitled‘‘T-LYMPHOCYTE SUB POPULATIONS, CYTOKINES AND PREGNANCY SPECIFIC BETA-1 GLYCOPROTEIN IN ECLAMPSIA IN KADUNA STATE, NIGERIA’’ is a record of my work that was carried out and documented by me under the supervision ofDr B.O.P. Musa of the Immunology Unit, Department of Medicine, Professor S. O. Shittu (Department of Obstetrics and Gynecology) and Dr A. A. Babadoko (Department of Haematology). The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for the award of another degree or diploma at any University.

Jim Monday BANDA

_____ _____ _____

Name of studentSignature Date

The declaration is confirmed by

_____ _____ _____

Dr B.O.P.Musa Signature Date
Chairperson, Supervisory Committee

CERTIFICATION

This dissertation entitled: ‘‘T-LYMPHOCYTE SUBPOPULATIONS, CYTOKINES AND PREGNANCY SPECIFIC BETA-1 GLYCOPROTEIN IN ECLAMPSIA IN KADUNA STATE, NIGERIA’’ by Jim Monday BANDA meets the regulations governing the award of Doctor of Philosophy, Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to God Almighty who empowered me with strength in the ‘inner man’ to start and finish this work.To Him alone be glory, honor and adoration. To my wife, Mrs. Omolade Folasade Banda, to my daughters: Faith Saignom, Hope Simnom and Grace Kpeinom; my mother Mrs. Martina Swad Banda and my late father Mr. Banda Kwasau Nyam Gizo.

ACKNOWLEDGEMENTS

To God be the glory, honor and adoration without whom I can do nothing; 'for *by strength shall no man prevail*'. I want to use this medium to acknowledge the help of God Almighty and the people He used as 'destiny helpers' on my way to the top; making this study a dream –come- true.

To the members of the supervisory team, I am sincerely grateful toDr (Mrs.) B.O.P. Musa for her support, encouragement, patience,skilful supervision and commitment in making this study a success. My unreserved gratitude goes to Professor S. O. Shittu for the benefit of impactful discussions, skilful supervision and encouragement, as well as the provision of access to pregnant women at the Antenatal Clinic (ANC), eclamptic wards and Family Planning Units of the Obstetrics and Gynaecology Department. I am equally indebted to Dr A. A. Babadoko, the head of Haematology Department, for assistance, encouragement and wise counsel to me in the course of this study.

I am highly indebted to Professor G.C. Onyemelukwe; his experience in research, suggestions and encouragement birthed this study.

I must not forget to appreciate the contributions of: Dr (Mrs) Z. Sarkin-pawa, (ABUTH Zaria), Dr H. Muazu, of Hajjiya Gambo Sawaba General Hospital (HGSGH) Zaria, and Dr J. Adze, (Barau Dikko Specialist Hospital (BDSH) Kaduna). Dr M. S. Dangaru, (Yusuf Dantsoho Memorial Hospital (YDMH) Tudun Wada Kaduna), and Dr J. G. Gajere, (General Hospital (GH) Kafanchan) were helpful in patient selection, and exchange of ideas in the course of the study.

I am highly indebted to Professor A. G. Bakari, the current Dean, Faculty of Medicine andformer Head of Department (Medicine) for his support and encouragement as

well as Prof B. G. Sani (current Head, Department of Medicine). Dr J.A. Randawa, Head of Obstetrics and Gynaecology and Dr N.D, Chom of Radiology Department of ABUTH Zariaalso appreciated for their encouragement and support.

I wish to express my gratitude to Professor (Mrs.) J.U. Okpapi, and Professor (Mrs) A. I. Mamman, Deputy Dean Clinical post graduate (PG) school and Professor M. A. Abdul, department of Obstetrics and Gynaecology forsupport and encouragement.

My thanks go to my Father Banda Kwasau Gizo Nyam of blessed memory, and my mother, Mrs. Martina Swad Banda, who taught me hard work,a 'never-give-up' attitude and to trust in God.Tomy elder brother Mr. F.G. Banda without whose support I would not have known the four corners of a class room. I cannot thank my wife Deaconess (Mrs.) Omolade Folasade Banda enough for her encouragement and unreserved support to me in the course of my study. I also thank my children Miss Faith Saignom Banda, Miss Hope Simnom Banda, Miss Grace Kpeinom Banda and Mr. Alex O Fadipe for their patience and prayers towards the successful completion of my study.

My profound gratitude goes to Mr. Philip Jalo and Julius Okojokwu Ocheme for helping out in the area of data analysis and interpretation.Mrs.Lillian Okonkwo of Immunology Unit and Mrs Aisha Abubakar in the ART Laboratory are highly appreciated for the technical support given to me during the course of my study. To other members of Immunology Unit, my thanks especially go to Mr. Joseph Egbe and Mr. Lasisi Araba for their encouragement and goodwill.

Banda, Jim Monday.

ABSTRACT

A comparative cross-sectional study of eclampticand normal healthy pregnant womenconductedin Ahmadu Bello University Teaching Hospital (ABUTH),Shika Zaria to determine Tcell subpopulations (CD3+, CD4+ and CD8+T lymphocytes); cytokines (Tumor necrosis factor[TNF]-α, Interleukin [IL]-2, IL-4, and IL-10) and Pregnancy Specific beta-1 Glycoprotein (PSG-1) levels in the peripheral blood ofthird trimester women with eclampsia (EC; N=38), normal healthy pregnant and non pregnant women controls(PC; N=38 and NPC; N=38 respectively), age and parity matched, attending labour rooms/wards and Antenatal Clinics (ANC) of Ahmadu Bello University Teaching Hospital Shika,Zaria and four other Hospitals in Kaduna state, Nigeria. Participants with smear positive malaria, seropositive for human immunodeficiency virus (HIV) or any other known clinical infection were excluded from this study. Complete Blood Count (CBC) and T lymphocytesubpopulationswere determined by Sysmex, Auto blood analyzer and Becton Dickinson (BD) FACScount machine using monoclonal anti-T lymphocyte antibody (FITC-Fluorescein isothiocynate labelled) respectively. Cytokines and PSG-1 levels were estimated using Quantikine ELISA kits. Data obtained were analyzed using analysis of variance (ANOVA) to evaluate the difference between parametric data (T-lumphocyte subpopulations) and Kruskal Wallis test to evaluate the difference between nonparametric data (PSG-1 and cytokines). A p-value of less than 0.05 was considered to be significant. Results showed reduction in the levels of mean total Tcells (CD3+ lymphocytes), T helper lymphocyte (CD4+lymphocytes) and ratio of helper-to-suppressor T cells in women with EC and PCcompared NPC($P<05$). Eclampsia was associated with a further significant decrease in mean CD3+and CD4+ lymphocytes levels ($P<05$) compared to PC. In relation to cytokines, women with EC, had a significantly elevated mean serum level of pro-inflammatory cytokine TNF-α

and low levels of anti-inflammatory cytokine IL-10 compared to both PC and NPC ($P<0.05$). Mean IL 2 levels in the eclamptics were not significantly different from both controls. The mean levels of PSG-1 in the eclamptic women group were not significantly different from PC ($P>0.05$), while the total white blood cell count, and differential percentage neutrophils count were noted to be elevated among the eclamptic women compared to PC and NPC ($P<0.05$). The study suggested an association of EC with a dominant pro-inflamatory cytokine environment, which supports the hypothesis of Th 1 response in women with ECas opposed to Th2 response in normal healthy pregnant women. Further studies are required to determine the true status of Th1/Thh2 paradigm shift in established EC. The new information generated in this study provide a basis for the Clinicians, Researchers and the Government of Nigeria to explore, in terms of the strategies for improving and meeting up with health standards as stipulated in the Millenium Development Goals (MDGs).

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ABBREVIATIONS AND ACRONYMS

ThI	T-helper 1
Th2	T helper 2
CMI.	Cell Mediated Immunity
HLA	Human Leucocytes Antigen
WBC	White Blood Cell
IFN-y	Interferon gamma
IL-2	Interleukin-2
IL-4	Inter leukin-4
IL-10	Interleukin 10
TNF-a	Tumor Necrosis Factor alpha
GVD.	Graft Versus host Disease
PSG-1	Pregnancy Specific beta-1 Glycoprotein-1
βHCG..	Beta Human Chorionic Gonadotrophin
HPL	Human Placental Lactogen
PE	Preeclampsia
EC.	Eclampsia
PC	Pregnant Control
NPC	Non-Pregnant Control
CD	Clusters of Differentiation
FACS count.	Fluorescence Activated Cell Sorter count
AIMLT	Associate Institute of Medical Laboratory Technology
FIMLT	Fellows Institute of Medical Laboratory Technology

MMLS	Masters Degree in Medical Laboratory Science
NVRI	National Veterinary Research Institute
ANOVA	Analysis of Variance
PLT	Platelets
Neut.	Neutrophils
Lymph.	Lymphocytes
COX	Cyclooxygenase
MHC	Major Histocompatibility Complex

CHAPTER ONE

1.0 INTRODUCTION

Hypertensive disorders complicating pregnancy are common and along with haemorrhage and infection contributes greatly to maternal morbidity and mortality. Preeclampsia (PE), the precursor of eclampsia (EC) is a human-pregnancy-specific disease defined as the occurrence of hypertension and significant proteinuria in a previously healthy woman on or after 20th week of gestation. It is a common medical complication of pregnancy occurring in 2-8% of all pregnancies (Moodley, 2004; Ghulmiyyah and Sibai, 2012), and incidence has continued to increase worldwide due to inadequate and poor utilization of maternal health care facilities (Dolea and AbouZahr, 2003),It is a life threatening disease, (Oladokun, *et al.,* 2000)accounting for about 50,000 maternal deaths annually world wide (WHO, 2011). Therefore, it is a public health issue (WHO, 2002).Unfortunately, there has been little progress in EC compared to advances made in eliminating other major obstetric complications (Sibai, 1998).Reduction in maternal mortality by 75% between 1990 and 2015 has been considered part of the millennium development goal of the World Heath Organization (WHO) (Osungbade and Ige, 2011).

PE and EC are not distinct disorders but manifestation of the spectrum of clinical features of the same condition (Mahmoudi, 1999; Shah 2009). The mildest disorder in this continuum is pregnancy-induced hypertension (PIH). In PE, hypertension and proteinuria are present and when convulsions occur in addition to these signs, the condition is referred to as EC (Shah, 2009).

Eclampsia has been identified as a clinical condition since the time of Hippocrates but it was not until the 17^{th} century that the convulsions in pregnancy were

distinguished from tonic clonic fits in the non-pregnant state (Chesley, 1980) and the term eclampsia appeared in a Treatise on Gynaecology written by Varandaeus in 1619 (Sibai, 1996) and clonic spasms in association with pregnancy were described by Dew in 1694 (Sibai, 1996).

How pregnancy excites or aggravates hypertension remains unresolved despite decades of intensive research. Eclampsia is thought to be a derangement from the normal physiological immunological mechanism that exempts the fetus (semi allograft) from being rejected by the mother (Raghupathy, 2001; Chaouat, 2007). Some features suggest that PE/EC may have an immune pathology (Saito *et al.*, 2007): these include the first pregnancy preponderance of the disorder, short sexual cohabitation period, diminished exposure to sperm (assisted reproduction technique) and an increased incidence of EC when a woman changes partner in secondary pregnancy (Trupin*et al.*, 1996; Salomon and Seely, 2004). Also susceptibility to EC varies from one woman to another, indicating genetic and immune factors, (Molvarec *et al*., 2010; Aggarwal *et al.*, 2010).

Sargent *et al.*, (1982) reported that normal pregnancy but not preeclamptic pregnancies, did not elicit any cell mediated immunity to fetal human leucocyte antigen (HLA) indicating an abnormal immune response in pregnancy complicated by PE and EC.

According to the cytokine hypothesis of hypertension in pregnancy, the reduction in the utero-placental perfusion pressure and the ensuing placental ischemia are thought to increase the release of cytokines such as tumor necrosis factor (TNF)-α from the placenta into the maternal circulation. The increase in plasma would then lead to

maternal endothelial cell dysfunction, generalized vascular changes and hypertension (Comrad*et al.,* 1998).

Maternal immunologic factors such as cytokines, Natural Killer cells (NK)-cells, activated macrophages and lymphocytes have been shown to be associated with several pregnancy complications such as recurrent spontaneous miscarriages, PE and preterm delivery (Raghupathy, *et al.*,2001and Saito *et al.,* 2007). Cytokines have been shown to play a vital role in normal pregnancy both in maintenance of placental growth and in the modulation of maternal immune reactivity to prevent rejection of the conceptus (Wegmann, *et al.*, 1993).

The maternal immunologic state that is most conducive to successful pregnancy is said to be maintained by local secretionof Thelper (Th) 2 cytokines while Th1 reactivity is associated with EC (Azizieh *et al.,* 2005; Saito *et al.,* 2007), recurrent spontaneous miscarriage and preterm delivery(Romero *et al.,* 2006; Saito, *et al.,* 2007).

TheCD4+T helper cells polarizes majorly along Th1and Th2 cytokine productionprofiles in response to activation by an antigen.Th1 cells secrete the pro-inflammatory cytokines IL-2, IFN-γ, TNF-α and TNF-β which activate macrophages and cell-mediated reactions relevant to cytotoxic reactions and delayed-type hypersensitivity (Mosmann and Sad, 1996). Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 which induce vigorous humoral immunity (Mosmann and Sad, 1996).Th1 cytokines tend to be inflammatory cytokines while Th2 tend to have anti-inflammatory properties. There seems to be a balance between inflammatory cytokines and anti-inflammatory cytokines in healthy condition. Deviations from this balance may be associated with disease conditions like PE and EC.

Also numerical imbalances between helper and suppressor T lymphocytes have been demonstrated in various immunologically based diseases by using monoclonal antibodies that identify subsets of Tcells (Bach and Bach 1981). It thus seemed reasonable to examine the peripheral blood lymphocytes subsets using monoclonal antibodies to determine if any numerical imbalance exists in the immunologically compromised mother during normal pregnancy and pregnancy complicated by EC.This is necessary in order to know whether there is a shift or not in the T cell subsets and cytokines of cellular immunity.

1.1 Definition of the Research Problem

Normal, successful pregnancy is associated with a Th2phenomenon due to a shift in cytokine pattern from Th1 to Th 2 (Piccini, 2010).There is a natural homeostasis between the fetus and the mother, to avoid a feto-maternal graft rejection. An altered adaptation of the maternal immune system to the fetus might be responsible forpregnancy complications like EC, preterm delivery, anemia or infection in pregnancy. A current theory is that women who developedEC have abnormal immunological responses to the fetus and placenta and that hypertension and proteinuria represent clinical signs of a mild form of fetal rejection, while severe forms of EC represent spontaneous abortion and fetal death

What are the alterations or changes that may occur in lymphocyte sub populations, cytokines, and pregnancy specific beta-1 glycoprotein levels in pregnancies complicated by eclampsia? This question therefore, forms the basis of this study.

1.2 Justification for the Study

Preeclampsia and EC has remained a significant public health threat in both developed and developing countries, contributing to maternal and perinatal morbidity

and mortality globally (Shah, 2009). However, the impact of the disease is felt more severely in developing countries including Nigeria (Acquaah-Arhin and Kwawukume, 2003; Adamu *et al.*, 2003; Igberase and Ebeigbe, 2006; Olufemi*et al.*, 2006) with a range of between 700 and 1000 per 100,000 deliveries. World Health Organization (WHO) reported that in Nigeria, 37,000 women die annually due to PE and EC related complications. It accounts for up to 40% of maternal death in Northern Nigeria (WHO, 2004).

Hypertensive disorders remain among the most significant and intriguing unresolved problems in obstetrics. In their study, Dekker and Sibai (1998) reported a possible role of immune maladaptation in the pathophysiology of PE. They indicated that women who are most likely to develop PE/EC have significantly lower proportion of Th cells compared to women who remain normotensive. With CD4+T heper cells being the major producers of cytokines, it is believed that the equilibrated balance of Th1 versus Th2 cytokines defines the welfare of an organism (Miossec, 1993; Charlton and Lafferly 1995). An important role in the maintenance of pregnancy is played by cytokines, where successful fetal outcome is correlated to the production of Th2 cytokines and fetal rejection to Th1 cytokines and this has been demonstrated in mice (Kohchi, *et al*., 1991; Chaouat, *et al.*, 2007) and needs to be investigated in human pregnancy. This suggests that there is an immune etiology in pregnancy complicated by EC (Dekker *et al.*, 1998).

Multiple lines of evidence also suggest an immunomodulatory function of Pregnancy Specific Glycoprotein (PSG) in preventing rejection of the allotypic fetus (Majumdar *et al.*, 1982). Low PSG levels in human maternal circulation are associated with threatened abortions (Bohn *et al.*, 1976, Hau *et al.*, 1985). Reports show that PSG induces production of anti-inflammatory cytokines from monocytes and macrophages

in vitro (Wessells *et al.,* 2000; Snyder *et al.,* 2001) consistent with the observation of PSG-mediated switching of the immune system from a predominantly Th1 response to Th2 response, which is considered more compatible with successful pregnancy (Motran *et al.,* 2003).

The role ofTlymphocytes subpopulations, cytokines as well as PSG-1in pregnancies complicated by EC in Nigerian pregnant women has not been well studied. Pertinent studies include '*the role of T lymphocytes and cytokines in acute crisis states'* (Musa*et al.,* 2008) and the other on '*Pregnancy Specific beta-1 glycoprotein (PSG-1) in normal pregnancy*' (Onyemelukwe *et al.*, 1985). This study is therefore, necessary to determine Tcell subpopulations, serum levels of pro-inflammatory cytokines (TNF-α, IL-2), anti-inflammatory cytokines (IL-4, IL-10) and PSG-1 in eclamptic womenin Kaduna State, Nigeria.

This research work will provide information that will assist in clinical decision and intervention towards effective management of women with EC; it will also assist in the understanding of the mechanism of immunopathology, prevention and overall reduction in the incidence of maternal mortality due to EC. In respect to policy making, research and millennium development goals (MDGs), this study will contribute to advancement in the medical industry by adding to the knowledge of the health professionals in the management of EC.

1.3 Aim and objectives

1.3.1 Aim

The aim of the study is to evaluate the cell mediated immune system through the study of T lymphocyte subsets, cytokines and pregnancy specific beta-1 glycoprotein (PSG-1) in normal pregnancies and those complicated by EC.

1.3.2 Objectives

i) To determine the CD3+, CD4+ and CD8+T- lymphocyte populations in the peripheral blood of patients with eclampsia, normal healthy pregnant and non pregnant women controls.

ii) To analyze for serum TNF-α, Interleukin (IL)-2, IL-4, IL-10 and PSG-1 in patients with EC, normal healthy pregnant and non pregnant controls.

iii) To compare the levels of Tcell sub populations, cytokines and PSG-1from patients with EC, PC and NPC..

1.4 Research Hypothesis

1.4.1 Null hypothesis

T cells subsets; cytokines and PSG-1 are not altered in pregnancy complicated by EC.

1.4.2 Alternate hypothesis

T cells subsets; cytokines and PSG-1 are altered in pregnancy complicated by EC.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Immunity and Importance in Clinical Practice.

Recent discoveries in immunology have revolutionized clinical practice. Beyond vaccine production, immunology has found wide application in medicine in the area of diagnosis, treatment and research (Bach and Bach, 1981; Eghtesad *et al.*, 2010and Zingone *et al.*, 2013). Advances in immunology and indeed cell mediated immune responses offer better understanding of the mechanism of diseases and provide targets for possible therapeutic intervention (Keiser *et al*., 2009). Manipulation of the immune system has aided in successful organ transplantation and immunotherapy with anti-inflammatory cytokines in patients with various inflammatory disorders including organ transplant recipients, rheumatoid arthritis Crohn's disease, and psoriasis (Asadullah *et al*., 2003). Investigators reported that failure in successful organ transplantation is traceable to cellular immunity and suppressing the immune response can prevent transplant rejection (Barry *et al*., 2007; Eghtesad et *al.*, 2010).

2.1.1 Clusters of differentiation (CD) and T-lymphocytes subpopulations

The immune system works through leukocytes interacting with each other, with other cells, with tissue matrices, with infectious agents and with other antigens. These interactions are mediated by cell-surface glycoproteins and glycolipids. Antibodies against these leukocyte molecules have provided powerful tools for analysis of their structure, function, and distribution. The associated Clusters of Differentiation (CD) nomenclature is commonly used when referring to leukocyte surface molecules and antibodies against them. Clusters of differentiation provide an essential classification for diagnostic and therapeutic purposes. Also, the discovery of CD provided a forum

for the study of function and distribution of molecules and epitopes, and catalyzed the development of an industry providing reagents for research, diagnosis, and now therapy (Boumsell,1996).The identification of clusters of antibodies with very similar patterns of binding to leukocytes at various stages of differentiation led to the CD nomenclature and provided a common nomenclature that allowed the scientific community to communicate results in a universal language.

T cells are now recognized as CD4+ and CD8+ T-subsets based on their function and T cell receptor surface proteins (Swain 1983). These are measured in the laboratory manually (rosette method) and more recently by immunophenotyping (flow cytometry) using sheep erythrocyte receptor technique and monoclonal antibody. The CD4+ and CD8+ Tcells are otherwise known as T 'helper'/'inducer'and 'suppressor'/'cytotoxic' based on their functional properties. A CD4/CD8 ratio of 1.8 ± 0.`6 has been reported in the peripheral blood of apparently healthy individuals and imbalances in immune disorders such as acquired immune deficiency syndrome (AIDS), autoimmune conditions, bacterial infections and helminth infections (Romagnani, 1991).

2.1.2 Th1/Th2 reactions and cytokines

The response from T-helper lymphocytes present in the human immune system can be divided into two separate and counterbalancing reactions, depending on the type of cytokines that are produced. Human CD4+T- lymphocytes were originally shown to secrete cytokines in two distinct profiles: Th 1 and 2 responses (Del Prete *et al*., 1991; Romagnani, 1994). However, this Th1/Th2 concept, originally described in mice (Mosmann and Coffman 1989), is not as distinct from humans.

T helper (Th) lymphocytes are traditionally classified into the Th1 and Th2 subsets based on their cytokine production pattern (Romagnani 1991). A naïve CD4 + T cell differentiates after activation by antigen into one of several phenotypes, although the main ones are termed Th1 and Th2 (Zhou and Paul, 2008). The major influence on determining which pathway a naïve CD4+T cell will become committed to, is the presence of IL-12 which promotes differentiation into a Th1 cell or IL-4 which promotes differentiation into a Th2 cell. The most important cytokines produced by Th1 cells are IL-2, TNF-α and IFN-γ. Considered to be the main effectors of phagocyte–induced host defense, these cells are highly protective against infections sustained by intracellular agents. On the other hand, Th2 cells produce IL-4, which stimulates Ig E and IgG1 antibody production, IL-5 which promotes the growth and differentiation of eosinophils, and IL-10 and IL-13, which together with IL-4 inhibit macrophage function. Th2 is mainly responsible for phagocyte–independent hostdefense against helminthic infections (Romagnani, 1991). The cytokines produced by these T-cells determine the effector function as well as participate in the development and expansion of each subset. As it is, IFN-γ produced by Th1 cells promotes further Th1 differentiation and inhibits Th2 cells. Vice versa, the IL-4 produced by the Th2 cells promotes Th2 differentiation and, accompanied by the anti-inflammatory IL-10, produced mainly by activated macrophages, inhibits the activation of Th1 cells. Thus, Th1 and Th2 cells amplify their own subset and each cross- regulates the other. Once an immune response develops along one pathway, it becomes increasingly polarized in that direction (Janeway 2005). However, immune cells other than Th1 and Th2 cells, as well as other cells in the body can produce cytokines. Therefore, in general terms of the entire immune system the Th1/Th2

responses are called type 1 and type 2 responses, but for simplicity, in this study these responses will be called Th1 and Th2.

2.1.3 Pro- and anti-inflammatory cytokines

The balance between the effects of pro- and anti-inflammatory cytokines is thought to determine the outcome of the disease (Dinarello 2000). The timing and the location of pro- and anti-inflammatory cytokines plays a role in the outcome of a disease, as shown by Widhe *et al.* (2002), where patients with non-chronic neuroborreliosis showed higher levels of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α in cerebrospinal fluid compared to patients with the chronic form of the disease. Furthermore, the non-chronic patients showed higher serum levels of the anti-inflammatory cytokine transforming growth factor (TGF-β) 2 than chronic patients. These findings can be interpreted as implying that an early pro- inflammatory reaction is needed in the central nervous system to get rid of the infection, but at the same time serum levels of TGF-β2 rise to control the systemic immune response. In line with this reasoning, the chronic patients failed to mount the initial necessary pro-inflammatory response and the subsequent systemic anti-inflammatory response (Widhe *etal*., 2002).

The damaging effects of excessive amounts of pro-inflammatory cytokines are also seen in sepsis. Normally, secreted TNF-α helps to contain an infection locally, but in the case of sepsis TNF-α is released systemically, which leads to vasodilatation and loss of plasma volume owing to increased vascular permeability, resulting in shock. In septic shock, disseminated intravascular coagulation is also triggered by TNF-α, leading to hypercoagulation in capillaries and massive consumption of clotting protein (Janeway, 2005).

As reviewed in Dinarello (2000), the concept of pro- and anti-inflammatory cytokines is based on whether genes coding for the synthesis of mediator molecules are up-regulated during an immune response. Pro-inflammatory genes are those coding for phospholipase A2 (PLA2), cyclooxygenase (COX)-2 and inducible nitric oxide (NO) synthase, since these are the enzymes that increase the synthesis of platelet-activating factor and leukotrienes and NO. The cytokines IL-1 and TNF (and in some cases IFN-γ) are effective in stimulating the expression of these genes. Chemokines, such as IL-8, are chemotactic and facilitate the passage of leukocytes into the tissue from the vascular compartment. In addition, they are also pro-inflammatory molecules. Anti-inflammatory cytokines block or suppress the intensity of the pro-inflammatory cascade. For example, IL-4, IL-10, IL-13 and TGF-β suppress the production of IL-1, TNF, IL-8 and vascular adhesion molecules. Therefore, the balance between the effects of the pro-inflammatory and anti-inflammatory cytokines is thought to determine the outcome of the disease.

2.1.4 Role of cytokines in pregnancy

Despite the unique ability of the immune system to highly specify antigen recognition, its susceptibility to cytokines allows these molecules to dominate all kinds of immune reactions. Cytokine is a term used to describe a broad range of structurally diverse molecular families and individual proteins best known for their many roles in immune system function.They are often pleiotrophic molecules with diverse and cell type specific activities including regulating cell activation, hematopoiesis, apoptosis, cell migration and cell proliferation (Oppenhhein, 2001). In this capacity, they are involved in virtually all aspects of both innate and adaptive immune responses. Cytokines are required for the initiation and progression of normal immune responses (Fearon and Locksley, 1996). Several disease conditions have their

onset and course influenced by cytokines (Keicolt-Glaser *et al.,* 2002). Cytokines are known toplay a major role in successful pregnancy development. Recently, it was discovered that for pregnancy to succeed, three immunologic phases are necessary: viz the event of implantation and first trimester require a strong inflammatory response to ensure the adequate remodeling of the uterine epithelium and the removal of cellular debris following the implantation of the blastocyst. The first trimester pregnancy is hallmarked by pro-inflammatory events. The second immunologic phase of gravidity consists of second and third trimesters. This is the phase of fetal growth and development when an anti-inflammatory state is established (Mor and Cardenas, 2010). Finally, delivery represents the third immunological phase of pregnancy, when pro-inflammatory events dominate again and promote uterine contractions to deliver the fetus and the placenta (Mor and Cardenas, 2010).

2.2Normal Pregnancy

For mammalian pregnancy to succeed, large physiological adjustments are required in the mother. These changes result from signals passing between the conceptus (especially the trophoblast) and the mother throughout pregnancy. Every system in the body is affected, including the immune system, which is part of a complex signaling system between cells that have developed the ability to recognize self and non-self. The immunology of pregnancy is the result of the combination of signals and responses originating from the maternal immune system and the fetal-placental immune system. The signals originated in the placenta will modulate the way the maternal immune system will behave in the presence of potential dangerous signals (Wegmann *et al*., 1993; Mor and Cardenas 2010).

Data from inbred mice and less good evidence from human pregnancy (Wegmann*et al.,* 1993) suggest that the maternal immune response may be modulated away from

cellular responses towards humoral immunity. For example, antibody production during pregnancy tends towards the IgG1 isotype and away from the complement fixing IgG2a isotype, particularly for antibodies against fetal alloantigens. In addition, cytotoxicity mediated by non-specific natural killer cells, seems to be dampened by inhibition of the Th1 helper cells, which produce cytokines such as interleukin-2 and interferon gamma. Activation of natural killer cells in pregnant mice is known to result in fetal resorption. This suggests a bias towards the production of helper cells in pregnancy and may be a protective mechanism tending to promote fetal survival (Wegmann *et al*., 1993). It has been observed that the cytokines produced by activated lymphocytes during murine pregnancy tend to favour antibody production over cytotoxic responses (Dudley *et al*., 1993). According to Dudley *et al.*, (1993), the effect is most prominent within the uterine decidua.

Another possible immune modification might be some adaptation to inhibit non - specific complement mediated damage to trophoblast. Holmes and Simpson (1992) have shown that this is achieved at the level of the trophoblast itself without requiring any systemic dampening of the complement cascade, which presumably needs to remain intact if the mother is to combat infections adequately. The human trophoblast expresses three membrane bound complement regulatory proteins, which protect it from maternal complement mediated damage arising not only from activation of the classic or alternate pathways but also after systemic complement activation in response to microbial infection.

Understanding what normal healthy pregnancy is will help in appreciating pregnancy complications.

Successful pregnancy depends on adaptation of the woman's immune system to tolerate a genetically foreign fetus (Hunt *et al.,* 2005). Although the mechanisms that promote the survival of the conceptus are still largely unknown, it has become increasingly clear that the maternal immune tolerance towards the fetus is the interaction of a jigsaw puzzle of actors - cells, serum protein, hormones, cytokines, enzymes and neurotransmitters.

The lack of HLA antigens on the syncytiotrophoblast and the presence of only the non-classic HLA G antigen on the cytototrophoblast cells precludes the fetal trophoblast from playing any part in currently recognized types of allogeneic immune reactions (Billington, 1992*).* All these reactions depend on the cellular recognition processes associated with the major histocompatibility complex classes (MHC) I and II. Not only, therefore, will the maternal immune system fail to be stimulated by allogeneic trophoblast, but allogeneic trophoblast cannot be the target for otherwise armed maternal cytotoxic T cells. Furthermore, according to current understanding of the phenomenon of ''MHC restriction'', the absence of classic major histocompatibility complex antigens on the trophoblast will prevent the co-recognition of any other form of cell surface antigens that it might express. The mother is not "immunodeficient"; in terms of classic mechanisms dependent on T cells she remains immunocompetent throughout pregnancy (Manyonda *et al*., 1993*).* However, redeployment of T lymphocytes and altered Tcell subpopulations in the maternal immune response in normal pregnancy has been documented (Jenkins, 1977; Hunt *et al*., 2005).It has also been postulated that the adaptive immune response skews towards Th2-type response during normal pregnancy (Wegmann *et al*., 1993). The resultant shift in the cytokine pattern from Th1toTh2 is reported to exhibit immunosuppressive properties important for the survival of the fetus (Maksheed *et*

al., 2000). The T helper (Th2) cells secrete specific cytokines (IL-4, IL-6, IL-10, and IL-13) that promote implantation and their dysfunction may favor EC (Whitecar *et al.,* 2001, Hayashi *et al.,* 2004).

Records show that high concentrations of pregnancy associated hormones in blood such as progesterone and Human Chorionic Gonadotrophin (HCG), Human Placental Lactogen (HPL) and pregnancy specific beta-1glycoprotein (PSG-1) inhibit Thl-driven cellular immune responses in normal pregnancy (Snyder *et al*., 2001; Majumdar*et al.,* 1982; Khil *et al.,* 2007). Multiple lines of evidence suggest an immunomodulatory function of pregnancy specific protein (PSG) in preventing rejection of the allotypic fetus (Majumdar *et al.,* 1982). Low PSG levels in human maternal circulation are associated with threatened abortions (Bohn and Weinmann, 1976; Hau*et al.,* 1985). Reports show that PSG induces anti-inflammatory cytokines production from monocytes and macrophages *in vitro* (Wessells *et al.,* 2000, Snyder *et al.,* 2001) consistent with the observation of PSG-mediated switching of the immune system from a predominantly Th1 response to Th2 response, which is considered more compatible with successful pregnancy (Motran *et al.,* 2003).

2.2.1 Maternal –fetal interface

In a pregnant woman, local adaptation of the maternal immune system allows for successful co-existence between the mother and the semi-allograft that is the fetus and placenta expressing both maternal (self) and paternal (non-self) genes (Hunt 2006; Mor and Abraham, 2009). Cytotoxic adaptive immune responses are diminished, bypassed or even abrogated while regulatory adaptive immunity is enhanced (Guerin *et al*., 2009; Leber *et al*., 2010).By contrast, innate immunity remains intact serving two purposes: to continue to provide host defense against infection and to interact

with fetal tissues to promote successful placentation and pregnancy (Naganta and Schust, 2010 and Zhang*et al.,* 2011).

The fetal allograft is exposed to the maternal immune system at the placenta and fetal membranes (the amnion and chorion), collectively described as the maternal-fetal interface. On the fetal side of the interface, the placenta and membranes enclose the fetus and are derived entirely from fetal tissue forming a specialized epithelial surface within the placenta; fetal syncytiotrophoblast cells directly contact maternal blood for nutrient exchange. On the maternal side of the interface, the uterine tissues in contact with the placenta and fetal membranes, the decidua, is rich in specialized maternal immune cells including lymphocytes and macrophages (Guant and Ramin, 2001). Despite the prolonged direct exposure of decidual leukocytes and maternal blood to fetal antigens, the immune system does not recognize the fetus as foreign. Several mechanisms underlie this maternal tolerance of fetal tissues.

During pregnancy the maternal immune system must perform the double task of protecting the mother against infections while not rejecting the fetus (Manyonda *et al*., 1993). While numerous mechanisms are proposed to be working locally at the feto-maternal interface (Mellor and Mum, 2000; Cauchetenx *et al*., 2003), others appear to act on the maternal immune system as a whole, and in particular at the level of T and B lymphocytes. It has been shown that the considerable increase in steroid hormone levels associated with pregnancy has a negative impact on the production of new clones of maternal T and B lymphocytes, through thymic involution (Clarke and Kendall, 1994; Tibbetts *et al.,* 1999) and down regulation of B lymphopoiesis in the bone marrow (Medina and Kincade, 1993), respectively. Reports show that pregnancy estrogen levels cause a major decrease in the production of B cells (Medina *et al.,*

1994). This same group has shown that hormonal levels maintained during lactation prolong this effect.

Another mechanism involves the cytokines. There is much evidence that cytokines play a very important role in the maintenance of pregnancy by modulating immune and endocrine systems. Placental tissue produces cytokines and hormones that are essential to the regulation of the feto-maternal unit. Decidual lymphocytes express cell surface markers for activation such as CD69 and HLA-DR, and these cells secrete many cytokines. Recent studies suggest that in pregnant women, cytokines produced by Th2 cells predominate over those produced by Th1 cells, resulting in the maintenance of pregnancy.The Th2 derived cytokines, IL-4 and IL-6, induce the release of HCG from trophoblasts, and the HCG stimulates progesterone production from corpus luteum in pregnancy. Progesterone stimulates the secretion of Th2 and reduces the secretion of Th1 cytokines. Thus Th2 type cytokines appear to contribute to the maintenance of pregnancy by controlling the immune and endocrine systems and promoting the function of the trophoblasts at the implantation site (Miyaura and Iwata 2002, and Khil *et al.,* 2007). Therefore, the immune system has adaptive strategies tomaintain the Th 2 response in pregnancy.

It has been demonstrated that immune cells were dominant within the decidua in early pregnancy in humans. During normal pregnancy, the human decidua contains a high number of immune cells, such as macrophages, natural killer (NK) cells and, regulatory T cells (Treg) (Aluvihare *et al*., 2004; Mor *et al.,* 2006). B cells are absent from the adaptive immune system, but T lymphocytes constitute about 3–10% of the decidual immune cells (Wicherek, 2009). During the first trimester, NK cells, dendritic cells, and macrophages infiltrate the decidua and accumulate around the invading trophoblast cells (Ashkar, *et al.*, 2000; Shimada, 2006). Interestingly,

depletion of immune cells, instead of helping the pregnancy, terminates the pregnancy. Thus, deletion of macrophages, NK cells, or dendritic cells (DC) has deleterious effects on placental development, implantation, or decidual formation (LeBounteller and Piccini 2008). In other studies, it has been shown that in the absence of NK cells, trophoblast cells are not able to reach the endometrial vascularity leading to termination of the pregnancy (Hanna *et al.,* 2006). These studies suggest that uterine NK (uNK) cells are critical for trophoblast invasion in the uterus. Similarly, depletion of DCs prevented blastocyst implantation and decidual formation (Bimberg 2007). Indeed, the study suggests that uterine detritic cell (uDC) are necessary for decidual formation and may affect the angiogenic response by inhibiting blood vessel maturation (Bimberg, 2007).

2.2.2 Humoral immunity

Humoral immunity (‘Antibody mediated’ immunity), results from recognition of pathogens by specific antibodies. It is most effective against extracellular pathogens and is essential for fighting many bacterial infections. Majority of microbial antigens are endocytosed by antigen-presenting cell (APC), which include macrophages, dentritic cells, and B lymphocytes processed and presented preferentially in association with MHC class II for the production of antibody by the B cells. Extra cellular microorganisms are coated by antibodies, which then mediate uptake of the pathogens by phagocytic cells, including neutrophils and macrophages. Presentation of the bacterial antigens on the surface of the macrophage then stimulates lymphocytes specific to the pathogen, and the B cells to produce more antibodies to control the infection. This humoral immune response is augmented by T-helper 2 (Th2)-type lymphocytes which provide co-stimulation and induces replication of the

B cells. The Th2 response predominant during pregnancy results in adequate antibody-mediated immunity to pathogens (Gaunt and Ramin 2001).

It has been shown that the considerable increase in steroid hormone levels associated with pregnancy has a negative impact on the production of new clones of maternal B lymphocytes, through thymic involution (Clarke and Kendall, 1994; Tibbetts *et al.*, 1999) and down regulation of B lymphopoiesis in the bone marrow (Medina and Kincade, 1993). In pregnancy estrogen levels cause a major decrease in the production of B cells (Medina *et al.*, 1994).

2.2.3 Cell mediated immunity (CMI)

The CMI is essential for controlling intracellular pathogens. It involves lymphocyte recognition of cell-associated foreign antigens, followed by destruction of the infected cell. In contrast to humoral immunity, this arm of response is stimulated by Thelper 1 (Th1) lymphocytes and the cytokines they release. The most important effectors of the CMI response, cytotoxic T lymphocytes, are the immune cells that recognize foreign antigens on the surface of the infected ‘’self’’ cells. Cells infected with viruses or other intracellular pathogens are cytotoxic T lymphocytes’ most common targets. The cell mediated immune response is critical for controlling such pathogens because their intracellular location shelters them from antibody binding.

The activated CD4+ lymphocyte is a very strigently controlled process and only occurs in the presence of both antigen (peptides/class 1 MHC) and costimulatory molecues on the surface of professional antigen presenting cells (APCs). The most potent Tcell co-stimulatory molecules are CD80 and CD86 and are expressed mainly on matured dentritic cell (DC), activated macrophages and B cell lymphocytes. Naïve CD4+ Tcells can differentiate after activation by antigen into one of several

phenotypes, although the main ones are termed Th1 and Th2 (Mosmann and Sad, 1996).These subsets are defined by the particular cocktail of cytokines produced, and therefore their effector functions in host defense.

The CD4 positive T helper subsets include Th1, Th2 and Th17 cells (Saito *et al.,* 1999). The polization of T helper response along a Th1 or a Th2 pathway has aided in the understanding of numerous basic immunologic pathways and disease. As more knowledge is gained, it is apparent that these subsets represent the tip of the iceberg in the multiple checks and balances that are characteristic of the immune system. After the discovery of the Th1/Th2 dichotomy, research was dedicated to understanding their cytokine polarization. The emphasis is then shifted to examining the signals required for Th1/Th2 differentiation and has shifted yet again to examining the role of dentritic cell subsets, rediscovered regulatory T cells, and newly discovered Th17 cells in modulating immune responses.

There is evidence that each of these subsets is involved in the defense against a certainmicroorganisms. Th1 are pivotal in defense against intracellular microorganisms in general and mycobacteria in particular. Patients with mutations in the IFN-γ receptor or IL-12 receptor of the Th1 path present with recurrent infections with mycobacteria and Salmonella. Th1 and Th17 cells play major roles in autoimmunity, whereas Th2 cells are the hallmark of atopic disease. T regulatory (Treg) cells represent a major subset of CD4+ T cells that may be involved in regulating and attenuating the activity of the three T helper subsets.

T cells may be classified as Th1 cells, which synthesize interleukins (IL-2), interferon (IFNγ) and tumor necrosis factor (TNF)β and induce cellular immunity, or Th1 cells, which synthesis IL- 4, IL-5, IL-6 and IL- 10, and induce antibody production.

Wegman *et al.* (1993) suggested that in pregnant women, cytokines produced by Th2 cells predominate over those produced by Th1 cells resulting in the maintenance of pregnancy.

2.2.4The Th 1 / Th 2 paradigm shifts in pregnancy

Emphasis on cell-mediated immunity versus humoral immunity changes according to the type of T-helper lymphocytes response to an infectious threat. Multiple factors, including the cytokine environment and co-stimulatory molecules present during activation of the T-helper cell, determine the development of either Th1 or Th2 helper phenotype. The hypothesis is that in addition to hormonal factors that affect the Th1/Th2 balance, macrophages present at the maternal-fetal interface release predominantly Th2-stimulating cytokines and contribute to the overall dominance of humoral immunity during pregnancy (Szekere-Bartho, 2002). In addition to stimulating B lymphocytes, Th2 cells suppress the cytotoxic T lymphocyte, decreasing the robustness of cell-mediated immunity. In the uterine deciduas, the Th2 cytokine environment favors activation of B lymphocytes, resulting in stimulation of antibody secretion and suppression of cell – mediated immunity (Szekere-Bartho, 2002). This phenomenon is often referred to as the Th1 – Th2 shift of pregnancy and is thought to contribute to maternal tolerance of the fetus by suppressing the anti-fetal cell-mediated immune response. Th2 family cytokines such as IL-4, IL-6, IL-10, and IL-13 exhibit immunosuppressive properties and are of importance for survival of the fetus (Szekere-Bartho, 2002).

An evolving model of pregnancy-associated immune changes suggests that the hormonal environment of pregnancy contributes to local suppression of cell-mediated immunity at the maternal-fetal interface while mediating a systemic change toward

Th2 dominance (Wegman *et al*., 1993). That the local Th1/Th2 shift may also influence the systemic maternal immune response during pregnancy is evidenced in pregnant patients with autoimmune disorders. Women with rheumatoid arthritis, a predominantly cell-mediated autoimmune disorder, tend to experience remissions during pregnancy (Elenkove *et al.,* 2001). Similarly; patients with multiple sclerosis have fewer exacerbations while pregnant but worsening symptoms during the postpartum period (Rumarker and Anderson 1995). Systemic lupus erythematosis, however, a predominantly antibody-mediated automimmune disorder, often worsens during pregnancy, perhaps due to increased immunoglobulin synthesis and decreased clearance of immune complexes resulting from robust Th2 activity. These well-studied changes in severity of autoimmune disorders during pregnancy illustrate systemic immune alterations that occur in conjunction with the Th1/Th2 shift and contribute to increased susceptibility to preeclampsia, (Bardequez *et al*., 1991) as well as infections with intracellular pathogens (Okoko *et al*., 2003).

2.2.5 The immunological phases of 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 (Wegmann *et al*., 1993; Mor and Cardenas 2010) 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 (Loke and King, 2000). The reason for these contradictory results may be due to 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, 2007).

Implantation, placentation, the first and early second trimester of pregnancy resemble "an open wound" that requires a strong inflammatory response. During this first stage, the blastocyst has to break through the epithelial lining of the uterus in order to implant; damage the endometrial tissue to invade; followed by the trophoblast replacement of the endothelium and vascular smooth muscle of the maternal blood vessels in order to secure an adequate placental-fetal blood supply (Dekel *et al.*, 2010). All these activities create a veritable "battleground" 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 (Mor and Cardenas, 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"). Thus, the first trimester of pregnancy is a proinflammatory phase (Morand Cardenas, 2010).The second immunological phase of pregnancy 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.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 the 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. In conclusion, pregnancy is a pro-inflammatory and anti-inflammatory condition, depending upon the age of gestation (Mor, 2009; Mor and Cardenas, 2010).

2.2.6 The role of Pregnancy Specific beta-1 glycoprotein (PSG-1)

Pregnancy specific beta-1-glycoproteins (PSGs-previously known as SP), are members of the carcinoembryonic antigen (CEA) family and are highly glycosylated proteins secreted by the placenta (Bohn, 1971). Human PSGs have been detected in the maternal serum as early as three (3) days post fertilization, coinciding with the attachment of the blastocyst to the uterine wall (Dimitriadou *et al.,* 1992), and during normal pregnancy, PSG molecules are released into the maternal circulation reaching 200-400µg/ml in the serum at the end of gestation (Lin *et al*., 1976). It is thought to play a crucial role in supporting gestation and fetus protection against the maternal immune system (Blanchon *et al.,* 2001). Sacks *et al*. (1999) proposed that pregnancy-specific factors induce the suppression of a specific arm of the maternal response and assumes the central role in maternal immunological adaptation.

During normal pregnancy, a state of systemic suppression of the maternal immune system seems to be present (Matthiesen *et al.,* 1996; Bermasand Hill,1997).PSG isolated from human placenta have an inhibitory effecton phytohaemaglutinin or allogenetically stimulated lymphocytes (Majumdar *et al*., 1982; Harris *et al.,* 1984). PSG could modulate the immune response by inducing the secretion of anti-inflammatory cytokines such as IL-10, IL-6 and TGF-β by human and murine cells **(**Bebo and Dveksler 2005 and Martinez *et al.,* 2012). Previous studies have reported

abnormal levels of PSGs in complicated pregnancies and have shown their importance for the maintenance of a successful pregnancy (Grudzinskas *et al*., 1983).

2.3 Preeclampsia (PE) and Eclampsia (EC)

Preeclampsia and EChave been identified as clinical conditions since the time of Hippocrates. The term preeclampsia, sometimes referred to as toxaemia of pregnancy, describes the development of new onset hypertension (140/90mmHg or a single recording of a diastolic blood pressure of 110mmHg) and proteinuria (300mg/24hours) or ≥ 2+ on dipstick testing of random urine samples after the 20th week of gestation (Chesley, 1985, Ramsy, 2003; Weinstein, 2005). Preeclampsia, if left untreated, can develop into EC, the life-threatening occurrence of seizures during pregnancy (Shah, 2009).

It was not till the 17th Century that the convulsions in pregnancy were distinguished from tonic clonic fits in the non- pregnant state (Chesley, 1980).The term eclampsia appeared in a treatise on gynecology written by Varandaeus in 1619 (Sibai, 1996) and clonic spasms in association with pregnancy were described by Dew in 1694 (Sibai, 1996)

Globally, PE/EC remains one of the leading causes of maternal morbidity and mortality with higher incidences in developing than developed countries due to inadequate and poor utilization of maternal health care facilities (Dolea and Abouzahr, 2003; Vanderjagt *et al.*, 2004). Therefore it is a public health issue. Unfortunately, there has been little progress in preventing the disorder compared to advances made in eliminating other major obstetric complications (Sibai, 1998).

Although the cause of PE/EC remains elusive, the origin of the condition is recognized as lying in the placenta. Evidence for this lies in the case that PE/EC occurs only in the presence of pregnancy and resolves after delivery of the placenta. Delivery of the placenta is the 'cure' (Poston *et al.*, 2006). Various prevention strategies including administration of calcium supplementation, antioxidants (vitamin C and E) and aspirin showed initially promising results in small trials, but these findings were unable to be replicated in larger clinical studies. In fact, supplementations with vitamin C and E have now been associated with low birth weights (Poston *et al.*, 2006).

2.3.1 Diagnosis of preeclampsia/eclampsia

Pre-eclampsia the precursor of EC is diagnosed when a pregnant woman develops high blood pressure (two separate readings taken at least six hr. apart of 140mmHg or more in systolic blood pressure and/or 90mmHg or more in diastolic blood pressure) and≥300 mg of protein in a 24-hr. urine sample. A rise in baseline blood pressure (BP) of 30 mmHg, systolic or 15 mmHg diastolic, while not meeting the absolute criteria of 140/90mmHg, is still considered important to note, but is not considered diagnostic."Severe PE" involves a BP over 160/110 mmHg with additional symptoms. Edema (especially in the hands and face) was originally considered an important sign for a diagnosis of PE, but in current medical practice only hypertension and proteinuria are necessary for a diagnosis. However, 'Pitting' edema (unusual swelling, particularly of the hands, feet, or face, notable by leaving an indentation when pressed on) can be significant, and should be reported to a health care provider.

Preeclampsia is often asymptomatic, and so its detection depends on signs or investigations. In general, none of the signs of PE are specific, and even convulsions

in pregnancy are more likely to have causes other than EC in modern practice. Diagnosis, therefore, depends on finding a coincidence of several pre-eclamptic features, the final proof being their regression after delivery.

Some women develop high blood pressure without proteinuria (protein in urine), which is called pregnancy-induced hypertension (PIH) or gestational hypertension. Both PEand PIH are regarded as very serious conditions and require careful monitoring of mother and baby as prevention of any disease process requires the ability of methods for prediction of those at high risk for the disorder.

Although numerous clinical and biochemical tests have been proposed for prediction or early detection of PE, most remain unrealistic for general use in most developing countries. At present, there is not a single reliable and cost-effective screening test for PE which can be recommended for use in most developing countries (Wagner, 2004). Studies on uterine artery Doppler and first-trimester maternal serum markers for early detection of PE have shown some promise (Kharb, 2009; Audibert and Boucoiran, 2010; Yale, 2010). But there is not enough evidence to suggest their routine use in clinical practice in resource poor settings (Papageorghiou and Campbell, 2006).

In developed countries, promising blood tests for quantification of the substances of placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 with a high sensitivity and specificity have been developed (Andraweera *et al*., 2012). It has been reported that Vascular endothelial growth factor (VEGF) family proteins do not have sufficient statistical power to accurately predict late-onset pre-eclampsia, but may be useful in detection of early-onset PE (Andraweera *et al.*, 2012).

2.3.2Epidemiology and risk factor

Preeclampsia affects approximately 2-8% of all pregnancies worldwide (Moodley, 2004, Weinstein, 2005 and, Ghulmiyyah and Sibai, 2012) with onset of symptoms in the late second or third trimester, most commonly after the 32nd week. Some women will experience PE as early as 20 wk, though this is rare. It is much more common in women who are pregnant for the first time (Hauth *et al.,* 2000). In these women, the disease is mostly mild, the onset mostly near term or intrapartum (75% of cases), and only conveys a negligible increased risk for adverse pregnancy outcome (Hauth *et al.,* 2000). By contrast, frequency and severity of the disease are substantially higher in women with multifetal gestation (Wen *et al.,* 2004), chronic hypertension (Sibai, 2002), previous PE (Sibai, 2002), pregestation diabetes mellitus (Caritis *et al.,* 1998) and per-exiting thrombophilias (Alfirevic *et al*., 2002).

Several risk factors have been identified with increased risk of PE (Dekker and Sibai 2001). Generally, PE is a disease of first pregnancy(Hauth *et al.,* 2000). The risk increases in those with limited sperm exposure with the same partner before conception (Dekker and Sibai 2001; Einarsson *et al.,* 2003; Dekker and Robillard, 2003). The protective effect of long term sperm exposure with the same partner may explain the high risk of PE in women younger than twenty 20 years. A previous abortion (spontaneous or induced)or healthy pregnancy with the same partner is associated with reduced risk of PE, but this protective pregnancy effect is said to be lost with a change in partner-so called‘dangerous fathers’ (Oudejans *et al.*,2004). Report shows that men who fathered a preeclamptic pregnancy were nearly twice as likely to father a preeclamptic pregnancy in a different woman, irrespective of whether she had already had a preeclamptic pregnancy or not (Lie *et al.*, 1998). Thus, mothers have a substantially increased risk in their second pregnancy if they become

pregnant by a man who had fathered a preeclamptic first pregnancy in another woman. This risk is as high as the average risk in first pregnancies (Lie *et al.,* 1998).

Advances in assisted reproductive technology (ART) have introduced several challenges for the maternal immune system that also increased the risk of PE. These include women who are older than 40 years, infertile and pregnant by donated gametes, which are donor insemination, oocyte donation, or even embryo donation. The use of gamete donation will affect the maternal-fetal immune interaction, and many of these women will have multifetal gestation (Wen *et al.,* 2004).

Obesity is a definite risk for PE. Risk increase with body mass index (Dekker and Sibai, 2001; O'brien *et al*., 2003). The worldwide increase in obesity is likely to raise the frequency of PE (Dekker and Sibai, 2001; Cedergren, 2004). Obesity has a strong link with insulin resistence, which is a risk factor for PE (Dekker and Sibai, 2001; Wolf *et al*., 2002). The exact mechanism by which obesity or insulin resistance is associated with the disorder is not completely understood. Possible explainations are; increased shear stress, associated with a hyperdynamic circulation; dyslipidemia or enhanced cytokine mediated oxidative stress; amplified sympathetic activity and increased tubular sodium resorption, as well as direct interference of insulin resistance and therefore hyperinsulinaemic state with placentation (Dekker and Sibai, 2001).

Healthy pregnancy itself is a state of inflammation, at least in the third trimester (Mor and Cardenas, 2010). Based on this concept, PE is not a separate entity, but simply the extreme end of a range of maternal systemic inflammatory response engendered by the pregnancy itself. The corollary is that any thing that would enhance this factor would predispose to PE (Redman and Sergent 2003). Any factor that increases the

maternal inflammatory response such as infections and rheumatic diseases will also predispose women to PE (von Dadelszen and Magee, 2002; Boggess *et al*., 2003).

Eclampsia may also occur in the immediate post-partum period. This is referred to as "postpartum EC". The most dangerous time for the mother is the 24–48 hrs postpartum and careful attention should be paid to ECsigns and symptoms (Munjuluri *et al.*,2005).

The impact of PE/EC is felt more severely in developing countries (Acquaah-Arhin and Kwawukume, 2003; Adamu *et al.*, 2003; WHO, 2004; Igberase and Ebeigbe, 2006; Olufemi *et al.*, 2006) includingNigeria with a range of between 700 and 1000 per 100,000 deliveries. The WHO (2004) reported that in Nigeria, 37,000 women die annually due to PE and EC related complications. It accounts for up to 40% of maternal deaths in Northern Nigeria.

2.3.3 Etiology of preeclampsia

The PE syndrome is thought in many cases to be caused by a shallowly implanted placenta which becomes hypoxic leading to an immune reaction characterized by secretion of up-regulated inflammatory mediators from the placenta acting on the vascular endothelium (Burne, 2006). The shallow implantation is thought to stem from the maternal immune system’s response to the placenta and refers to evidence suggesting a lack of established immunological tolerance in pregnancy. This results in an immune response against paternal antigens from the fetus and its placenta (Burne, 2006). In some cases of PE it is thought that the mother lacks the receptors for the proteins the placenta uses to down regulate the maternal immune system's response to it (Moffett and Hiby, 2007).

Report shows that many miscarriages are as a result of immunological disorderswhere the mother's immune system "unleashes a destructive attack on the tissues of the developing child" (Wegmann *et al*., 1993). In many cases of the PE syndrome, however, the maternal response to the placenta appears to have allowed for normal implantation. It is possible that women with higher baseline levels of inflammation stemming from underlying conditions such as chronic hypertension or autoimmune disease may have less tolerance for the inflammatory burden of pregnancy (Redman *et al.,* 1999).

2.3.4 Eclampsia and hypothesis

Writings describing EC have been traced as far back as 2200B.C (Lindheimer *et al*, 1999) yet the precise of EC is not known and many hypotheses have been advanced to explain the potential causes of EC.The current one include the following:

2.3.4.1 Abnormal trophoblastic invasion

Pregnancy is a normal physiologic process that requires a synchronized adaptation of multiple organ systems. Increased perfusion is characteristic of the hemodynamic alterations commonly seen in successful pregnancy (Magness *et al*., 2000). Blood vessels are remodeled to form a low-resistance arteriolar system, which serves to promote delivery of a greatly increased blood and oxygen supply to the developing fetus (Osol and Cipolla, 1993). Increased endothelium-mediated release of vasodilators in response to vasoconstrictors serves to promote perfusion to the fetus under potentially adverse conditions (Bird *et al.,* 2000). Inadequate remodeling and cytotrophoblast invasion of spiral arteries are believed to promote the abnormal placental development seen in EC (Zhou *et al.,* 1993; Fisher, 2004).

In normal implantation, the uterine spiral arteries undergo extensive remodeling as they are invaded by endovascular trophoblasts. In EC, however, there is incomplete trophoblastic invasion. In this case, decidual vessels, but not myometrial vessels, became lined with endovascular trophoblast. Meekins *et al.*(1994) described a continuum in the number of spiral arteries with endovascular trophoblast in placentas of normal women and in those with EC. Studies showed that the magnitude of the defective trophoblastic invasion of spiral arteries correlates with the severity of the hypertensive disorder (Madazli *et al.*, 2000).Using electron microscopy;DeWolf*et al.* (1980) examined arteries taken from the utero-placental implantation site. They observed that early preeclamptic changes included endothelial damage, insudation of plasma constituent into the vessels wall, proliferation of myointimal cells and medial necrosis. They found that lipids accumulated first in myoitimal cells and then in macrophages. Typically the vessels affected by atherosis develop aneurismal dilatation and are frequently found in association with spiral arterioles that have failed to undergo normal adaptation (Khong, 1991). Obstruction of the spiral arteriolar lumen by atherosis may impair placental blood flow. It is thought that these changes cause placental perfusion to be pathologically diminished which eventually lead to the EC syndrome (Lain and Roberts, 2002; Redman and Sargent, 2003).

2.3.4.2 Immunological factors.

There is evidence to support the theory that EC is immune mediated.Certainlythemicroscopic changes at the maternal – placental interface are suggestive of acute graft rejection (Labarere, 1998).

Report shows that the risk of EC appreciably enhanced in circumstances where formation of blocking antibodies to placental antigenic sites might be impaired (Beer, 1978). Risk factors for EC related to paternal antigen exposure suggest that

immunologic dysfunction at the fetal-maternal interface may contribute to EC. More over, women without an intact immune system because of untreated human immunodeficiency virus (HIV) rarely develop PE, whereas HIV treatment and resulting immune reconstitution bring the risk of PE to levels seen in the general population (Brettle, 1992).

Formation of blocking antibodies may arise in situations in which effective immunization by a previous pregnancy is lacking, as in first pregnancies; or where the number of antigenic sites provided by the placenta is usually great compared with the amount of antibody as with multiple fetuses (Beer, 1978). "Immunization" from prior abortion does not seem to occur. Strickland *et al*. (1986) analyzed outcome of over 29,000 pregnancies at Parkland Hospital and reported that hypertensive disorders were decreased only slightly in women who previously had miscarried and were now having their first advanced pregnancy. The immunization concept was supported by their observations that EC developed less often in multiparas who had a prior term pregnancy. Other studies have shown that multiparous women impregnated by a new consort have an increased risk of EC (Mostello *et al*, 2002; Trupin *et al*., 1996).Risk factors for EC related to paternal antigen exposure suggest that immunologic dysfunction at the fetal-maternal interface may contribute to EC.

Bardeguez *et al.*, (1991) reported that beginning from an early trimester women who are most likely to develop EC have a significantly lower proportion of helper T cells (Th) compared with that of women who remain normotensive.Reports have shown that T helper cells secrete cytokines (IL-4, and IL-10) that promote implantation and their dysfunction may favor EC (Whitecar *et al*., 2001; Hayashi *et al*., 2004).

In women with anticardiolipin antibodies, placental abnormalities and EC develop more commonly. This however, was recently challenged by Brauch *et al.*(2001).According to Katano *et al*. (1996), antibodies associated with beta 2-glycoprotein 1 appear most relevant. Immune complexes and anti-endothelial cell antibodies may also be involved (Taylor and Robert, 1999).

These studies provide compelling evidence that immune dysregulation is involved in EC pathogenesis, but the mechanisms by which this occurs have thus far not been well elucidated.

2.3.4.3 Thevasculopathy and the inflammatory changes.

In many ways, inflammatory changes are a continuation of the placental cause (s) discussed in the abnormal trophoblastic invasion above. In response to placental factors released by ischemic changes, or any other inciting cause, a cascade of events is set in motion (Redman and Sergeant, 2003). The decidua also contains an abundance of cells that when activated, can release noxious agents (Staff *et al.*, 1999). These then serve as mediators to provoke endothelial cell injury.

Redman *et al*., (1999) have proposed that the endothelial cell dysfunction associated with EC can result from a "generalized perturbation of the normal, generalized maternal intravascular inflammatory adaptation to pregnancy''. In this hypothesis, EC is considered a disease due to an extreme state of activated leukocytes in the maternal circulation (Faas *et al,* 2000; Gervasi *et al.*, 2001). Cytokines such as tumor necrosis factor-α (TNF-α) and the interleukins may contribute to the oxidative stress associated with EC. Oxidative stress is characterized by reactive oxygen species and free radicals that lead to formation of self propagating lipid peroxides (Manten *et*

al.,2005) these in turn generate highly toxic radicals that injure endothelial cells, modify their nitric oxide (N0) production and interfere with prostaglandin balance.

Diedrich *et al*. (2001) did not detect any lipid hydro peroxides in 38 preeclamptic women, 10 of whom had HELLP syndromes (Hemolysis, elevated liver enzymes, and low platelets). Other consequences of oxidative stress include production of lipid laden foam cells seen in atherosis, activation of microvascular coagulation seen in thrombocytopenia, and increased capillary permeability seen in edema and proteinuria. These observations on the effect of oxidative stress in EC have given rise to increased interest in the potential benefit of antioxidants to prevent EC (Chappell *et al.,* 1999; Zhang *et al.*, 2002). Antioxidants are a diverse family of compounds that function to prevent over production of and damage caused by noxious free radicals. Examples of antioxidants include vitamin E, vitamin C (Ascrobic acid) and beta - carotene.

2.3.4.4 Nutritional factors

A number of dietary deficiencies or excesses over the centuries have been blamed as the cause of EC. Dietary taboos that have included meat, protein, purines, fat, dairy products, salt, and other elements have been advocated at times. Observations and theories led to studies of dietary deprivation of various sorts that many times were models of absurdity. In recent times, sanity and a scientific approach has prevailed, for example, blood pressure in non pregnant individuals is affected by a number of dietary influences, including minerals and vitamins. Some studies have shown a relationship between dietary deficiencies and the incidence of EC (Zhang *et al.,* 2002). As such, studies of supplementation with various elements such as zinc, calcium, and magnesium to prevent EC have followed. Other studies, such as the one

by John *et al.*(2002), showed that in the general population a diet high in fruits and vegetables that have antioxidant activity is associated with decreased blood pressure. This is related to the case-control study by Zhang *et al.*, (2002) in which the incidence of EC was doubled in women whose daily intake of ascorbic acid (Vitamin C) was less than 85mg.

Obesity in non-pregnant individuals causes endothelial activation and a systemic inflammatory response associated with atherosclerosis (Ross, 1999).1n the study of pregnant women by Wolf *et al.* (2002), C-reactive protein, an inflammatory marker was shown to be increased in obesity, which in turn was associated with EC.

2.3.4.5 Genetic factors

The predisposition to hereditary hypertension undoubtedly is linked to EC (Ness *et al*., 2003), and the tendency for PE/EC is inherited. Chesley and Cooper.(1986) studied sisters, daughters, granddaughters, and daughters-in-law of eclamptic women and concluded that PE/EC is highly heritable. The single-gene model, with a frequency of 0.25, best explained their observation. Other investigators cited possibilities of polygenic inheritance (Trogstad *et al*. 2004). A study that included almost 1.2 million births reported a genetic component for gestational hypertension as well as EC (Nilson *et al*., 2004). They reported 60 % concordance in monozygotic female twin pairs which was much higher than that found by Treloar *et al*.(2001) and Kilpatrick *et al*. (1989), but Hayward *et al*.(1992), reported an association between the histocompatibility antigens HLA-DRA4 and protein hypertension. Hoff *et al*. (1992) concluded that a maternal humoral response directed against fetal anti-HLA-DR immunoglobulin antibody might influence the development of gestational hypertension. A number of single-gene mutations have been studied in women with

PE. Ward *et al*. (1993) reported that women heterozygous for angiotensinogen gene variant T235 had a higher incidence of PE and fetal growth restriction.

Morgan *et al*., (1995, 1999) did not confirm this, but they did find that women homozygous for this mutation had abnormal trophoblastic invasion. Some of the inherited thrombophilias predispose women to PE. Polymorphisms of the gene for TNF, lymphotoxin-α andinterleukin-1β have been studied with varying results (Lachmeijer*et al.*,2001; Livingston, 2001).

2.3.4.6 Infection factor

Ekwempu (1980) reported that‘as far back as 1899 Stroganoff, thought that PECas an infective fever because it was common under conditions of overcrowding and lying-in wards; its incidence varied from year to year just like infection; some people were more susceptible than others, implying some form of immunity and it was contagious as crops of cases always come from neighborhood’. Ekwempu. (1980) observed a high incidence of EC with rise in incidence of infection. He observed that about 60 to 70 %of patients with EC were febrile on admission and therefore postulated that infection of any type may be related in a casual way to EC. Report showed no scientific evidence that pregnant women are more susceptible to infectious diseases than nonpregnant women (Hart, 1988). There is in general, neither viral nor other infections (such as tuberculosis) that neither seem to occur more commonly in pregnancy, nor are localized infections more likely to become generalized, as occurs in immunosuppressed patients. Rather, reports show that falciparum malaria is more common and severe in first rather than subsequent pregnancies (Bruce-Chwatt, 1983). The potential effects of pregnancy on human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS) are of growing concern. Evidence

does not suggest that pregnancy makes any difference to the natural course of HIV infection in women (Brettle, 1992). In summary, the current hypothesis for the pathogeneses of EC is that an immunologic disturbance causes abnormal implantation resulting in decreased placental perfusion. The abnormal perfusion stimulates the production of substances in the blood that activate or injure endothelial cells. The vascular endothelium provides a single target for these blood-borne products, which explain the multiple organ system involvement in EC.

2.4. Pathophysiology of PE/EC

The placenta appears to be central in the aetio-pathogenesis of EC. Selective fetocide in a pregnancy with discordant twins, resulting in involution of the affected placenta, has been reported to reverse PE (Heyborne and Porreco, 2004). Molar pregnancies, in which there is no fetus, increase the risk of EC. Placentae affected by EC often show signs of vascular disease such as infarcts, sclerotic narrowing of arteries and arterioles, and fibrin deposition with thrombosis (Karumanchi *et al.,* 2005; Baumwell and Karumanchi, 2007). Although lacking in sensitivity and often more predictive of PE when employed in the second trimester, abnormal Doppler flow can be visualized in some cases of PE (Cnossen *et al*., 2008). Together, these findings suggest that the placenta and its abnormal vascular development are fundamental to EC. Once proposed as the inciting event in EC abnormal placentation is now more often viewed as part of the disease evolution.

In normal pregnancy, maternal uterine spiral arteries are remodeled by invading fetal cytotrophoblastic cells. These invading cytotrophoblasts undergo adhesion receptor switching, shedding those molecules displayed by epithelial cells and taking on the phenotype of endothelial cells (Zhou *et al*., 1997b). Narrow, high-resistance blood

vessels are thus transformed into dilated high-capacitance vessels, as vascular smooth muscle is replaced by fibrinoid. Aided by the effects of oestrogen, proximal portions of the spiral arteries dilate and there is an overall increase in uterine blood flow from approximately 45 ml/min during menstruation to 750 ml/min at term (Gabbe *et al.*, 2007).

In PE, fetal cytotrophoblastic cells only invade the decidual portions of the maternal arteries, not the myometrial segments. The cytotrophoblasts do not undergo the adhesion receptor switching characteristic of normal pregnancy (Zhou *et al*., 1997a). This shallow invasion and aberrant pseudovasculogenesis result in decreased placental perfusion. As pregnancy progresses, abnormal placentation produces placental hypoxia and ischemia as the compromised uterine blood flow is unable to maintain the demands of the growing placenta and fetus. Placental factors are then potentiated that may in part be responsible for the maternal syndrome of PE. The end result is generalized endothelial dysfunction manifested by hypertension, proteinuria, and thrombotic microangiopathy (Davison *et al*., 2004). Proponents of a primarily immunologically mediated mechanism for the pathogenesis of EC have proposed a more proximal event triggering the development of PE. Following blastocyst formation, syncytiotrophoblast shedding into the maternal circulation is a normal part of pregnancy, but is increased and abnormal during PE (Sibai *et al*., 2005). Normally, older and naturally apoptotic syncytiotrophoblasts form syncytial knots that are released and travel to the maternal pulmonary bed where they are ingested and processed by macrophages (Sibai et al., 2005). In PE this process of syncytio-trophoblast renewal is overactive and complicated by necrosis and aponecrosis of the syncytiotrophoblast particles. These smaller necrotic particles, fragments and nucleic acids can enter into the maternal circulation and provoke a systemic inflammatory

response including an activated endothelium (Ilekis *et al*., 2007). This theory explains why markers of inflammation are elevated as early as the first trimester in PE (Mor and Cardenas 2010).

Historically, studies have focused on cytokine elaboration, indicators of endothelial dysfunction, markers of oxidative stress, and the imbalance between vasoactive substances. Endothelin-1, nitric oxide, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1 α, IL-1β, Fas ligand, neurokinin B, asymmetric dimethyl L-arginine (ADMA), and thromboxane A_2 among others, have been evaluated. None of these factors has been clearly proven to be causae of PE/ EC (Roberts and Cooper, 2001 and Karumanchi*et al*., 2005).

An imbalance between vasoconstrictor and vasorelaxant factors was proposed as a possible cause of the hypertension accompanying EC. The vasoconstrictor thromboxane A_2 is increased out of proportion to prostaglandin I_2 in PE but administration of aspirin, a thromboxane inhibitor, does not consistently prevent EC in pregnant women (Caritis *et al.*, 1998).

2.4.1 Placentation abnormalities

On the basis of the observation that the only definitive cure for EC is delivery of the placenta and that women who experience a molar pregnancy, in which a placenta develops without a fetus, frequently develop severe PE, it is reasonable to assume that the placenta plays a central role in the pathogenesis of the disease. Pathologic examination of placentas from preeclamptic pregnancies generally reveals placental infarcts and sclerotic narrowing of arteries and arterioles, with characteristic diminished endovascular invasion by cytotrophoblasts and inadequate remodeling of the uterine spiral arterioles (Lin *et al.*, 1997). Although gross pathologic changes are

not always seen in the placentae of women with EC, placental profiles including abnormal uterine artery Doppler and placental morphology have been used to identify a subset from a cohort of high-risk women who go on to develop the syndrome (Hershkovtz *et al*, 2005). Uterine artery Doppler studies that assess the pulsatility index (PI) reveal increased uterine vascular resistance well before the clinical signs and symptoms arise (Li *et al*., 2005; Gomez*et al*., 2006). Mechanical constriction of the uterine arteries produces hypertension, proteinuria, and, in some species, glomerular endotheliosis, supporting a causative role for placental ischemia in the pathogenesis of PE (Alexander *et al*., 2004).

Mammalian placentation requires extensive angiogenesis to establish a suitable network for the supply of oxygen and nutrients in the fetus. A variety of pro- and antiangiogenic factors are elaborated by developing placentas. It is believed that placental angiogenesis is defective in PE, as evidenced by failure of the cytotrophoblasts to convert from a more epithelial to endothelial phenotype, based on cell surface marker studies(Zhou *et al.*,1997b; Alexander *et al*., 2004). Normally, invasive cytotrophoblasts downregulate the expression of adhesion molecules that arecharacteristic of their epithelial cell origin.They adopt a cell-surface adhesion phenotype that is typical of endothelial cells, a process that is referred to as pseudovasculogenesis (Zhou *et al.,* 1997b). In PE, cytotrophoblast cells fail to undergo this switching of cell-surface integrins and adhesion molecules (Li *et al*., 2005). This abnormal cytotrophoblast differentiation is an early defect that may eventually lead to placental ischemia. Others have demonstrated that hypoxia-inducible factor-1 is up regulated in PE and suggest that it and its target genes may play a central role in the abnormal differentiation phenotype of PE (Cowden*et al*., 2005; Nevo *et al*., 2006). Whether this lack of conversion of cytotrophoblasts to an

endothelial phenotype in women with PE is a primary or secondary event remains uncertain (Fisher, 2004)

2.4.2 The maternal syndrome

The abnormal placentation that results from failure of trophoblast remodeling of uterine spiral arterioles is thought to lead to the release of secreted factors that enter the mother's circulation, culminating in the clinical signs and symptoms of PE. All of the clinical manifestations of PE can be attributed to glomerular endotheliosis, increased vascular permeability, and a systemic inflammatory response that results in end-organ damage and/or hypoperfusion. These clinical manifestations typically occur after the 20th week of pregnancy (Chesley 1985).

2.4.2.1 Hypertension

Accommodation of normal pregnancy includes a decrease in both systolic and diastolic blood pressure (BP) as a result of a decrease in systemic vascular resistance secondary to vasodilatation. Relaxin, which is released from the ovaries under the influence of human chorionic gonadotrophin, up regulates nitric oxide synthase (NOS) (Fujiyama *et al*., 2001), the enzyme that generates nitric oxide (NO) from arginine, *via* the endothelial endothelin B receptor (Jeyabaan *et al*., 2003). In EC, derangement of endothelial-derived vasoactive factors is thought to result in the predominance of substances that are vasoconstrictors (endothelin, thromboxane A_2) over vasodilators (NO, prostacyclin). Hypertension, defined as repeat BP measurements ≥140/90 mmHg, results from abnormal vasoconstriction.

Normal pregnancy is accompanied by increased production of NO and its second messenger, cyclic guanosine 3′5′ monophosphate (Conrad and Vernier, 1989), with a parallel increase in renal expression of constitutive NOS (Conrad *et al.,1993*;

Fujiyama *et al*., 2001) . In the pregnant rat, an infusion of NG-nitro-l-arginine methyl ester (L-NAME), an exogenous inhibitor of NOS, has been shown to replicate some of the hemodynamic features of EC (Danielson and Comrad, 1995). L-Arginine supplementation reversed these adverse effects of L-NAME on pregnancy, attenuating hypertension, significantly decreasing proteinuria, and reducing the proportion of injured glomeruli (Helmbrecht *et al.,* 1996). However, in humans, evidence to support a role of NO deficiency in the pathogenesis of the hypertension in EC has been conflicting. Although elevated circulating levels of asymmetric dimethyl arginine, an endogenous inhibitor of NOS, has been a consistent finding in pregnancies that are complicated by PE, plasma concentrations are typically very low with a narrow distribution among healthy adults, making quantification extremely challenging and the clinical significance of the finding uncertain (Holden *et al.,* 1998; Pettersson *et al.,* 1998; Teerlink, 2006). Furthermore, L-arginine supplementation has not conferred significant benefit in women with pregnancies that are complicated by PE (Staff *et al.,* 2004; Hladunewich *et al.,* 2006).

Another hypothesis considered the possibility that an early gestational exaggeration of the normal accommodation to pregnancy can be used to identify and may be pathogenic condition in PE (Bosio *et al.,* 1999). A longitudinal study that used Doppler echocardiography in 400 primigravidas throughout pregnancy noted a significantly increased cardiac output without any difference in peripheral vascular resistance in the 24 women who eventually developed PE compared with healthy control subjects (Bosio *et al*., 1999).This increased cardiac output was followed by a marked reduction in the cardiac output and increased peripheral vascular resistance with the onset of the clinical syndrome. This notion of a crossover in the hemodynamic profile in women who develop ECresulted in a handful of studies that

used β blockers in a preventive manner (Easterling, *et al*., 1999; Carr *et al*., 2001; Easterling*et al*., 2001). These studies were typically small and/or uncontrolled. Furthermore, reduced fetal growth was noted in the women who received the β blockers, possibly because of an overaggressive decrease in the cardiac output (Easterling*et al*., 2001).

Attention has again turned to the renin-angiotensin system (RAS) to provide a pathophysiologic understanding for the hypertension of PE. In normal pregnancy, all components of the RAS are upregulated, but resistance to the pressor effects of AngII allows for normal to low BP (Holden *et al*., 1998). Similarly, reduced sensitivity of the renal circulation has been demonstrated in pregnant rats as AngII infusion failed to decrease GFR, renal plasma flow (RPF), and urine flow (Novak *et al.,* 1997). One explanation may be increased plasma levels and urinary excretion rates of Angiotensin a potent counterregulator of AngII, documented in human pregnancy (Merrill *et al.,* 2002). Angiotensin was demonstrated to be significantly decreased in women with PE compared with normal pregnant control subjects (Merrill *et al.,* 2002). It is interesting that evidence supports decreased levels of renin, AngI, and AngII in women with EC compared with normal pregnancy (Brown *et al*., 1997). Despite this, enhanced vascular sensitivity to components of the RAS is seen in women with EC. Enhanced vascular sensitivity to an angiotensin infusion can identify women who are at increased risk for the development of PE (Brown *et al*., 1990).

Studies have identified an autoantibody of the IgG subclass in the plasma of women with EC that is capable of stimulating the AT1 receptor (Wallukat *et al.,* 1999). When serum from preeclamptic women was added to cultured neonatal rat cardiac myocytes, the chronotropic effect could be blocked by losartan, confirming the effect was mediated via the AT1 receptor (Wallukat *et al*., 2003).Furthermore, the

autoantibody might stimulate heterodimerization between the AT1 receptor and the B2 receptor for bradykinin (Wallukat *et al*., 2003). This may play an important role in the enhanced vascular sensitivity to angiotensin. It might also induce the production of reactive oxygen species, which block cytotrophoblast invasion *in vitro* and may relate to shallow trophoblastic implantation, thus accounting for several of the clinical features of EC (Xia *et al*., 2003). This autoimmune activity wanes after delivery (Wallukat *et al*., 1999). More recently, these antibodies also were found in patients with acute vascular rejection (Dragun *et al.*, 2005), suggesting that they may play a role in other forms of endothelial injury as well.

2.4.2.2 Decreased glomerular filtration rate (GFR)

Healthy pregnant women exhibit marked glomerular hyperfiltration peaking above normal, nongravid levels by 40 to 60% (Chapman *et al.*, 1998).This hyperfiltration seems to result primarily from depression of the plasma oncotic pressure in the glomerular capillaries. The reduction of the plasma oncotic pressure in pregnancy is attributable to two phenomena. The first is a hypervolemia-induced hemodilution that lowers the protein concentration of plasma that enters the glomerular microcirculation. The second is an elevated rate of RPF. Hyperperfusion of glomeruli blunts the extent to which the oncotic pressure can increase along the glomerular capillaries during filtrate formation. In PE, variable degrees of renal insufficiency are associated with a characteristic glomerular lesion, "glomerular endotheliosis."Precise physiologic measurements in conjunction with immediate postpartum biopsies were used to examine the determinants of the GFR in women with PE as compared with healthy gravid control subjects (Lafayette *et al.*, 2003).

Study used a semiquantitative scale to grade the endotheliosis that was present on biopsy specimens that were taken from women with EC approximately one week before delivery (Strevens *et al*., 2003). They noted moderate to severe endotheliosis in all women with significant hypertension and proteinuria before delivery. Of interest, women with nonproteinuric gestational hypertension and normal pregnant women also exhibited endotheliosis but to lesser degrees, suggesting that pregnancy-induced hypertension may in some cases reflects an earlier or milder form of the same pathology (Fisher *et al*., 1981).

2.4.2.3 Proteinuria

Preeclampsia is differentiated from gestational hypertension by the presence of proteinuria and is the most common cause of nephrotic syndrome in pregnancy (Brown *et al.*, 2001). The quantity of protein that is excreted in the urine varies widely. Significant protein excretion is defined as ≥300 mg in a 24-hr. urine collection or 1+ or greater on urine dipstick testing of two random urine samples that are collected at least 4hrs apart (Brown *et al.*, 2001).

Numerous studies have used a variety of methods to examine the biochemical constitution of preeclamptic urine, including protein selectivity indices, with variable results. Generally, urine from EC has demonstrated poor selectivity and has not differed significantly from other forms of primary renal disease (Simanowowitz and MacGregor, 1974). Glomerular proteins of intermediate size, such as albumin, have been identified alone or in combination with varying degrees of tubular proteins, such as B_2-microglobulin, reflecting the tubular damage that can occur in severe PE (Quaas *et al*., 1987; Winkler *et al.*, 1988).

2.4.2.4 Coagulopathy andhaemolysis elevated liver enzymes and low platelet count(HELLP) syndrome

In EC, endothelial injury may also become manifest as a low-grade coagulopathy with increased fibronectin, increased platelet aggregation, shortened platelet survival, and depressed antithrombin III levels (Roberts, 1998). The heamolysis elelavated liver enzymes and low platelets count (HELLP) syndrome develops in up to 10% of pregnancies with severe PE, and evidence exists to suggest that it is not simply an epiphenomenon of extreme hypertension. Plasma concentrations of cellular fibronectin have been shown to be consistently higher throughout pregnancy in woman who develops EC compared with healthy control subjects. In addition, markers of platelet activation, including β-thromboglobulin, as well as assays of platelet aggregation has been demonstrated to precede the clinical manifestations of the disease (Balleger *et al.*, 1992; Felfernig-Boehm *et al.*, 2000). The HELLP syndrome is more common, probably about 1 in 500 maternities, but may be as dangerous as eclampsia itself. These two major maternal crises can present unheralded by prodromal signs of PE. Cerebral hemorrhage is a lesion that can kill with EC. In that cerebral hemorrhage is a known complication of severe hypertension in other contexts, it must be assumed that this is a major predisposing factor in this situation, although this has not been proven. Uric acid levels may help to predict maternal complications among patients with EC according to a systematic review and decision analysis (Koopmans *et al.,* 2009). In very rare cases, temporary vision loss can occur due to partial or complete cortical blindness by petechial hemorrhages and focal vasogenic edema in the occipital cortex (Cunningham, *et. al*., 1995; Do, *et al*., 2002).

2.4.2.5 Seizures

Seizures with other neurologic symptoms, including headache and visual disturbances (Koopmans *et al*.,2009), complicate approximately 5 of every 10,000 live births, with

a declining incidence as a result of improved prenatal care with expedited delivery and, possibly, the widespread use of magnesium sulfate (Altman *et al.*,2002). The precise mechanism that is responsible for the development of seizures is not clear, but proposed theories include cerebral vasospasm, edema, and the possibility that severe hypertension might disturb cerebral autoregulation and disrupt the blood–brain barrier

2.4.2.6 Circulating angiogenic factors in eclampsia

Recently, two endogenous anti-angiogenic proteins of placental originsoluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (Ito *et al*., 2002) have been suggested, on the basis of rodent models, to play a causal role in the pathogenesis of EC (Maynard *et al.*,2003; Venkatesha *et al.*,2006). SFlt1 is a secreted protein, a splice variant of the vascular endothelial growth factor (VEGF) receptor Flt1, which lacks the transmembrane and cytoplasmic domain of the membrane-bound receptor. Circulating in the blood, it acts as a potent antagonist to VEGF and placental growth factor (PlGF). Both VEGF and PlGF are made by the placenta and circulate in high concentration during pregnancy. Circulating sFlt1 levels are greatly increased in women with PE even before the onset of clinical symptoms (Levine *et al.,* 2004). Consistent with the action of the circulating protein to bind PlGF, free PlGF levels are also decreased in preeclamptic women before the onset of clinical symptoms (Levine *et al.,* 2004). When administered to pregnant and non-pregnant rats, sFlt1 produces a syndrome of hypertension, proteinuria, and glomerular endotheliosis that resembles PE (Maynard *et al.,* 2003). It has also been shown that VEGF induces endothelial fenestrae *in vitro*, and the loss of 50% of VEGF production in the mouse glomerulus leads not only to glomerular endotheliosis but also to loss of glomerular endothelial fenestrae similar to what is noted in human PE (Eremina *et al*., 2003). Antagonists of

VEGF, used in-antiangiogenic oncology trials, sometimes produce hypertension and proteinuria in humans (Yang *et al*., 2003). Finally, higher circulating levels of the chromosome 13–encoded gene product sFlt1 in pregnancies with trisomy 13 may explain the increased risk for EC in women who carry fetuses with trisomy 13 (Bdolah *et al.,* 2006).

In addition to its role in the pathogenesis of EC, circulating concentration of sFlt1 and PlGF may have important predictive and diagnostic implications. The concentration of sFlt1 starts to rise near the end of the second trimester in women who are destined to have EC; a full 4 to 5 week before clinical manifestations are first detected (Levine *et al*., 2004). By the time preeclamptic manifestations are pronounced, plasma concentrations of sFlt1 are greatly elevated, from two to four times the levels found in normal pregnancy, and are greatest in patients with severe PE. In women who develop EC, there is a modest but significant decrease in PlGF levels beginning as early as the first trimester. From mid-pregnancy onward, the concentration of unbound PlGF in plasma falls significantly lower at the time when sFlt1 levels are rising. Unbound PlGF is also freely filtered into the urine and thus may also serve to predict the subsequent development of EC (Buhimschi *et al.,* 2005).

Endoglin (Eng) is an angiogenic receptor that is expressed on the surface of endothelial cells and placental syncytiotrophoblasts. Eng acts as a co-receptor for TGF-β, a potent proangiogenic molecule. Endoglin messenger ribonucleic acid (mRNA) is upregulated in the preeclamptic pacenta (Venkatesha *et al.,* 2006). Moreover, the extracellular region of Eng is proteolytically cleaved, and soluble Eng (sEng) is released in excess quantities into the circulation of preeclamptic patients. In pregnant rats, sEng exacerbates the vascular damage that is mediated by sFlt1, resulting in severe PE-like illness, including the development of a HELLP-like

syndrome and fetal growth restriction(Venkatesha *et al*.,2006). In explants cultures of trophoblasts from 5 to 8 wk of gestation, monoclonal antibody (mAb) to Eng and antisense Eng oligonucleotides stimulated trophoblast outgrowth and migration (Caniggia *et al*., 1997). TGFβ1 and/or TGF-β3 inhibit trophoblast migration and invasion, and it seems that Eng mediates this effect. Therefore, it has been speculated that production of sEng by the placenta may be a compensatory mechanism to limit the effects of surface Eng. In recent clinical studies, sEng was elevated not only during the disease but also before onset of symptoms (Levine *et al*., 2006). Elevations in sEng were particularly pronounced and, therefore, potentially most useful for prediction in women who developed preterm PE or EC with an infant who was small for gestational age. Although the gestational pattern of sEng concentration tended to parallel the trajectory of the sFlt1/PlGF ratio, multivariate analysis indicated that each was significantly associated with EC. Indeed, a composite measure that incorporated all three angiogenic molecules (sFlt1, sEng, and PlGF) was more strongly predictive of PE than the individual biomarkers (Levine *et al.,* 2006).

2.5Treatment and Prevention

The only known treatments for EC or advancing PE are abortion or delivery, either by induction of labor or Caesarea section. However, post-partum EC may occur up to six weeks following delivery even if symptoms were not present during the pregnancy. Post-partum EC is dangerous to the health of the mother since she may ignore or dismiss symptoms as simple post-delivery headaches and edema. Hypertension can sometimes be controlled with anti-hypertensive medication, but any effect this might have on the progress of the underlying disease is unknown (Wide-Swensson 1995).

2.5.1 Antihypertensive therapy

Antihypertensive may reduce maternal and fetal mortality among pregnancy patients with hypertension as compared to placebo according to a randomized controlled trial (Wide-Swensson, 1995).

2.5.2 Magnesium sulfate

Magnessium sulphate is the anticonvulsant drug of choice in eclamptics (Fathima and Moodley, 2000). In some cases, women with EC can be stabilized temporarily with magnesium sulfate intravenously to forestall seizures while steroid injections are administered to promote fetal lung maturation. When induced delivery needs to take place before 37 weeks gestation, it is accepted that there are additional risks to the baby from premature birth that will require additional monitoring and care.

2.5.3 Dietary and nutritional factors

Studies of protein and calorie supplementation have found no effect on EC rates, and dietary protein restriction does not appear to increase EC rates (Kramer and Kakuma, 2003). No mechanism by which protein or calorie intake would affect either placentation or inflammation has been proposed studies conducted on the effect of supplementation with antioxidants such as vitamin C and E found no change in EC rates (Rumbold *et al.,* 2006).

A study giving calcium supplementation in women with low-calcium diets found no change in EC rates but did find a decrease in the rate of severe preeclamptic complications (Villar *et al.*, 2006). Low selenium status is associated with higher incidence of EC (Rayman *et al.,* 2003).

2.6 Immunology of Preeclampsia/Eclampsia

2.6.1 An imunological insight into the origin of preeclampsia

Although many factors impact EC, including some not yet identified, evidence suggests an association with impaired angiogenesis (Rajkumar *et al.,* 2001; Rana *et al*., 2007), as well as changes in local oxygen tension (Roberts and Gammil, 2005), and immunological alterations in the early placental microenvironment (Redman and Sargent, 2005; Roberts and Gammil, 2005; Sargent, *et al*., 2006).

The early success of pregnancy relies on appropriate implantation and placentation (Red-Horse *et al.,* 2004; Sargent *et al.* 2006). Changes or alteration in these two events can cause a number of immediate or long-term problems including infertility, spontaneous miscarriage, intrauterine growth restriction or pre-term birth, in addition to EC. For instance, deficient implantation may lead to infertility, whereas if placentation is inappropriate, first trimester pregnancy loss or second or third trimester growth restriction may result in EC resulting from placental dysfunction. It alters maternal systems (Sargent *et al.*, 2006), progressing through a pre-clinical stage (early placental developmental arrest) to a clinical stage (deficiencies in late placental development). These placental inadequacies contribute to hypoxic conditions and cause the manifestation of the disease: hypertension, proteinuria, coagulopathy and hepatic dysfunction (Redman and Sargent, 2005).

In the PE syndrome, one of the most important observations that support a diminished placental perfusion is poor vascular remolding of the uterine spiral arteries that irrigate the intervillous space (Roberts and Gammil, 2005). Normal vascular remodeling is achieved by the migration of the differentiated cytotrophoblast; from eight weeks of gestation, two different villous cytotrophoblastic precursors exist which are programmed to differentiate into separate sub populations (James *et al*.,

2005): the syncytiotrophoblast, which is formed by the merging of villous trophoblast, and the extra villous columns. The extravillous trophoblast consists of all the trophoblast located outside the placental villi. It is highly migratory, proliferative and invasive (Zhou *et al.*, 1997a). The cytotrophoblasts that differentiate into the invasive phenotype express new membrane bound molecules, among them the chemokine receptor, CCR10 (Saito and Sakai,2003), and adhesion molecules. In PE, the cytotrophoblast does not acquire the vascular adhesion phenotype since it cannot express the adhesion molecules characteristic of the differentiated invasive extravillous cytotrophoblast [& VB3, & IBI, VE-Cadherin/CD/44, VCAM-1 (Vascular cell adhesion molecule – 1] /106 and PECAM-ICP laterate endothelia. It also maintains the molecule – 1/CD31 cell adhesion phenotype of the cytotrophoblast of the proximal column region expressing & 6B4, & VB6 and E- Cadrin/CD 324 (Zhou *et al.,* 1997b). This contributes to altered trophoblast invasion and failure of vascular remodeling in EC (Roberts and Gammil, 2005).

The invasion of cytotrophoblast involves, in addition to vascular adhesion molecules, a physical approximation of these cells to the extracellular matrix, the degradation of this matrix and their subsequence migration (Lyall, 2006). In order to degrade the extracellular matrix, matrix metalloproteinases (MMPS) are required. MMPS 1, 2, 3, 9 and 14 participate in the invasion in normal human pregnancy with MMPS – 9 being the most abundant due to greater expression during weeks 3 – 9 of human gestation(Huppertz, 2008). In EC, the cytotrophoblast are deficient in MMP – 9 productions and also have a decreased expression of MMP-1 and MMP-7 (Lyall, 2006) the nuclear transcription factor that mediate the signaling of the MMP-3, MMP-9 and MMP-14 genes. ETS2 [V-ets erythroblastosis virus E26 oncogene homolog 2 (Avian)], is considered essential for normal placental function (Yamamoto *et al*.,

1998), although no report has so far demonstrated a deficiency in MMP-1 due to inappropriate expression of ETS2 in EC.

In normal human pregnancies, the differentiation and invasion process of trophoblasts are regulated by the paracrine factors, activin and transforming growth factors β TGFβ. Activin A stimulates the invasive potential of placental cytotrophoblasts. However, since the invivo expression of activin is low, it is more likely that activin that stimulates the trophoblast is derived from maternal tissue. On the other hand, TGF-β isoforms which are mainly inhibitors of trophoblast growth are co-expressedwith their receptors by the cytotrophoblast (Jones *et al.*, 2006). TGF-BI and TGF β-2 are present in the decidua, whereas the extravilloustrophoblast expresses solely TGF β2 (Lyall *et al.,* 2001). TGFβ3 is present only in cells of the immune system, and it is expressed in large concentrations in cases of placental pathology (Jones *et al.*, 2006). TGFB-1 may diminish MMP-9 production.

2.6.2Role of cytokines in preeclampsia/eclampsia.

Normal pregnancy trophoblast displays insensitivity to interferon (IFN), possibly a mechanism to promote feto-placental survival. This insensitivity is due to a lesion in IFN signal transduction (predominantly IFN-α) in the trophoblast owing to a lack of expression of proteins related to the transcription factor-ISGF-3 (IFN stimulated gene factor 3). This may be one of the reasons the normal trophoblast does not express classical polymorphic HLA antigens, such as HLA-A and HLA-B (Cross *et a1.,* 1999),

and only HLA-C molecules have been found in extra villous trophoblast (King *et al.,* 1996).

Evidence shows that INF-γ inhibits the migration of human cytotrophoblasts in explant cultures in a NK dose-dependent manner (Hu *et al.,*2006), and it is found in

high levels in eclamptic women (Arriaga-Pizano *et al.*, 2005; Banerjee *et al.*,2005; Murphy *et a1.*,2009). However, its role during normal pregnancy is still controversial. For instance, primiparous, but not multiparous, IFN-γ knockout mice experience fetal loss (Ashkar *et al*., 2000) and this cytokine can trigger spiral artery modifications (Monk *et al., 2005*).

Interlukin-12 is another cytokine that represses human cytotrophoblast proteases in an IFN--γ-dependent manner and up-regulates other inhibitors, such as tissue inhibitor of metalloproteinases-I (Karmakar *et al.*, 2004).Monocytes of the pre-eclamptic spontaneously synthesize greater amount of IL-Iβ, IL-6 and IL-8 (Luppi and DeLoia, 2006). TNFα and IL-6 are also increased in eclamptic women compared to normal pregnant women (Benyo *et al*., 2001; Gu *et al*., 2008). TNF-α is associated with the increase in CD65+ cells in the eclamptic decidua in humans, and since TNF-α and IL-1β induce the production of monocyte chemotactic protein-I in decidua celIs, they promote the persistence of macrophages at the implantation site and probably participate in the poor endovascular invasion of the trophoblast observed in EC (Lockwood *et al.*,2006).

A cytokine that contributes to protecting the fetus from rejection during pregnancy is IL-10. The IL 10 produced by the trophoblast is able to activate HLA-G expression in trophoblasts in the feto-maternal interface and in monocytes; an effect that is selective for these MHC class-1 molecules (Moreau *et al.*, 1999). Interleukin-I0 levels are decreased both in placenta and in serum of women with EC (Hennessy *et al*., 1999; Makris *et al*., 2006). The lowered levels of IL-I0 in this disease have been associated with the 1082G allele of the IL-I0 gene, which is more frequently found in pre-eclamptic patients (Kamali-Sarvestani *et a1.*,2006), and to an AA genotype in the

promoter region of IL-I 0 in the placenta (Makris *et a1.*,2006). These could be considered as genetic susceptibility factors for the development of EC.

Eclampsia is a two-stage disease, thus it is important to clar.ify that many cytokines could be involved in the pathophysiology of both stages.

2.6.3 Th 1/Th 2 paradigm shift and its relationship with preeclampsia/eclampsia

For many years, the model of immune regulation during pregnancy has been based on a shift of the maternal immune response from Th I toward a Th2 response (Lin *et al.*, 1993; Saito *et al*., 1999; Tsuda *et al.*, 200I). This model is based on the observation that in a non pregnant woman, the response that elicits an antigen presented to a Th 0 lymphocyte will depend in part on the cytokine microenvironment in which this lymphocyte is present. For instance, a cytokine microenvironment rich in IL-12, IL-18 and IFN-γ will favor the production of Th I cells that secrete TNF-α, IL-2 and IFN-γ and promote activation of other cells such as macrophages. T lymphocytes on the other hand in a microenvironment rich in IL-10 and IL-4 will promote the production of Th2 lymphocytes. Th 1 cytokines inhibit Th2 responses and vice-versa. In healthy non- pregnant women there is a balance between Th I and Th2 responses (Sargent *et al.*, 2006), however in pregnancy this balance is significantly altered by the presence of the placenta, since the progesterone, PSG-1 and cytokines it produces modulate the immune cells via the Th2 response (Onyemelukwe *et al.*, 1985; Piccinni *et al.*, 2000).
It has been proposed that in EC the shift toward Th2 probably does not occur, or it is reverted in very early stages of the disease. In consequence, Th I responses are not suppressed and production is normal (Sargent *et al*., 2006). In women with EC, the cytokine profiles in peripheral blood are mainly Th 1 showing an increase in IFN-γand a decrease in IL-4 (Arriaga-Pizano *et al*., 2005). Also, there are increased levels of IL-

10 in serum and placenta in these patients (Hennessy *et al*., 1999).Th2 cells are predominant in the second and third trimesters in normal pregnancy, whereasTh 1subpopulations are most common in eclamptic patients (Saito *et al*., 1999).

It should be emphasized that the concept of Th I / Th2 responses in pregnancy appears to be rather simplistic since the precise nature of the changes in the cytokine profiles is still controversial (Hviid, 2006). Chaouat *et al.*(2004) have addressed the limitations of the Th I ITh2 paradigm in pregnancy since IFN-γ cannot be, by itself, the decisive element because this cytokine is required at low doses for vascular development and the transformation of the uterine spiral arteries necessary for normal implantation. Since normal implantation requires the participation of certain inflammatory responses, this paradigm could be over-simplified especially in pregnancy diseases. Therefore, the role of NK cells and other cytokines such as IL-12, IL-8, etc secreted by immune cells and by other cells should also be considered (Chaouat *et al*., 2004).

Other cytokines also modulate Th1/Th2 responses, such as the TGF-β cytokine family which has been implicated in Th1/Th2 regulation, although the exact role remains controversial (Conboy *et al*., 1997). Prior to studies in knockout mice, it was thought that the three mammalian isoforms of TGF-β (1, 2 and 3) had redundant functions. New evidence has shown that although they participate in a diversity of biological functions, each subtype plays particular roles in immune system regulation (Ingman and Jones, 2008). In mouse studies, TGF-β2 romotes Th 1 cell development in the presence of high concentrations of IL-2 (Hoehn *et al.*, 1995) and IL- 4 (Lingnau *et al.*, 1998). Conversely, Wen *et al*. (2002) have shown in human bronchial epithelial cells that IFN-γ antagonizes the release of TGF-β2, but it also blocks TGF-β1 to a lesser extent.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Area of Study

The study was conducted in Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, General Hospital (GH) Kafanchan, Barau Dikko Specialist Hospital (BDSH), Yusuf Dantsoho Memorial (YDM) Kaduna, and Hajiya Gambo Sawaba General Hospital (HGSGH) Zaria.These sites representSouthern, Central and Northern senatorial zones of Kaduna State, Nigeria. Kaduna State lies between latitude 09°2´N and 11°23´ N and longitude 061°5´E and 08°60´E and shares boundaries with, Plateau and Bauchi States in the East, Nasarawa State and Federal Capital Territory in the South, Katsina, Kano and Zamfara States in the North and Niger State in the West. The State consist of 23 Local Government Areas and is divided into three senatorial zones namely: zone 1 corresponding to northern zone, zone 2 corresponding to central zone and zone 3 corresponding to southern zone (Appendix I).

3.2 Study Design

The study was a comparative cross sectional study and patients were enrolled as they presented. The study was controlled for confounders by matching for age and parity.

3.3 Ethical Approval

Ethical clearance was obtained from the Scientific and Health Research Ethics Committee of the Ahmadu Bello University teaching hospital Shika– Zaria and the Kaduna State Ministry of Health (KSMOH) before commencing the study (Appendix II). Patientsretained the right to deny consent for or opt out of the study at any stage. Patient's confidentiality was maintained throughout the study (Appendix I and II).

3.4 Patients and Controls.

The Patients and controls were recruited from Labour wards, Eclamptic site wards and the ANC, Labor ward/room and Family Planning Unit, after being assessed by competent clinicians within the facilities.

3.4.1 Sample size

The minimum sample size in the study was determined by the formula (Cochran1963):

$n = \frac{Z^2 pq}{d^2}$ = sample size

Where

n= Minimum sample size

Z = is the value of the normal curve corresponding to 95% confidence interval = 1.96

q = 1 – P = 1- 0.02=0.98

p = prevalence of eclampsia=2%, (Olopade and Lawonyin, 2008).

d = is the level of significance or error margin=5%

$$n = \frac{Z^2 pq}{d^2} = \frac{1.96^2 \times 0.02 \times 0.98}{0.05^2}$$

n = 30.1

Therefore the minimum sample size for eclampsia at 2% was 30 and this was made up to 38 to control for attrition.

3.4.2 Clinical evaluation and selection of patients and controls.

After obtaining an informed written consent (Appendix III and IV) study participants were evaluated and blood pressure measured by the collaborating clinician at the ANCs of the Obstetrics and Gynaecology Department of the respective hospitals.

Participants who fulfilled the entry criteria (detailed below) were enrolled for the study. Participants' personal data such as age and parity, etc were sourced from each subject in addition to the data resulting from the clinical and laboratory examination and were entered into the study profoma (Appendices V, VI and VII).

3.4.3 Inclusion criteria

3.4.3.1 Group I (Eclampsia-EC)

This group included 38 pregnant women selected according to the following criteria: pregnant women diagnosed by the clinician as eclamptic in their third trimester with identifying features of high blood pressure (≥140/90), proteinuria (2+ dip stick testing of random urine) and tonic-clonic convulsion, who were previously normotensive and nonproteinuric after 20 weeks of gestation(Chesley, 1985; Anim-Nyame et al., 2003; Ramsy *et al.*, 2003). Their height, weight, blood pressure, body temperature and medical history were documented. A standard study protocol was drawn up to capture these data (Appendix V).

3.4.3.2 Group II (Pregnant Control-PC).

The group consisted of 38 third trimester normotensive, nonproteinuric normal healthy pregnant women with no known medical disorders (Teran *et al*, 2001), age

and parity matched with eclamptic women in group 1. Their height, weight, blood pressure, body temperature and medical history were documented. A standard study protocol was drawn up to capture these data (Appendix VI)

3.4.3.3 Group III (healthy non pregnant control-NPC)

This group was made of 38 Healthy normotensive, nonproteinuric normal healthy pregnant women with no known medical disorders (Teran *et al*, 2001), age matched with women in group 1 and II. Their height, weight, blood pressure, body temperature and medical history were documented. A standard study protocol was drawn up to capture these data (Appendix VII).

3.4.4 Exclusion criteria

- Non consenting clients
- **S**ero-negative for human immunodeficiency virus (HIV) and were blood smear negative for malaria.
- Women on hormonal pills (oral/ injectable contraceptives).

3.5 Materials

The following materials were used for the study:

- Urinalysis strip: Medi-test combi-2 urinalysis stripes (Macherey-Nagel GmoH &Co. KG German). Components at time of impregnation; Protein: (tetrabromophenol blue 10 μg) Glucos and glucose (oxidase =7 U

  Peroxidase = I U, tetramethylbenzidine = 96 μg). For protein the minimum sensitivity of the test strip is 10 mg protein/dL urine. The color fields correspond to the following ranges of albumin concentrations: negative, 30, 100 and 500 mg/dL or negative,

- Rapid HIV tests Kits: Three test kits used for HIV test were Uni-Gold ™ Recomegen HIV 1 / 2 (Trinity Biotech, Ireland), Stat-Pak(Trinity Biotect, Japan) and Determine™ Trinity Biotect, Japan.
- Malaria test reagents: Geimsa's and leishman stains were used for the detection of malaria parasites in thick and thin blood films respectively. Giemsa was acquired commercially from Merck/BDH BH151 TD, United Kingdom (UK).
- A commercially obtained pair of ready-to-use reagent tubes (BD, USA) containing monoclonal anti-Tlymphocytes antibodies (FITC–flourescein isothiocyanate-labeled) for the measurement of absolute and percentage CD3+ Tcells, CD4+ T cells and CD8+ Tcells. Functionally CD4/CD3 defines CD4+ T cells; CD8/CD3 defines CD8+ Tcells, while both tubes define CD4/CD8/CD3 (CD3+ Tcells).
- FACScount controls containing four standard reference beads concentration (zero, low, medium and high) used to set up the instrument and check for linearity (BD FACScount USA).
- Enzyme linked immunosorbent assay (ELISA) kits for assay of cytokines and PSG-1. These are highly sensitivity Quantikine ELISA kits obtained commercially (R&D Systems Inc. UK) for measurement of the serum levels of IL-2, IL-4, IL-10, TNF- α and PSG-1.The assay kits were stored in the dark at 2-8 ºC.

3.6 Methods

3.6.1 Measurement of blood pressure

Blood pressure was measured by the attending Clinician or Nurse. Patient's blood pressure was measured in a supine position with syphygmomanometer after at least 10

minutes of rest. Systolic blood pressure was recorded at the appearance of sounds, and diastolic blood pressure was recorded at the disappearance of the fifth-phase (Wichman *et al.,* 1984).

Cuff size was 12x22cm and measurements were repeated after 30 minutes for 12 hours; the average was used in the analysis (Duley, 1992).

3.6.2 Detection of urinary proteins (albumin)

A clean–catch urine or urine from the catheter was collected into clearly labeled urine container with patient's name and identification number. Urinalysis was done at bedside immediately after urine collection according to manufacturer's instruction. The test was based on the protein 'error' principle of indicators. The test zone was buffered to a constant pH value and changed color from yellow to greenish blue in the presence of albumin. Other proteins were indicated with less sensitivity. The color change of the reagent pad was then compared with the corresponding color chart on the bottle label

3.6.2.1 Procedure

The test strip was immersed in to the urine sample for 1 sec, removed, briefly drained and matched within 30 sec with the color chart on the bottle container label of the test strip (Appendix VIII).

3.6.3 Collection and processing of blood.

With the assistance of the collaborating clinicians at the respective hospitals a total of 8 mls of blood were taken from each participant on receipt of consent, after confirmation of diagnosis and before the administration of any drugs. Five (5) mls were dispensed into plain test tubes to extract serum for cytokine and PSG-1 assay, and 3 mls into liquid ethylenediaminetetraacetic acid (EDTA) tubesfor the

determination of CBC and Tcell subsets. Blood samples were also collected from normal healthy controls after obtaining consent and treated similarly. Serum samples for the measurement of TNF-α, IL-2, IL-4 and IL-10 were stored in pre-labeled serum vials containing drops of trasylol^{TM} (aprotinin)-Sigma USA and stored at -20 ^{o} C to inhibit degradation of the cytokine before immunoassay.

3.6.4 Detection of malaria parasites

Malaria plasmodium parasites were detected by standard haematological procedure outlined by Dacie and Lewis. (1991) as obtained in the respective Hospitals' Standard Operating Procedure (SOP).

3.6.4.1 Procedure

Well mixed drops of whole blood were placed on dried clean gress free slides to prepare thick and thin blood films. Air dried thick and thin blood films were stained using Geimsa stain and leishman stains respectively. Dried stained blood films were examined microscopically for malaria parasite under x100 oil immersion. Blood samples of participants that were blood smear positive for malaria plasmodium organisms were excluded from the study.

3.6.5 Laboratory-based HIV screening test.

Nigerian established National algorithm for rapid serial HIV screening was used to rule out HIV infections in participants in this study. Comercially acquired test kits: Determine® HIV1/2 (Trinity Biotect, Japan) and Unigold® Recomigen HIV1/2 (Trinity Biotech, Ireland) and Stat-Pak® HIV 1 / 2 (Trinity Biotect, Japan)tests were used according to manufacturer's instructions as obtained in the hospital's Standard

Operating Procedure (FMOH, 2008). Serum samples reactive for HIV by these tests were excluded from this study.

3.6.5.1 Procedure

Twenty (20) µL of sera were used for HIV antibodies test using the Determine HIV 1/2 rapid test kits. Samples that were reactive by Determine were further tested by Uni- gold and those with discordant results from the two assays were retested using Stat-pack. All the test procedures were carried out according to the manufacturers' recom- mendations.

3.6.6 Complete blood count (CBC)

Complete blood count was performed using a KX-21N Hematology auto Analyzer (Sysmex, Kobe, Japan), a three-part a 3-part differential hematology analyzer to differentiate white blood cells into Neutrophils, Lymphocytes, and mixed population which consists of eosinophils, monocytes and basophils. Standardization, calibration of the instrument, and processing of the samples were done according to the manufacturer's instructions.

3.6.6.1 Procedure CBC by sysmex.

Twenty (20) µL of well mixed EDTA blood were introduced into the machine through the sample probe and the stat botton pressed down to start switch to commence analysis. The procedure was repeated for controls and standards. All patients' samples and controls were analyzed according to manufacturer's instruction.

3.6.7 Immunophenotypingof CD3+, CD4+ and CD8+ T lymphocytes using flow cytometry.

Flow cytometry of lymphocytes was carried out using FACScount flow cytometer (BD Biosciences) equipped with FACScount software for analysis. The lymphocyte population was gated on the scatter plot as determined by the characteristic forward scatter (FS) and side scatter (SC) which indicates the cell size and shape respectively. The analysis was on absolute CD3, CD4, CD8 count and percentage CD3, CD4 and CD8 Tcells.

3.6.7.1 Procedure for FACS counts technique

The reagent tube pairs for CD4 and CD8 were labeled with patient identifier (accession number) and vortexed upside down for 5 sec. and the upright for another 5 sec.

Fifty (50) µl of well mixed whole blood were introduced using pipette into the reagent tube, vortexed for 5 sec. and incubated at room temperature (20-25℃) for 60 min. The contents of the tubes were vortexed and 50µl of the fixative added to each reagent tube. Another 5 secvortex was done before running samples on FACScount machine. The procedure was repeated for control and standard. All patients' samples and controls were analyzed according to manufacturer's instruction.

3.6.8 Determination of Cytokines and Pregnancy Specific beta-1 Glycoproteins by Enzyme-linked Immunosorbent Assay.

3.6.8.2 Procedure for ELISA technique:

Cytokines (TNF-α, IL-2, IL-4, IL-10) and PSG-1 were assayed on batched serum samples stored at -20^{o}C in trasylol (SIGMA, USA) by ELISA kits following the outlined protocol by Pathare *et al.*, (2004). Frozen (-20^{o}C) serum samples were

thawed once and brought to room temperature at the time of assay. Serum samples were dispensed along side with dilutions of standards (recombinant cytokines) into wells of the microtitre ELISA plates precoated with monoclonal antibodies against the human cytokines (IL-2, IL-4, IL-10, TNF-α) and PSG-1 to be assayed and incubated at room temperature for 2 hr. The plates were washed four times with buffer (phosphatebuffered saline-0.05% and Tween 20). Conjugates (polyclonal antibodyagainst IL-2, IL-4, IL-10, and TNF-α or PSG-1) to horseradish peroxidase-HRP) was added and incubated for 2 hr at room temperature. Unbound enzymes were washed out while the bound enzyme was then detected by incubation in the dark with substrate solutions (stabilized hydrogen peroxide and tetramethylbenzidine-TMB). The plates were scanned using a microplate reader (Bio-Rad, USA) set at 450 nm with wave length correction set at 570nm. A standard curve was then generated from the known standards.

Concentrations of IL-2, IL-4, IL-10, and TNF-α or PSG-1 in the specimen was determined by comparing sample optical density with the values on the standard curve.

3.7 Method of Statistical Analysis

The data obtained were analyzed using the SPSS version 20.0 (Chicago, USA) and GraphPad Prism 6.0. Results were expressed as mean± standard deviation. ANOVA was used to evaluate the difference between parametric data (T-cell subsets) and Kruskal Wallis was used to evaluate the difference between non parametric data. Comparisons were made between eclampsia (EC), normal healthy pregnant controls (PC) and non pregnant controls (NPC). A p-value of less than 0.05 was considered to be significant

CHAPTER FOUR

4.0 RESULTS AND ANALYSIS

4.1 Demographic Characteristics and Clinical Features of EC, PC and NPC

The mean age and standard deviation of the patient and controls were: EC(25.0±5.9 years), PC (24.9±5.7years) and NPC (25.1±5.9 years) Table 4.1.

The mean gestational age and parity were EC (37.2±2.2 wks and 4±2.4) and PC (37.1± 2.0 wks and 1.5 ± 2.5) respectively Table 4.1.

In table 4.1, the mean values of blood pressures (systolic; diastolic) and standard deviation were noted to be higher in EC(171.6±25.2mmHg; 110.0±10.7mmHg) compared with PC (111.6±7.1mmHg; 79.5±11.3 mmHg) and NPC (109.6±6.6 mmHg; 84.2±6.4 mmHg). There was significant differences between EC, PC and NPC ($p<0.05$).

The mean BMI and standard deviation recorded was: EC (26.4±4.3 $Kg/m^{2)}$, PC(25.8±3.7 $Kg/m^{2)}$ and NPC (25.2±4.3 $Kg/m^{2)}$. There was no statistical difference between EC, PC and NPC ($P>0.5$) Table4.1.

Only 55.3% of the eclamptic women were found to have booked in the facility at the time of study. While (100%) of the pregnant women control had been booked who served as controls. Majority of the unbooked patients were said to have registered at Private Clinics, Nursing homes or Primary Health Care Centres (PHC), but there were no records to authenticate their claims. In Table 4.1, urinary proteins (albumin), ≥2+ were recorded in all eclamptic women but not in pregnant and nonpregnant women controls.

Other clinical features of eclampsia recorded were pitting edema, blurred vision, severe frontal headache and tonic clonic fits that occurred antepartum16 (42.1%) and intrapatum; 22 (57.9%) and in the eclamptic women, respectively (Table 4.1).

4.2The Haematological Parameters of the EC, PC and NPC

The data in Table 4.2 shows the haematological parameters in patient with EC, PC and NPC:

4.2.1 Haemoglobin (Hb).

The mean Hb and standard deviations were: EC (10.9±1.9 g/dL), PC (10.9±1.4 g/dL) and NPC (11.2±1.2 g/dL).There was no significant difference between EC, PC and NPC ($P>0.05$).

4.2.2 The total white blood count cell (WBC) count.

The mean total white blood cell count in EC (9.8± 4.9 $x10^9$/L) was significantly higher compared to PC (6.9±3.6 $x10^9$/L) and NPC (5.6±2 $x10^9$/L) $P<0.05$. The white blood cell count further increased significantly in EC compared to PC ($P<0.05$)

4.2.3 The differential lymphocyte count.

The mean differential lymphocyte count displays increase from patient in EC (25.5±18.3 %) to PC (28.5±9.4%) and NPC (43.0±10.8%). EC is significantly different from NPC ($p< 0.05$)

4.2.4 The differential neutrophil counts.

The mean differential neutrophil count displays a significant increase from NPC (48.5±10.7%) to PC (64.6±8.1%) andthen to EC (70.3±12.0%), $P< 0.05$. A further

increase in mean differential neutrophil count was noted in EC compared to PC ($P<0.05$).

4.2.5 Platelet count (10^9/L)

The mean platelet counts progressively decreased from NPC (225.2±63.7 x10^9/L) to PC (221.9±53.4 x10^9/L) and to EC (195.8±91.6 x10^9/L), however, the decrease was not significant ($p>0.05$).

4.3 The Subpopulations of T lymphocytein EC, PC and NPC.

Total T cell subpopulation values in patient with EC as well as PC and NPC are shown in Table 4.3

4.3.1 Percentage and absolute total T cells (CD3 +T lymphocyte)

The mean percentage and absolute values of total T cells (CD3+ T cells) decreased from NPC (74.9 ±11.8 %; 1617.9±519.5 cell/µL) to PC (72.2±10.5 %; 1290.2±251.4 cell/µL) and to EC (65.6±15.5 %; 1225.5±401.5 cell/µL), respectively. The CD3 % decrease in EC is significantly different from both PC and NPC at $P<0.05$, while the decrease in mean absolute values were significantly different from NPC at $P<0.05$.

4.3.2 Percentage and absolute CD4+ T lymphocytes (T 'helper'/ 'inducer' cells).

The percentage and absolute values of CD4+T helper cells also decreased from NPC (45.7±8.7%; 999.9±278.4 cell/µL), to PC (40.2±7.2%; 718.5±158.7 cell/µL) and to the EC (36.1±8.7%; 657.1±189.9 cell/µL), respectively. The mean values of CD4 % decrease in EC was significantly different from both controls at $P<0.05$, while the mean absolute value of CD4+ Tcells was significantly different from NPC at $P<0.05$.

4.3.3. Percentage and absolute CD8+Tlymphocytes (T‘suppressor’/’cytotoxic’ cells.)

In table 4.3, the mean percentage and absolute CD8+T lymphocytes were: EC (26.6±7.4%; 492.9±196.1 cell/µL) while those of PC and NPC were (28.3±5.4%; 495.9±124.7 cell/µL) and (25.2±6.6%; 574.9±242.2 cell/µL) respectively. There was no significant difference between EC, PC and NPC at P>0.05.

4.3.4 T helper–to- T suppressor cells (CD4/ CD8) ratio.

The mean values for CD4+/CD8+ ratio in EC was (1.4±0.5) while for PC and NPC was 1.5±0.3 and 1.9±0.6 respectively. There was significant decrease in women with EC compared with NPC (p<0.05) Table 4.3.

4.4 Serum levels of PSG-1 in EC, PC and NPC

In Figure 4.1, the mean serum (log value) of PSG-1 in women with EC was 2.53±0.11 pg/ml, while those for PC and NPC; were: 2.56±0.03 pg/ml and l0.62±0.20 pg/ml respectively.

While there was no significant difference between EC and PC women (P>0.05), women with EC had significantly higher mean serum values compared to NPC (P<0.05).

Table 4.1: The demographic and clinical characteristics of women with eclampsia and controls (Mean ± SD)

Characteristics	EC (n = 38)	PC (n = 38)	NPC (n =38)
Age (years)	25.0±5.9	24.9±5.7	25.1±5.9
Gestational age (wks)	37.2±2.2	37.1±2.0	NA
Parity	1.4±2.4	1.5±2.4	NA
BMI (Kg/m^2)	26.4±4.3	25.8±3.7	25.2±4.3
Systolic BP(mmHg)	171.6±25.2*	111.6±7.1	109.6±6.6
Diastolic BP(mmHg)	110.0±10.7*	79.5±11.3	84.2±6.4
Proteinuria (Albumin)	≥2+	ND	ND
Antenatal booking	55.3%	100.0%	NA
Antepartum convulsion	16(42.1%)	NA	NA
Intrapartum convulsion	22(57.9%)	NA	NA

KEY: EC= Eclampsia, PC=Pregnant Control, NPC=Non Pregnant Control, SD Standard deviation, BMI=Body Mass Index, BP= Blood Pressure, ND =not detected, NA= not applicable.*Bpin EC is significantly different from both controls at $p < 0.05$ using ANOVA

Table, 4.2: The haematological Parameters of EC, PC and NPC (Mean ±SD).

Parameters.	EC (n = 38)	PC (n = 38)	NPC (n =38)	p-value
Haemoglobin g/Dl	10.9±1.9	10.9±1.4	11.2±1.2	0.578
White blood cell x 10^9/L	9.8±4.9	6.9±3.6	5.6±2.0*	0.001
Lymphocyte (%)	25.5±18.3	28.5±9.4	43.0±10.8**	0.001
Neutrophil (%)	70.3±12.0	64.6±8.1	48.5±10.7*	0.001
Platelet x 10^9/L	195.8±91.6	221.9±53.4	225.2±63.7	0.148

Key: EC=Eclampsia, PC=Pregnant control, NPC=Non-pregnant, %=percentage, L=Litre, n= number, SD= Standard deviation.

*Neutrophil count in EC is Significantdifferent from and both controls (PC and NPC) at $p < 0.05$ (ANOVA) ** Lymphocyte count EC is significantly different from NPC at $p < 0.05$(ANOVA)

Table 4.3: T-lymphocyte sub populations of EC, PC and NPC (Mean ±SD)

Parameters	EC (n = 38)	PC (n = 38)	NPC (n =38)	p-value
CD3 (%)	65.6±15.5	72.2±10.5	74.9±11.8*	0.006
CD3 (cell/µl)	1225.5±401.5	1290.2±251.4	1617.9±519.5**	0.001
CD4%	36.1±8.7	40.2±7.2	45.7±8.7*	0.001
Abs CD4 (cell/µl)	657.1±189.9	718.5±158.7	999.9±278.4**	0.001
CD8 (%)	26.6±7.4	28.3±5.4	25.2±6.6	0.122
Abs CD8 (cell/µl)	492.9±196.1	495.9±124.7	574.9±242.2	0.116
CD4/CD8 ratio	1.4±0.5	1.5±0.3	1.9±0.6**	0.001

Key: CD= Clusters of differentiation, EC=Eclampsia, PC=Pregnant control, SD=Stanard deviation, n= number of participants.

*Significant value exist betweenEC and both controls (PC and NPC) at p <0.05. **Significant values exist between EC and NPC at p<0.05(ANOVA

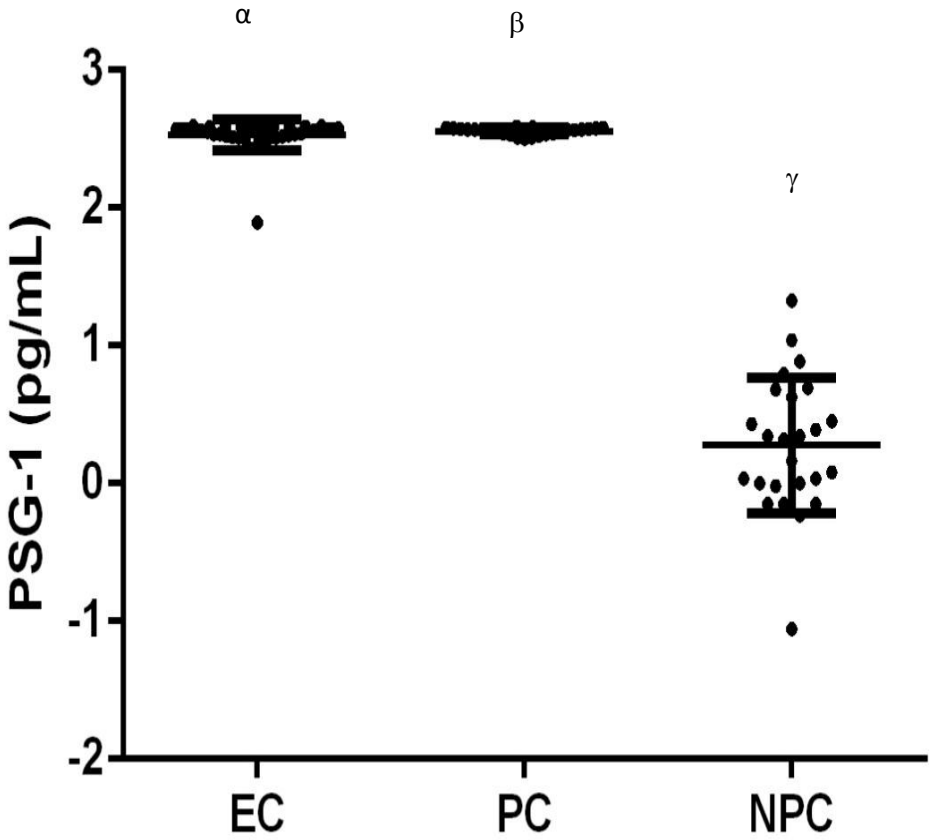


Figure 4.1: Serum levels of Pregnancy Specific beta-1 Glycoprotein (PSG-1) in patients with eclampsia (EC; n=38), normal healthy pregnant controls (PC; n=25) and healthy nonpregnant controls (NPC; =25). Pg= Picogram. Bars represent mean ± SD.

$^{\alpha\beta}$ p>0.05 EC versus PC. $^{\alpha\gamma}$p< 0.05 EC versus NPC.$^{\beta\gamma}$p< 0.05 PC versus NPC

4.5 Serum levels of Pro- and Anti-inflammatory Cytokines in EC, PC and NPC

4.5.1 Tumor necrosis factor alpha (TNF)-α

In Figure 4.2, the mean serum levels (log value) of TNF-α in PC (2.25±0.07pg/ml) was significantly elevated compared to NPC (2.24±0.10pg/ml), and furher deviated from EC (2.34±0.13 pg/ml) at $p<0.05$(Kruskal-Wallis test).

4.5.2 Interleukin (IL)-2

The mean serum levels (log mean ± SD) of IL-2 in women with EC and PC were 1.69±0.17 pg/ml and 1.71±0.09 Pg/ml respectively, while that of NPC was 1.72±0.13 pg/ml. There was no significant difference in mean serum values between EC, PC and NPC $p>0.05$ (Kruskal-Wallis test). Figure 4.3

4.5.3 Interleukin (IL)-4

The mean serum value of IL-4 and in EC, PC, and NPC were: 2.45±0.10 Pg/ml, 2.40 ±0.06 Pg/ml and 2.45±0.06 Pg/ml respectively. There was no statistical significant difference between EC, PC and NPC at $P>0.05$ (Kruskal-Wallis test). Figure 4.4

4.5.4 Interleukin (IL)-10

In Figure 4.5, the mean serum (log value) levels of IL-10 in women with EC (1.28±0.54 pg/ml) was significantly lower between PC (1.58±0.61 Pg/ml) and NPC(2.06±0.08 Pg/ml) at $P<0.05$ (Kruskal-Wallis test). There was further significant decrease in mean level of IL-10 in the eclamptic women compared with PC ($P<0.05$). Figure 4.5

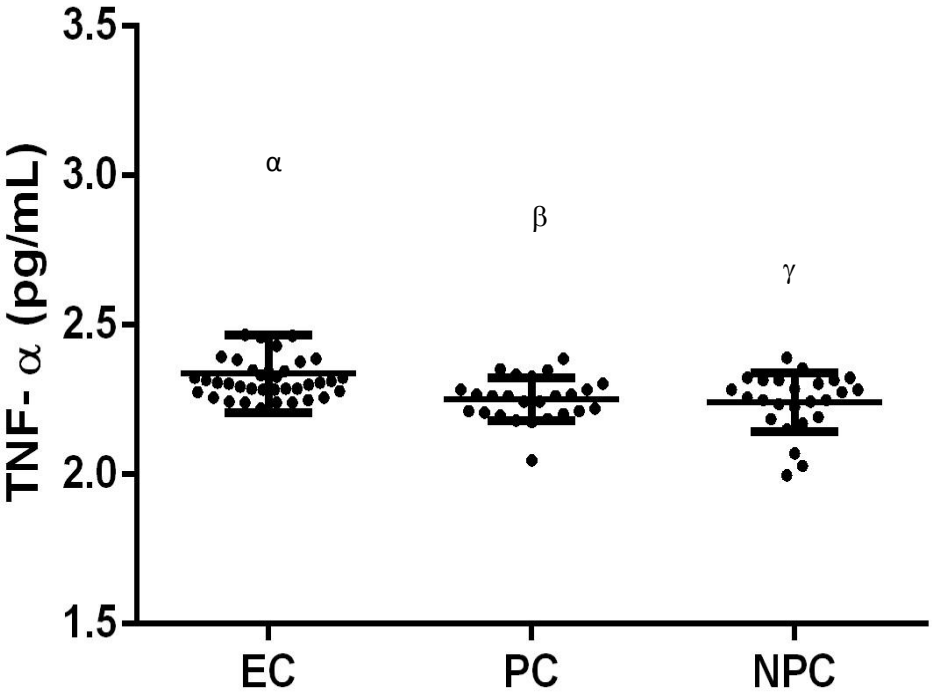


Figure 4.2: Serum concentration of tumor necrosis factor (**TNF)-α** in patient with eclampsia (EC; n=38), normal healthy pregnant control (PC; n=25) and healthy nonpregnant control (NPC; n=25). Pg=Picogram. Bars represent mean ±SD.

$^{\alpha\beta}$p <0.05EC versus PC. $^{\alpha\gamma}$p> 0.05 EC versus NPC.$^{\beta\gamma}$p> 0.05 PC versus NPC (Kruskal Wallis test).

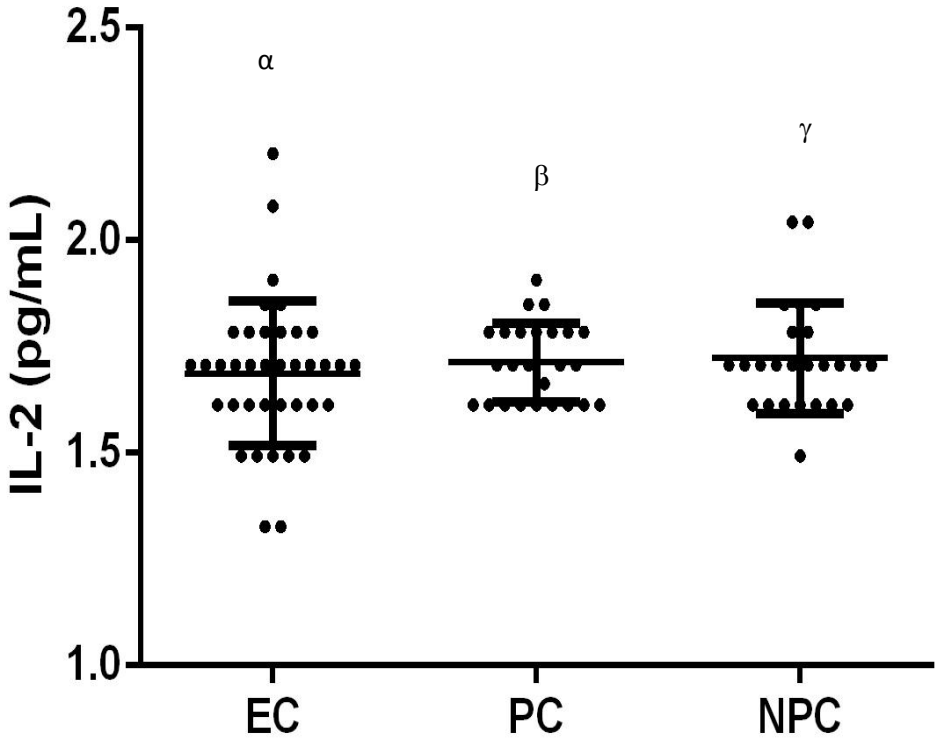


Figure 4.3: Serum levels ofIL-2 in patients with eclampsia (EC; n=38), normal healthy pregnant controls (PC; n=25) and healthy nonpregnant controls (NPC; n=25). Pg= Picogram. Bars represent mean ±SD.

$^{\alpha\beta}$P>0.05 EC versus PC.$^{\alpha\gamma}$P> 0.05 EC versus NPC. $^{\beta\gamma}$P> 0.05 PC versus NPC (Kruskal Wallis test)

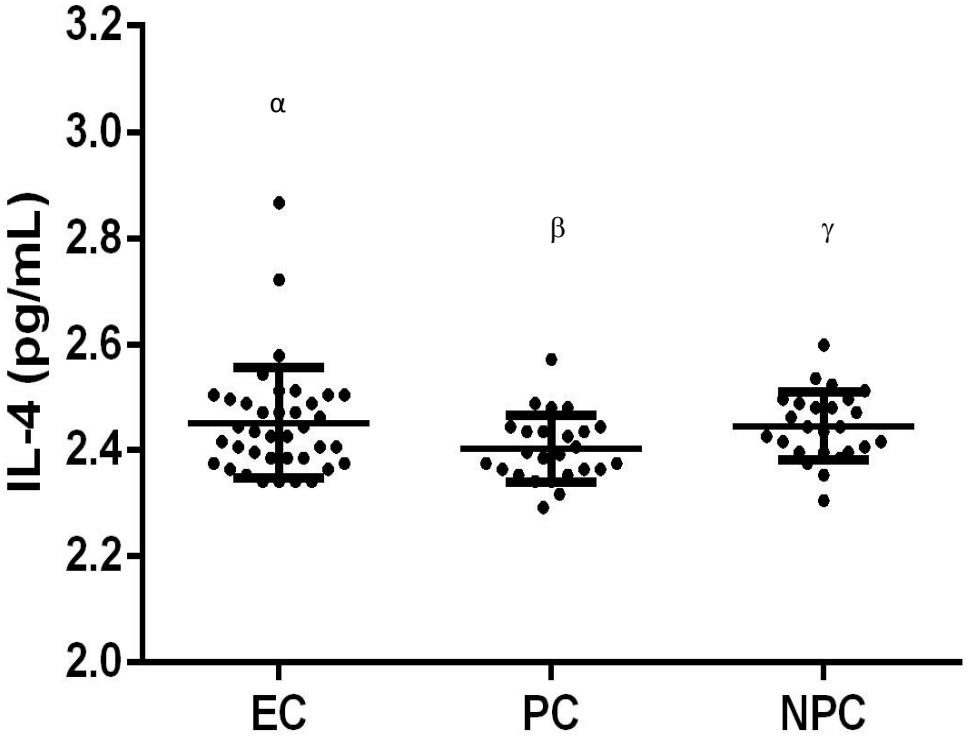


Figure 4.4: Serum levels of IL-4 in patients with eclampsia (EC; n=38), normal healthy pregnant controls (PC; n=25) and healthy nonpregnant controls (NPC; n=25). Pg= Picogram. Bars represent mean ±SD.

$^{\alpha\beta}$p>0.05 ECversus PC. $^{\alpha\gamma}$p> 0.05 EC vs NPC. $^{\beta\gamma}$p> 0.05 PC vs NPC (Kruskal Wallis test)

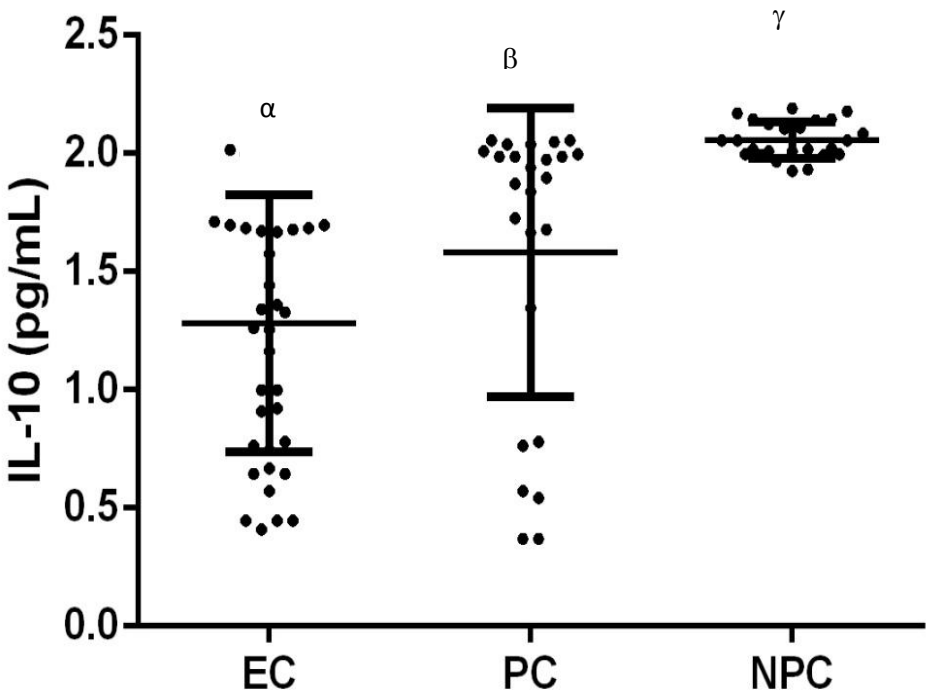


Figure4. 5 Serum levels of IL-10 in patients with eclampsia (EC; n=38), normal healthy pregnant controls (PC; n=25) and healthy nonpregnant controls (NPC; n=25). Pg= Picogram. Bars represent mean ± SD.
$^{\alpha\beta}p < 0.05$ EC versus PC. $^{\alpha\gamma}p < 0.05$ EC versus NPC; $^{\beta\gamma}P<0.05$ PCvresus NPC (Kruskal Wallis test)

CHAPTER FIVE

5.0 DISCUSSION

5. 1 Complete Blood Count

5.1.1 Haemoglobin (Hb) levels

Haemoglobin results in this study (Table 4.2) showed no significant difference between women withEC, PC andNPC (P>0.05). Akingbola *et al*., (2006) found a progressive decline in Hb concentration in healthy pregnant women from the first to the third trimester which may not be unconnected with the increase in growth of the baby with advancing pregnancy. The progressive decline in Hb concentration from the 1^{st} to 3^{rd} trimesters might be due to an increase demand for iron as pregnancy progresses. More iron is required to meet the expansion of the maternal Hb mass and the need of fetal growth. The additional progesterone and oestrogen that are secreted by the placenta during pregnancy cause a release of renin from the kidneys. Renin stimulates aldosterone-renin-angiostesin mechanism, leading to sodium retention and increase plasma volume. The increase in the 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. The women with and without eclampsia in this study at late 3^{rd} trimester with near normal Hb corroborates with those found by Shen and Jang, (2010) among the Chinese women.

5.1.2 White blood cell (WBC) and differential counts.

The WBC count was elevated in PC and significantly increased in EC compared with NPC. Differential analyses further demonstrated that the increase in neutrophils, and not lymphocytes accounted for the total leukocytes in EC and PC (Table 4.2)

This finding is consistent with that of Lurie *et al.*, (1998) in which increase in neutrophils differential count was reported in severe PE. The increase observed in WBC count in normal healthy pregnancy in this study is consistent with the findings of Akingbola *et al*., (2006) and Onwukeme and Uguru, (1990).

Normal pregnancy involves many changes in the maternal physiology including alterations in haematological parameters. In general, white blood cell counts rise during pregnancy with the occasional appearance of myelocytes or metamyelocytes in the blood (England and Bain, 1976).This increase in WBC primarily due to an increase in neutrophils may represent a response to stress due to redistribution of the WBCs between the marginal and circulating pools. Previous literature reveals that pain, nausea, vomiting, and anxiety have been reported to cause leukocytosis in the absence of infection in pregnancy (Milhorat, *et al*., 1942). A rising WBC count in pregnancy is not a reliable indicator of infection rather, Leukocytosis is considered to be evidence of an increased inflammatory response in pregnancy and EC. An increased leukocyte count was observed in antenatal dexamethasone administration in pregnant women (Denison *et al*., 1997) and in premature infants (Barak *et al*., 1992). Terrone *et al.*, (2000) also found the total leukocyte count was even higher in preeclamptic patients with haemolytic anaemia, elevated liver enzymes, and low PLT count (HELLP syndrome) than those without HELLP syndrome, suggesting an association between increased leukocytes and worsening thrombocytopaenia in those patients (Terrone *et al*.,2000). In the present study, to eliminate those variables that may influence the leucocyte count, patients who had symptoms of infection or were on drug administration were excluded. Therefore, the increased neutrophil count in EC is reflective of the severity of inflammatory response during pregnancy.

5.1.3 Platelets (PLT)

A non significant decrease in PLT counts fromNPCto PC and to EC was documented in the study (Table 4.2) P>0.05. The effect of pregnancy on maternal PLT counts is somewhat inconsistent: some studies demonstrate a mild decline in PLT count over the course of gestation (Abbassi-Ghanavati *et al*., 2006; Jensen *et al*., 2011), while van Buul *et al*., 1995 reported an increase in PLT count in pregnancy. The mechanisms for decrease in PLTcount are thought to be due to dilutional effects and accelerated destruction of PLTs passing over the often scarred and damaged trophoblast surface of the placenta (Fay *et al.,* 1983). PLT counts may also be lower in pregnant women with twin compared with singleton pregnancies, possibly due to greater thrombin generation (Tsunoda *et al.,* 2002). Although most cases of thrombocytopaenia in pregnancy are mild, with no adverse outcome to the mother or baby, occasionally a low PLT count may be part of a complex disorder with significant morbidity and may be life-threatening.

Thrombocytopaenia can be induced acutely by EC resulting from PLT activation, aggregation and consumption accompanied by increased mean PLT volume and decreased life span (Harlow *et al*., 2002).

5.2 Subpopulation of T Lymphocytes in the EC, PC and NPC.

The mean absolute and percentage values of CD3+ and CD4+ T lymphocytes in this studyshowed a steady decrease from NPC to PC and EC. A further decrease was demonstrated in the eclamptic compared with PC (Table 4.3). Also the CD4/CD8 ratio in eclamptic and normal healthy pregnant women decreased significantly ($p<0.05$), butressing the increased cytotoxic CD8+ Tlymphocytes in pregnancy compared with NPC(Table 4.3).

This is similar to the reports of Musa *et al.*(2012) and Tallon *et al*. (1984) where absolute numbers of total T cells (CD3+T) cells and T helper (CD4+T) cells decreased significantly in the third trimester. Other previous work on T lymphocyte enumeration in pregnancy revealed inconsistent results showing decrease (Sulmer and Ranock, 1977), increase (Clements*et al.,* 1978) and no change (Dodson *et al*., 1977; Birkeland and Krisofferson, 1979).

The report of investigation by Sridama *et al.*(1982), using monoclonal antibodies, demonstrated a selective decrease in the relative absolute numbers of T helper cells in pregnancy that normalized post partum. This has been supported by Tallon *et al*. (1984) which showed a decline in absolute numbers of total T cells as well as of T helper cells during second and third trimesters of pregnancy normalizing postpartum. Bardequez *et al.*(1991) reported that beginning in the early second trimester, women who were most likely to develop PE; the precursor of EC demonstrated a significant lower proportion of helper T cells compared with that of women who remain normotensive.

Normal, healthy pregnancy isknown to be associated with low levels of T helper T cells which are compatible with fetal survival and the protection of the mother (Sridama *et al.,* 1982; Wegmann *et al.,* 1993). Helper T lymphocytes secrete specific cytokines that promote implantation and healthy growth of the fetus and their dysfunction may favor EC (Whitecar *et al*., 2001; Hayashi *et al.,* 2004).

The significant difference found in the T lymphocyte subsets in the peripheral blood of women with EC and PC compared to NPC in this study (Table 4.3) could be as a result of the immunosuppressive effect of placental products such as PSG-1 (Onyemelukwe *et al.,* 1985), progesterone or human chorionic gonadotrophin

(Jenkins, 1977). Current findings indicate that gravidity is both a pro-inflammatory and anti-inflammatory condition depending upon the stage of gestation. As such, pregnancy is associated with inflammatory processes (Mor and Cardenas, 2010), which may result in increased expression of cell-adhesion-molecules (CAMs) that facilitate leukocyte extravasation to the tissue site where activation of the immunesystem occurs. Some researchers have proposed a redeployment or redistribution of T lymphocytes in the maternal blood circulation in pregnancy (Jenkins, 1977). These may explain the decrease recorded among the pregnant women with and without EC in relation to the nonpregnant control in this study, since women with infections such as malaria and HIV which is associated with depressed levels of CD4+T lymphocytes were excluded.

5.3 Pregnancy Specific Beta-1 Glycoprotein (PSG-1) in EC, PC and NPC

The result of this study, showed that women with EC and PC had significantly highermean serum levels of PSG-1($P<0.05$) compared to NPC (Figure 4.1). Although, there was a decrease in the mean serum PSG-1 levels among the eclamptic women compared to PC, the difference was not significant ($P> 0.05$).

This result agrees with findings of Onyemelukwe *et al.* (1985) who studied 71 normal healthypregnant women in northern Nigeria. They documented a low rise in PSG-1 up to 24 wks.then a steep rise up to 36 wks followed by a gradual fall near term. These researchers in Nigeria did not however have the privilege of measuring PSG-1 levels in pregnancy complicated by EC.The result of this study however, did not support the findings ofSilver *et al.*(1993) who reported low PSG levels in PE.

Pregnancy specific beta-1 glycoprotein, the major placental glycoproteins are a group of highly similar proteins synthesized in large amounts by the placenta trophoblast

that together with the carcinoembryonic antigens comprise a subfamily within the immunoglobulin (Ig) super family (Horne *et al.,*1976;Watanbe and Chou, 1988). During normal pregnancy, PSG molecules are released into the maternal circulation, reaching 200-400µg/ml in the serum at the end of gestation (Bohn, 1971; Lin *et al*., 1976). PSG is thought to play a crucial role in supporting gestation and fetus protection against the maternal immune system. Sacks *et al*., (1999) proposed that pregnancy-specific factors induce the suppression of a specific arm of the maternal response and assumes the central role in maternal immunological adaptation. PSG could modulate the immune response by inducing the secretion of anti-inflammatory cytokines such as IL-10, IL-6 and TGFβ by human and murine cells **(**Snyder *et al*., 2001; Blois *et al.*, 2014). PSG also suppresses mixed lymphocyte reaction and T cell activation by mitogens (Harris*et al.*, 1984)

In this study, the slightly decreased mean PSG-1 value in the eclamptic women compared to the normal healthy pregnant women group might in part be associated with the pathogenesis of EC. Lower levels of PSG-1 have been associated with certain human, pathological conditions such as autoimmune disease, spontaneous abortion and EC (Masson *et al.*, 1983; Tamsen *et al*., 1983; Arnold *et al.*, 1999 and Blanchon *et al.*, 2001). Increased PSG-1 level in pregnancy is necessary for the maintenance of Th 2 response which is compatible with healthy pregnancy while low mean levels of PSG-1 were not recorded as reported by other researchers. EC was associated with an excessively dominated pro-inflammatory cytokine environment, which, supports the hypothesis of increased inflammatory responses in ECas illustrated by increase in TNF-α and decreased anti-inflammatory cytokine (IL-10). This may partly support the hypothesis of a Th1 shift in EC.

5.4 The Serum Cytokines levels in EC, PC and NPC

This study showed that TNF-α, levels in eclampsia was significantly higher than in normal healthy pregnant and non-pregnant control group (Figure 4.2) P <0.05.Decreased levels of IL-10 were also demonstrated in women with EC compared to both controls.This result is similar to the findings of Musa *et al.* (2012) who reported higher levels of TNFα in the sera of pregnant women with and without eclampsia compared to normal non-pregnant controls. Similarly their studyalso found lower levels of IL-10 in eclamptic women. This also agreed with the work of Anim-Nyame *et al*. (2003) who found elevated TNFα concentrations in 18 pregnant women with normal pregnancies compared to the non-pregnant status in the third trimester

Increased secretion of pro-inflammatory cytokines such as TNF-α and IL-6 have been reported in normal pregnancies, pregnant women in labour (Mor and Cardenas, 2010) and pregnancy complicated by EC (Anim-Nyame *e al*., 2003).

In normal pregnancy, there is a shift towards a Th 2-type immune response which protects the baby from a Th1-type (cytotoxic) response which could harm the baby with its products like IL-2, IL-12, IFNγ, and TNF-α. Thus inflammation appears to favor a preponderance of Th1-type reaction (Wegmann *et al.*, 1993). Redman *et al*., (1999) proposed that PE arises from an exaggeration of vascular inflammatory response

TNF otherwise known as cachexin is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. In normal pregnancy TNF-α is low in the first trimester and subsequently increases with advancing gestational age (Daher *et al*., 1999).TNF-α is produced chiefly by macrophages, although it can be produced by many other cell types such as CD4+

lymphocyte, NK cells and neurons. The primary role of TNF is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, apoptotic T cell death, cacthexia and inflammation. This may explain decrease helper CD4+ Tcells in patient with EC in this study. Dysregulation of TNF production has been implicated in variety of diseases and due to the pleiotropic effects, this cytokine has been intensely studied in PE in connection to various pathophysiological and cell immunological aspects found in this syndrome. Many studies have evaluated circulating TNF-α level in EC as a bioactive or immunoreactive agent, with different results on the chosen study method. The results obtained are inconsistent; increased, unchanged or even insignificantly decreased circulating TNF-α levels being reported in women with PE compared to normal pregnancy in the third trimesters (Mzammil *et al*., 2005; Jonsson *et al.,* 2006). The biological effect of TNF-α is seen after the attachment of the cytokine to one of two high affinity receptor initiated at the surface of the target cells: TNF-RI (TNF p55, CD 120a) and TNF-RII (TNF p 75, CD 120b) (Tartaglia 1992). It has been demonstrated that during gestation, soluble TNF-α receptor (sTNF-R) has a parallel evolution to that of TNF-α. Soluble receptors have a predictive value in the development of EC, increased concentrations of both receptors being detected in the third trimester of pregnancy in women who subsequently develop PE the precursor of EC (Williams *et al.,* 1998)

The source of high concentration of circulating levels of inflammatory cytokines, including TNF-α, in pregnant women with EC has not yet been identified. TNF-α is located in the placenta villous stromal cells, in the cytotrophoblast and the syncytiotrophoblast, as well as the invasive trophoblast. One of the most studied hypotheses is the excessive production and release of TNF-α from the preeclamptic placenta as a consequence of hypoxia (Rinchart *et al*., 1999). Another potential source

of TNF-α in EC is activated maternal leukocytes. As monocytes/macrophages are generally the main reservoir of pro-inflammatory cytokines and are the first cells to be activated in non specific immune responses, these can be good candidates for excessive TNF-α synthesis in eclampsia (Luppi and DeLoia, 2006). Activated neutrophils are another TNF-α source in normal and pathological pregnancy (Vaughan and Walsh, 2005). Decidual macrophages and neutrophils can be the source of this pro-inflammatory cytokine, these cells being activated and in higher number in EC (Peracoli *et al.*, 2007). Report shows that TNF-α directly controls endothelial dysfunction, this cytokine may be involved, the pathogenesis of PE (Salomon and Seely, 2001). Higher levels of inflammatory cytokines (TNF-α) which are associated with a Th1/Th2 paradigm shift towards Th1 type immunity in EC, may explain the fresh and the macerated still births recorded in this study.

In this study, there was no statistical significant difference in mean serum IL-2 level between EC and PC and NPC (Figure 4.3) P>0.05. However, Darmochwal-Kolarz *et al*.(1999) found increase IL-2 in PHA-stimulated PBMC cultures from women with EC compared to NPC. IL-2 is mainly produced by activated T cells. This finding could be due to inadequate antigen presentation in pregnancy and women on birth control methods who were NPC.

The mean serum level of IL-4 was significantly higher in the women withEC compared with the PC andNPC (Figure 4.4) P>0.05. This however, agreed with the work of Omu *et al*. (1999) who also found increased levels IL-4 in serum from women with PE, the precursor of EC when compared with PC. On the contrary, Arriaga-Pizano *et al*., (2005) observed increased in IFN-γ and a decreased in IL-4 in EC

The Th2 derived cytokines, IL-4 and IL-6, induce the release of βHCG from trophoblasts, and the βHCG stimulates progesterone production from corpus luteum in pregnancy.The progesterone then stimulates the secretion of Th2 and reduces the secretion of Th1 cytokines. Thus Th2 type cytokines appear to contribute to the maintenance of pregnancy by controlling the immune and endocrine systems and promoting the function of the trophoblasts at the implantation site (Khil *et al.,* 2007).

In this study, a statisticcally significant decrease of anti-inflammatory cytokines IL 10 level was documented in eclamptic women compared to PC (Figure 4.5) $P<0.05$.

This also agreed with the findings of Musa *et al.*(2012) who reported lower levels of IL-10 in the sera ofwomen with EC compared to PC. Also Freeman *et al*. (2004) recorded decreased IL-10 in PE, the precursor of EC.The cytokine that contributes to protecting the fetus from rejection during pregnancy is IL-10. Low anti-inflammatory cytokines (IL-10) is associated with Th1 cell reactivity and pregnancy complication while elevated concentration which is associated with Th 2 cell reactivity, is compatible with normal pregnancy.

Interleukin-10 is an immunomodulatory Th2 cytokine that exerts anti-inflammatory effects through several mechanisms (Moore *et al.,* 2001). These include inhibition of TFN-α, which results in the reduction of the inflammatory cytokines IL-1β, IL-6, and TNF-α, as well as the inhibition of tissue factor expression, IFN-induced gene transcription, IL-2, chemokines such as regulated on activation, normal T-expressed and secreted (RANTES), and metalloproteinases in monocytes/macrophages and CD4+ T cells (Asadullah *et al.*, 2003). IL-10 also suppressesinflammation by inducing suppressor of cytokine synthesis-3 as well as heme oxygenase-1 expression, both of which are decreased in PE the precursor of EC (Zhao *et al.,* 2008). Additionally, IL-10

plays a role in the differentiation of T cells, which aid in immunoregulation. With respect to pregnancy, serum and placental IL-10 levels have been reported to be increased in normal pregnancy (Freeman *et al.*,2004), however, reports in women with EC show decreased, increased, unchanged, or increased serum and placental levels of IL-10 (Hennessy *et al.*,1999;Freeman *et al.*,2004 ; Sharma *et al*.,2007)

The lowered levels of IL-I0 in EC have been associated with the 1082G allele of the IL-I0 gene, which is more frequently found in preeclamptic patients (Kamali-Sarvestani *et al.*,2006), and to an AA genotype in the promoter region of IL-I0 in the placenta (Makris *et al.*,2006). Thus reduced IL-10 levels could be considered as a genetic susceptibility factor for the development of EC.

IL-10 produced by activated Th2 cells during pregnancy, exerts its primary anti-inflammatory effects by inhibiting T cell pro-inflammatory cytokine production and the antigen-presenting capabilities of macrophages and dendritic cells (Sargent*et al*., 2006). Preeclampsia and EC are associated with a shift of Th1/Th2 toward Th1 (Azizieh *et al.*, 2005). The shift to a predominant Th1-type immunity in EC is reinforced by other experiments on intracellular cytokines measurements in T and NK cells, as well as by the assessment of cytokine secretion levels of PBMCs isolated from preeclamptic patients (Darmochwal-Kolarz *et al.*,2002; Rein *et al*.,2002; Azizieh *et al*.,2005).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECCOMENDATIONS

6.1 Summary of Findings

The study was primarily designed to address the assumption that Tcell subsets, cytokines and PSG-1 are altered in EC and that the immunopathology, pathogenesis or pathophysiology of EC is associated with **a**n altered adaptation of the maternal immune response to the fetus that exempts it from being rejected by the mother. The objective was to analyze for CD3+, CD4+and CD8+ T cell subsets, cytokines (IL-2, IL-4, IL-10 and TNF alpha) and PSG-1 levels in EC.

The following points summarize the changes in hematological, T cells, cytokines and PSG-1 levels:

6.1.1 Patient with eclampsia

The study revealed an association of eclampsia with increased markers of inflammation (WBC and neutrophils),depressed CD4+ T cells compensated by high levels of proinflammatory cytokine (TNF-α) and low levels of anti-inflammatory cytokine (IL-10). The deduction was that the low level of IL-10 could not counter the high levels of TNF-α culminating in Th1 reactivity, endothelial inflammation, fetal mortality, and Tcell apoptosisas well as decreased CD4+ Tcells and immunopathology in eclampsia. Statistically significant low levels of PSG-1 were not recorded as reported by other reseachers. Therefore, EC was not associated with low levels of PSG-1.

6.1.2 Normal healthy pregnant controls

A mild depression of CD4+T cells with increased markers of inflammation (WBC, and neutrophils), compensated by an increased level of the pro-inflammatory cytokine (TNF-α) were found. The increased levels of TNF-αwas counter regulated by increased serum levels of anti-inflammatory cytokine (IL-10) and high levels of PSG-1 resulting in relative immune suppression and immune tolerance as a result of Th1 reactivity counter regulated by Th2 type inflammatory response.

6.2 Conclusion

In this study, EC was associated with an excessively dominant pro-inflammatory cytokine environment, which supports the hypothesis of increased inflammatory responses in eclampsia as illustrated by increase in TNF-α and decreased anti-inflammatory cytokine (IL-10). This partly supports the hypothesis of a Th1 shift in EC. While significantly depressed CD4+ T lymphocytes and decreased IL-10, duly compensated by elevated amount of pro-inflammatory cytokine (TNF-α) were documented in this study, the presence of EC was not associated with low levels of PSG-1 as reported by other researchers. Elevated TNF-α in the maternal circulation might play a central role in the excessive systemic inflammatory response, as well as the generalized endothelial dysfunction characteristics of the maternal syndrome of EC. This emerging information will no doubt add to the body of knowledge and could be exploited in preventing adverse pregnancy outcomes and controlling immunopathology associated with EC.

6.3 Recommendations

From the findings in this study, the following recommendations are proffered to improve the quality of findings in furtherance of the research in EC:

6.3.1 Health Education and increased awreness:

Afew women in this study lost their babies to the ‘monster’ of eclampsia, and the women enrolled in this study formed part of the larger society where they came from, there is therefore the need to pay advocacy visits to the religious and community leaders and also embark on community sensitization in order to educate and create awareness of the consequences of PE and EC.

6.3.2 Anti-inflammatory cytokines as theraprutic agent:

The rising inflammatory cytokine (TNF-α) levels in normal healthy pregnant women adequately counter regulated by the anti-inflammatory cytokine (IL-10) helped to prevent EC and its debilitating effects. Therefore, further research is advocated to discover how anti-inflammatory cytokines could be exploited as a therapeutic agent for women at high risk of PE/EC.

6.3.3 Further study /research

This study points out the need for the following:

(a) A longitudinal study to capture alterations in T cell subsets, cytokines and PSG-1 as they manifest in the course of pregnancy.

(b) Further research should focus on discovering highly sensitive biomarkers that will facilitate early detection of PE the precursor of EC in the laboratory, during antenatal visits among our pregnant women as a strategy to avoid the debilitating effect of EC.

(c) The Th1/Th2 model is not limited to inflammatory cytokines (IL-2 and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) investigated in this study. There is

the need to study further other relevant cytokines such as IFN-γ and TGF-β for a clearer picture of the pathogenesis of the disease.

6.3.4 Importance in clinical decisions and decision making in the Nigerian government

Finally, the findings generated in this study provide a basis for the clinicians, researchers and government as part of strategies for improving and meeting up with health standards as stipulated in the Millennium Development Goals (MGDs) to be achieved by 2015.

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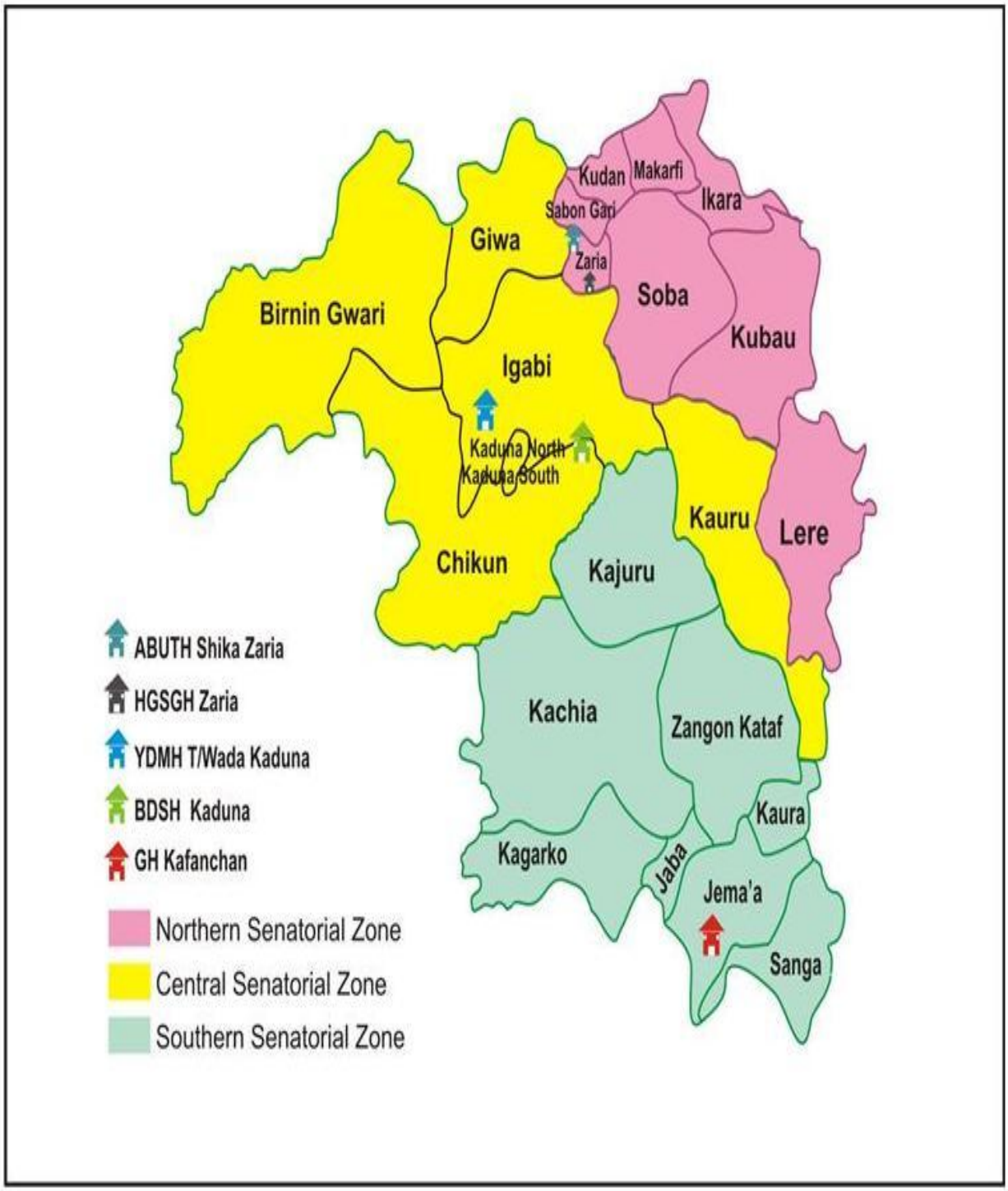
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APPENDICES

Appendix I: Map of Kaduna State highlighting the hospitals where samples were collected in the three Senatorial zones.



Appendix II: Ethical Approval

Ahmadu Bello University Teaching Hospital
P.M.B. 06, Shika - Zaria, Kaduna State, Nigeria. 069-876305
website: www.abuth.org abuthshika@yahoo.com Abuthshika@gmail.com

Chairman of Board:
Chief Medical Director: DR. LAWAL KHALID, *MBBS, FMCS, FWACS, FRCS(ED) mni*
Chairman, Medical Advisory Committee: DR. ABDULLAHI MOHAMMED, *MBBS, FWACP FICS*
Director of Administration: BARR. ISHAK BELLO, *LL.B, BL, LL.M, PGDM, AHAN, FCAI*

Our Ref:
ABUTH/HREC/TRG/36 12th July,2012

Banda Jim Monday
Dept of Immunology
ABUTH
Zaria.

ETHICAL CLEARANCE

Your application for ethical clearance on the research proposal titled. "**CELLULAR IMMUNITY IN NORMAL PREGNANCIES AND THOSE COMPLICATED BY PREECLAMPSIA.**" refers.

This is to convey ethical approval for you to commence the study. The ABUTH Scientific and Health Research Ethics Committee requires an annual update from the principal investigator.

Prof. J. U. Okpapi
Chairman, ABUTH HREC

MINISTRY OF HEALTH, KADUNA STATE

All Communications to be Addressed to:
THE HON. COMMISSIONER
Quoting Reference and Data
Tel: (062) 248084
(062) 248252

Independence Way,
P.M.B. 2014,
Kaduna,
Kaduna State, Nigeria.

MOH/ADM/744/Vol.1/190

25th September, 2012.

.....
.....
.....

ETHICAL APPROVAL

I am directed to convey the Honorable Commissioner's wish to grant approval to Banda Jim, Monday. Currently undergoing a PhD (Immunology) with the department of Medicine, Faculty of Medicine, ABU – Zaria. Topic: **CELLULAR IMMUNITY IN NORMAL PREGNANCIES AND THOSE COMPLICATED BY PREECLAMPSIA**

It is expected that necessary assistance be accorded to him in the process of his research, please.

However, it is mandatory for the researcher to submit a copy of his research finding(s) to the office of the Honorable Commissioner, Health as soon as it is concluded as you have the assurances of my highest regards.

27/9/12.

JACOB KAYIT MUSA
SECRETARY, ETHICAL COMMITTEE

Appendix III: Informed consent (English)

1DENTITY NUMBER…….

I ..... hereby allow Mr. Banda Jim Monday, Immunology Unit of the Department of Medicine, Ahmadu Bello University Zaria to take 8 mls of my blood for the purpose of research work on Cellular Immunity in normal Pregnancies andthose complicated by Eclampsia among women attending antenatal clinic at ABUTH Zaria,Barau Dikko Specialist Hospital Kaduna,Yusuf Dantsoho Memorial Hospital Kaduna and General Hospital Kafanchan Kaduna State, Nigeria.

I understand that the study will enable me to know my immunological status and its role in pregnancy. Also that some drugs will be given to help me recover my blood faster, and that bloodsample collection may cause me slight painbut will resolve with time and I do not have to miss work.

Name of patient……Thumb print/Sign…… Date……..…

Address of patient……

Mobile Phone number…….

Address of patient……

Name of witness……..…Thumb print/Sign…….Date……,,,….

Name of person administering the consent form……

Thumb print/sign…… Date……

Appendix IV: Consent Form (Hausa)

IDENTITY NUMBER……..……..

Ni, ...naba Mr Banda Jim Monday, na jimi'ar Ahmadu Bello (sashen tsare jiki daga chututuka da lafiyan jiki) izinin dibar mil takwas (8 mls) na jini na da niyyar binciken kwayoyin tsare jiki daga chututtuka. Ina sane da chewar diban jini zai haddasa zafin ciyo mara tsanani, kuma ina iya tafiyar da harkar rayuwa ta bada damuwa ba.

Sa hannu: …… Dangwala hannu…….Kwana wata…….

Adireshi……..

Lambar wayar ta-fi-da-gidanka……

Sa hanun mai lura da mara lafiya ko wani ma aikacin jinya. Tun daga Iyaye, miji ko Dan'uwa ko wani majibinci sa……...Dangwala hannu……Gwana wata…….

Lambar wayar ta-fi-da-gidanka…….

Adireshi……..

Appendix V: Study protocol

T LYMPHOCYTE SUB POPULATIONS, CYTOKINES AND PREGNANCY SPECIFIC BETA-1 GLYCOPROTEIN Studies in Eclamptic patients

Study Proforma:

IDENTITY NUMBER……

A. Patient Data

Name…….
Date…….……Height…… (m)

Age…….. Weight……Kg

Hospital No……. BP ……..……mmHg

Body temperature……

B. Patient's Obstetrics Data.

Age of the woman…….(Years)

Fundal height …… (m)

Gestational age ……..…… (Weeks)

Expected Date of Delivery…….

Last Menstrual Period (LMP)…….……

Parity……..

C. Laboratory Investigation

1. Hematological indices.

HB ( ) g/dL, PCV ( ) %,, WBC ( ) x 10^9/L

Neut,( ) %, Lymp( ).% Mono( )% Eos ( )% Basophiles ( )%

Platelets count ( ) x 10^9/L

2. Biochemistry: Proteinuria 2+ ( ), 3+ ( ), ≥4+ ( )

3 Immunological indices:

CD3 ( ) cell/µL, CD4 ( ) cell/µL, CD8 ( ) cell/µL

CD3 ( ) % CD4 ( ) % CD8 () %

IL-2 ( ) pg/mL, IL-4 ( ) pg/mL, L-10 ( ) pg/mL,

TNF-α, ( ) pg/mL

PSG1 ( ) pg/mL

AppendixVI:StudyProtocol

TLYMPHOCYTE SUBPOPULATIONS, CYTOKINES AND PREGNANCY SPECIFIC BETA-1 GLYCOPROTEINStudies in Normal Pregnant Controls

IDENTITY NUMBER……

A Patient's personal data

Name…….Date…….……

Height…….…… (m)

Age…….. Weight……(Kg)

Hospital No…… .BP ……..……mmHg

Body temperature……

B Patient's Obstetricsdata.

Age of the woman…….(Years)

Fundal height …… (m)

Gestational age …….. (Weeks)

Expected Date of Delivery…….

Last Menstrual Period (LMP)…….……

Parity……..

C Laboratory Investigations

1. Hematological indices.

HB ( )g/dL, PCV ( )%, WBC ( ) x 10^9/L

Neut, ( ) %, Lymp. ( ). % Mono ( ) % Eos ( ) % Basophil ( )%

Platelets count ( ) x 10^9/L

2. Biochemistry: Proteinuria2+ ( ), 3+ ( ), ≥4+ ( )

3 Immunological indices:

CD3 ( ) cell/µL, CD4 ( ) cell/µL, CD8 ( ) cell/µL

CD3 ( ) % CD4 ( ) % CD8 () %

IL-2 ( ) pg/mL, IL-4( ) pg/mL, L-10 ( ) pg/mL,

TNF-α, ( ) pg/mL

PSG-1 ( ) pg/mL.

Appendix VII: Study protocol.

T LYMPHOCYTE SUB POPULATIONS, CYTOKINES AND PREGNANCY SPECIFIC BETA-1 GLYCOPROTEIN Studies in Normal Healthy non pregnant controls

A Patient Data

Name……. Date……..…..…..…..

Height……..……

Age…….. Weight……..

Hospital No……. BP…….…

B. Laboratory Investigations

1. Hematological indices.

HB ( ) g/dL, PCV ( )%, WBC, ( ) x 10^9/L
Neut……..%Lymp…….% Mono……..% Eos……% Basophil……%
Platelet ( ) x 10^9/L

2. Biochemistry: Proteinuria 2+ ( ), 3+ ( ), ≥4+ ( )

3. Immunological indices:

CD3 ( ) cell/µL CD4 ( ) cell/µL CD8 ( ) cell/µL

CD3( ) % CD4 ( ) % CD8( ) %

IL -2( ) pg/mL, IL-4( ) pg/mL, .IL-10 ( ) pg/mL,

TNF-α ( ) pg/mL

PSG-1 ( ) pg/mL

Appedix VIII: Principle of Tests used in the Study.

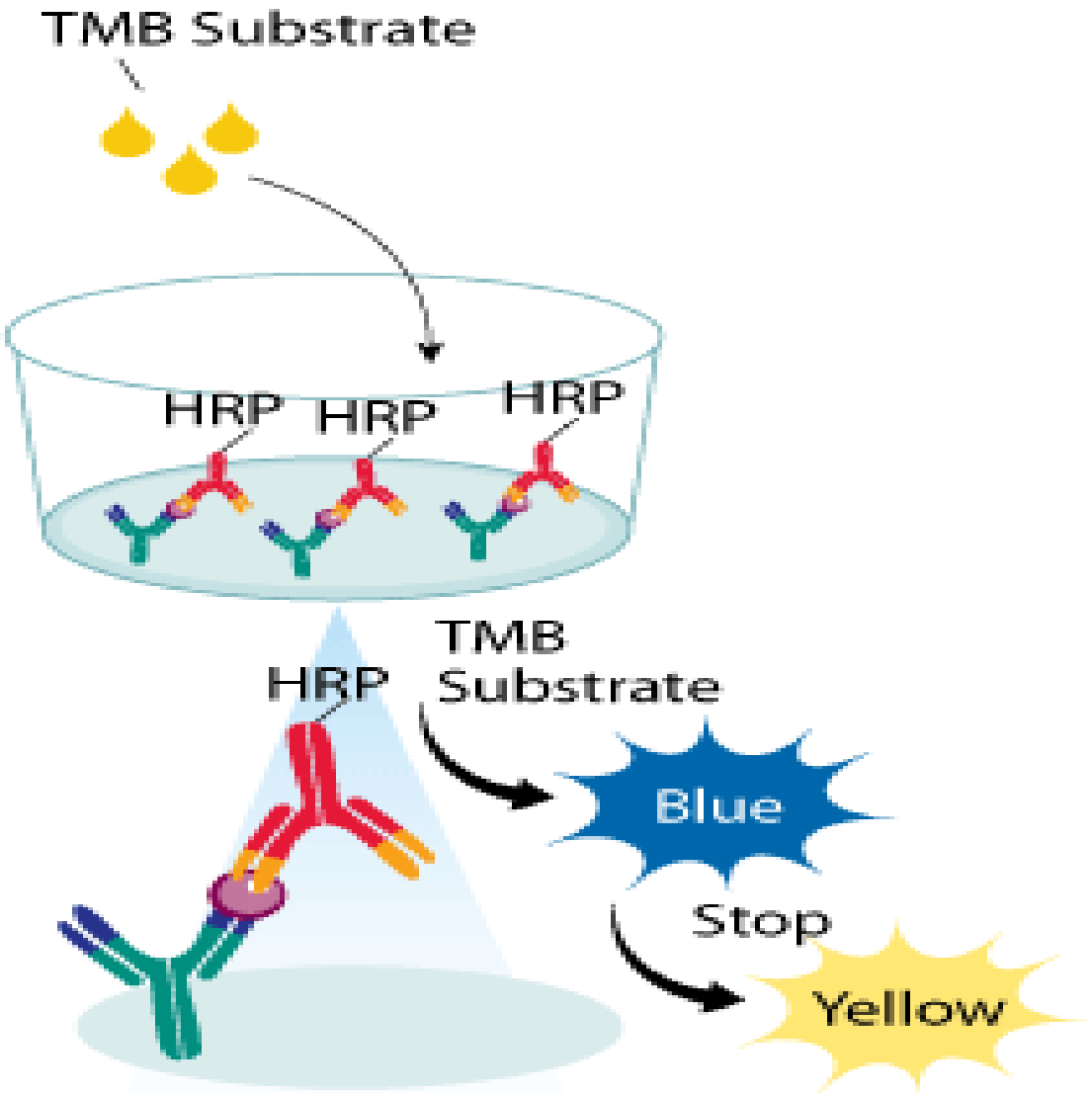
1. **Sysmex KX-21N**: The sample in the cytometer is a suspension of cells. In a flow cytometer, the suspension of cells is introduced in a fluid delivery system so that the cells move in a single file through a flow cell, where they are intersected by a narrow focused beam of laser light at a particular wave length. Once the beam of light hits a cell in this system, there are several important pieces of information that are gathered simultaneously. A forward angle light scatter (FALS) sensor detects light scatter by the cells in a forward direction. The forward angle light scatter (FALS) gives information about cell size. A sensor perpendicular to the beam of light gather information at 90^{o} also called side light scatter (SS), providing information about the complexity of the cell or particle; for example, neutrophils display more SS than lymphocytes. The combination of FALS and SS may be used together (in a scattergram) or in combination with specific antibodies to identify a cell of interest in a specimen. Fluorescent emissions from antibodies attached to the cells are measured by photomultiplier tubes (PMTs).
2. **FACS count machine (BD-BIOSCIENCE) principle of operation:** A single test requires one convenient, ready-to-use reagent tube pair; one tube determines the absolute number of 'helper'/'inducer' T lymphocytes (CD4/CD3), the other tube determines the absolute number of suppressor/cytotoxic T lymphocytes (CD8/CD3); both tubes measure the number of total lymphocytes (CD3).

   When whole blood is added to the reagents, fluorochrome- labeled antibodies in the reagent bind specifically to lymphocytes surface antigens. After a fixative solution is added to the reagent tubes, the sample is run on the

equipment. Here, the cells come in contact with laser light, which causes the fluorochrome- labeled cells to fluoresce. This fluorescent light provides the information necessary for the instrument to count the cells. In addition to containing the antibody reagent, the reagent tubes also contain known fluorochrome-intergrated reference beads. These beads function as fluorescence standard for locating the lymphocytes and also as well as quantitation standard for the enumeration of cells.

Analysis is automatic. The software identifies the T-lymphocyte populations and calculates the absolute counts. Results print immediately after sample is run and absolute cell counts for CD4 (helper/inducer T-lymphocytes) and CD8 suppressor/cytotoxic T-lymphocytes and CD3 (Total lymphocytes) as well as CD4/CD8 (Helper/ suppressor T-lymphocytes) ratio are reported.

3. **ENZYME LINKED IMMUNOSORBENT ASSAY:** The ELISA kits for TNF, IL-2, IL4, IL-10, and PSG I are solid phase ELISA based on sandwich principle. The microtitre wells are coated with a monoclonal anti TNF antibody, (anti-IL-2, anti -IL-4, anti-IL-10, anti-PSG 1 antibody depending on the one in use). An aliquot of patient sample containing endogenous TNF-α (IL-2, IL-4, IL-10, PSG I) is incubated in the coated well with a specific rabbit anti-TNF (anti-IL-2, anti-, IL-4, anti-IL-10, anti-PSG-I). A sandwich complex is formed. After incubation the unbound material is washed off and an anti-rabbit peroxidase conjugate is added for detection of the bound TNF-α (IL-2, IL-4, IL-10, and PSG 1). Having added the substrates solution, the intensity of color developed is proportional to the concentration of theTNF-α (IL-2, IL-4, IL-10, PSG I) in the sample.


TMB Substrate
HRP
HRP
HRP
HRP
TMB
Substrate
Blue
Stop
Yellow

Medi-Test Combi 2

en

IVD In vitro-diagnostics, suitable for self-testing

Test strips for rapid detection of protein and glucose in urine

Instructions for Use

Application

Screening test for early detection and monitoring of diabetes (diabetes mellitus) and metabolic anomalies.
The test is also suitable for use
- in preventive diagnosis (screening);
- for monitoring progress or relapse during therapy;
- for self-monitoring by the patient.

These urine test strips serve exclusively for detecting morbid (pathological) changes in human urine.

Notes

Discuss **any positive** or **any unclear** results with your doctor. You must also do this if this test does not give a positive result, but the ailments persist.
If you suspect a false negative result, please repeat the test with a test strip from an unopened container or ask your physician. Your doctor will carry out further investigations with the usual methods in his laboratory. In principle, individual test strip results enable definitive diagnosis and targeted therapy only in combination with other medical findings.
Use only clean, well-rinsed containers to collect urine.
Test urine preferably immediately after collection.
Always remove only the required number of test strips. Do not touch the test fields!
Tightly close container immediately after removal.
Do not remove the drying agent from the stopper, do not damage the stopper.
Protect test strips from sunlight and moisture. Store the container in a cool and dry place (do not refrigerate!) at a temperature between 4 ... 30 °C (39 ... 86 °F). The test strips can be used up to the imprinted expiry date if stored properly. Always discard damaged test strips or containers.

The stopper of the test strip container contains a non-toxic drying agent. If it is swallowed accidentally, drink plenty of water (if necessary contact a physician).
Dispose used test strips with domestic waste (use test strips only once!).
See below for an explanation of the symbols on box and container label.
Store test strips out of reach of children!

Explanation of symbols

CE Statement of Conformity (Product corresponds to the In-Vitro Diagnostic Medical Devices Directive 98/79/EC of the European Union)

Please read instructions for use!

Permitted storage temperature range

Use by

LOT Batch identification

REF Item number

Manufacturer

Do not reuse

Information and additional notes

Principle

Protein: The test is based on the „protein error" principle of indicators. The test zone is buffered to a constant pH value and changes color from yellow to greenish blue in the presence of albumin. Other proteins are indicated with less sensitivity.
Glucose: The detection is based on the glucose-oxidase-peroxidase-chromogen reaction. Apart from glucose, no other compound in urine is known to give a positive reaction.

Evaluation – Sources of error

Protein: The minimum sensitivity of the test strip is 10 mg protein/dL urine. The color fields correspond to the following ranges of albumin concentrations:
negative, 30, 100 and 500 mg/dL or negative, 0.3, 1.0 and 5.0 g/L.
Falsely positive results are possible in alkaline urine samples (pH > 9), after infusions with polyvinylpyrrolidone (blood substitute), after intake of medicaments containing quinine and also by disinfectant residues in the urine sampling vessel. The protein coloration may be masked by the presence of medical dyes (e.g. methylene blue) or beetroot pigments.
Glucose: Pathological glucose concentrations are indicated by a color change from green to blue-green. Yellow to weakly green test fields are to be considered as negative (or normal). The color fields correspond to the following glucose concentrations:
- 0 (negative), 20 (normal), 50, 150, 500 and ≥1000 mg/dL or
- 0 (negative), 1.1 (normal), 2.8, 8.3, 27.8 and ≥ 55.5 mmol/L.

The influence of ascorbic acid (vitamin C) has been largely eliminated. An inhibitory effect is produced by gentisic acid. False positive reactions can be caused by residues of peroxide -containing or other cleansing agents.

Quality Control in professional use

The performance of the test strips should be confirmed by use of positive and negative control solutions. Positive and negative controls should be analyzed once a day, whenever a new bottle of strips is opened, whenever a new lot of strips is started, and every 30 days to check storage conditions. Each laboratory should establish its own goals for adequate standards of performance, and should question handling and testing procedures if these standards are not met.

Reacting Substances

(Quantity resp. activity/cm² at time of impregnation)

Protein:	
tetrabromophenol blue	10 µg
Glucose:	
glucose oxidase	7 U
peroxidase	1 U
tetramethylbenzidine	96 µg

Notes

The effect of medications or their metabolites on the test is not known in all cases. In case of doubt it is therefore recommended to consult your physician.
Presentation: Packs of 50 and 100 test strips
Date of revision: 09/2014

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-0 · Fax: +49 24 21 969-199 · info@mn-net.com · www.mn-net.com

CE 0197

Durchführung des Harntests
Reihenfolge hierbei genau beachten:



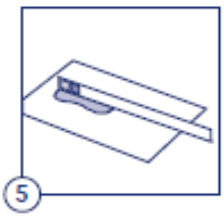
1. Uhr mit Sekundenanzeiger bereitlegen. Datum und Uhrzeit notieren.
2. Dose öffnen. Nur **einen** Teststreifen entnehmen. Reaktionszonen / Testfelder nicht berühren!
3. Dose nach der Entnahme sofort wieder fest verschließen.

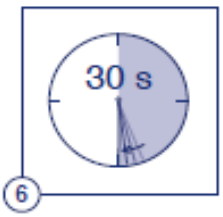


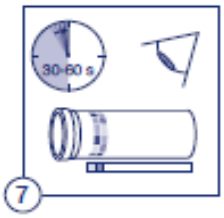




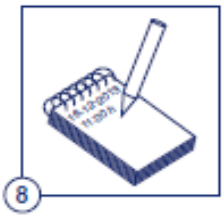
4. Teststreifen ca. 1 Sekunde in den Harn eintauchen. Die Testfelder müssen mit Harn benetzt werden.
5. Nach Herausziehen des Teststreifens aus der Harnprobe seitliche Kante auf Papier kurz abtupfen. Teststreifen nicht aus der Hand legen.
6. 30 Sekunden warten.







7. Mit der Auswertung beginnen:
   Auf dem Dosenetikett befindet sich für jedes Testfeld eine Farbreihe. Jedem Testfeld ist ein Farbwert zuzuordnen. Wenn keine Übereinstimmung gefunden wird, ist der Farbwert zu wählen, der dem Testfeld am nächsten kommt.
8. Ergebnis notieren und Ihrem Arzt beim nächsten Besuch vorlegen.
9. Bewertung spätestens 60 Sekunden nach dem Eintauchen beenden. Diese zeitliche Begrenzung beachten, da das Testfeld seine Farbintensität auch danach weiter ändert.





Test procedure
Follow this sequence exactly:

1. Provide clock with second hand. Note date and time.
2. Open container. Remove **one** test strip only. Do not touch reaction zones / tests field!
3. Close container tightly immediately after removing test strip.

4. Immerse test strip in the urine for approximately 1 second. All test fields must be submerged.
5. After removing the test strip from the urine sample, briefly dab the lateral edge on paper. Do not put the test strip down.
6. Wait for 30 seconds.

7. Start the evaluation: On the container label a color sequence for each test field can be found. Assign the test field to a color value of its sequence. Find a match or select the color value that comes as close as possible to the test field.
8. Note the result and submit it to your physician at your next visit.
9. Finish the evaluation within 60 seconds after immersion at the latest. Observe this time limit, since the test field still changes its color intensity after this period.

Teststreifen nach der Auswertung wegwerfen.
NICHT mehrfach verwenden!
Ändern Sie **nie** die Einnahme der verordneten Medikamente aufgrund eines Resultats dieser Teststreifen.

Discard test strip after evaluation.
DO NOT use more than once!
Never change the intake of prescribed medication as a result of this test.

Appendix IX: Results of statistical analysis

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Log (PSG-1 pg/mL)	Between Groups	89.678	2	44.839	572.266	.000
	Within Groups	6.582	84	.078		
	Total	96.259	86			
Log(IL-10 pg/mL)	Between Groups	9.095	2	4.548	19.348	.000
	Within Groups	19.978	85	.235		
	Total	29.073	87			
Log(TNF pg/mL)	Between Groups	.179	2	.089	7.816	.001
	Within Groups	.972	85	.011		
	Total	1.150	87			
Log(IL -2 pg/mL)	Between Groups	.021	2	.011	.538	.586
	Within Groups	1.680	85	.020		
	Total	1.701	87			
Log(IL-4 pg/mL)	Between Groups	.040	2	.020	2.828	.065
	Within Groups	.595	85	.007		
	Total	.634	87			

Kruskal-Wallis Test.

Table

Parameter	Subject			
	EC	PC	NPC	P
Log (PSG-1 pg/mL)	56.46	57.82	13.00	0.000
Log(IL-10 pg/mL)	29.21	42.00	70.24	0.000
Log(TNF α pg/mL)	56.71	34.42	36.02	0.000
Log(IL-2) pg/mL)	41.66	46.80	46.52	0.645
Log(IL-4) pg/Ml	47.49	33.56	50.90	0.035

One wayANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Age in years	Between Groups	.228	2	.114	.003	.997
	Within Groups	3776.763	111	34.025		
	Total	3776.991	113			
Gest.Age(weeks)	Between Groups	.329	1	.329	.075	.785
	Within Groups	325.079	74	4.393		
	Total	325.408	75			
SBP(mmHg)	Between Groups	94380.018	2	47190.009	193.918	.000
	Within Groups	27011.921	111	243.351		
	Total	121391.939	113			
DBP(mmHG)	Between Groups	20502.860	2	10251.430	109.444	.000
	Within Groups	10397.211	111	93.669		
	Total	30900.070	113			
BMI	Between Groups	24.813	2	12.407	.733	.483
	Within Groups	1878.598	111	16.924		
	Total	1903.411	113			

One wayANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Haemoglobin	Between Groups	2.723	2	1.362	.550	.578
	Within Groups	274.795	111	2.476		
	Total	277.519	113			
White Blood Cell	Between Groups	360.387	2	180.194	13.287	.000
	Within Groups	1505.349	111	13.562		
	Total	1865.737	113			
Lymphocyte	Between Groups	6668.940	2	3334.470	18.535	.000
	Within Groups	19968.903	111	179.900		
	Total	26637.844	113			
Neutrophil	Between Groups	9679.980	2	4839.990	44.687	.000
	Within Groups	12022.150	111	108.308		
	Total	21702.130	113			
Plateletes	Between Groups	19840.281	2	9920.140	1.945	.148
	Within Groups	566014.711	111	5099.232		
	Total	585854.991	113			

OnewayANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
CD3	Between Groups	3363786.386	2	1681893.193	10.209	.000
	Within Groups	1.829E7	111	164744.770		
	Total	2.165E7	113			
CD4	Between Groups	2540516.439	2	1270258.219	27.456	.000
	Within Groups	5135368.026	111	46264.577		
	Total	7675884.465	113			
CD8	Between Groups	164614.895	2	82307.447	2.192	.116
	Within Groups	4167399.211	111	37544.137		
	Total	4332014.105	113			
CD4/CD8	Between Groups	5.427	2	2.713	12.391	.000
	Within Groups	24.306	111	.219		
	Total	29.732	113			
CD3%	Between Groups	1744.390	2	872.195	5.368	.006
	Within Groups	18036.569	111	162.492		
	Total	19780.959	113			
CD4%	Between Groups	1737.017	2	868.508	12.913	.000
	Within Groups	7465.504	111	67.257		
	Total	9202.521	113			
CD8%	Between Groups	182.072	2	91.036	2.148	.122
	Within Groups	4705.441	111	42.391		

OnewayANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
CD3	Between Groups	3363786.386	2	1681893.193	10.209	.000
	Within Groups	1.829E7	111	164744.770		
	Total	2.165E7	113			
CD4	Between Groups	2540516.439	2	1270258.219	27.456	.000
	Within Groups	5135368.026	111	46264.577		
	Total	7675884.465	113			
CD8	Between Groups	164614.895	2	82307.447	2.192	.116
	Within Groups	4167399.211	111	37544.137		
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CD4%	Between Groups	1737.017	2	868.508	12.913	.000
	Within Groups	7465.504	111	67.257		
	Total	9202.521	113			
CD8%	Between Groups	182.072	2	91.036	2.148	.122
	Within Groups	4705.441	111	42.391		
	Total	4887.512	113			