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A STUDY OF THE ROLE OF CYTOKINES IMBALANCE AND OXIDATIVE STRESS IN THE PATHOGENESIS OF PREECLAMPSIA IN KHARTOUM TEACHING HOSPITAL

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DEDICATION

To my mother, my family

To my teachers in the medical school, to all members of biochemistry department

To the cooperative staff of the Institute of Brain Chemistry and Human Nutrition

London Metropolitan University

To my friends

To all pregnant women who participated in the study

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ABSTRACT

Preeclampsia remains a major cause of maternal and perinatal morbidity and mortality. Its exact aetiology is still obscure.

Objectives: The objective of this case control prospective study which was conducted in Khartoum Teaching Hospital during the period (2007-2008) was to study the role of cytokines imbalance and oxidative stress in the pathogenesis of preeclampsia.

Methodology: The levels of interferon gamma (IFN-γ), as Th1 cytokine and interleukin-4 (IL-4), interleukin-10 (IL-10) as Th2 cytokines were determined using Enzyme-linked immunosorbent assay (ELISA) in sera from 33 women with preeclampsia (at presentation and 7 days later) and 32 women with normal pregnancy as a control group.

In order to study the effect of the oxidative stress on pathogenesis of preeclampsia, the activity levels of the erythrocyte antioxidant enzymes; glutathione peroxidase, catalase and superoxide dismutase were determined in 37 women with preeclampsia compared to 38 women with normal pregnancy in the third trimester using ELISA, and omega-3 (Eicosapentaenoic acid ; EPA & Docosahexaenoic acid; DHA) and omega-6 (arachidonic acid) profiles in three erythrocyte membrane phospholipids fractions; phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin, using gas chromatography. Further more, the plasma antioxidant vitamins; retinol and α-tocoferol, were also determined using HPLC.

Results: at presentation, the levels of IFN-γ and IL-4 were slightly—not statistically significant—higher in the women with preeclampsia whereas IL-10 was significantly higher in the women with preeclampsia (P=0.002). Seven days later the women with preeclampsia had significantly lower levels of IFN-γ (P=0.035) and IL-4 (P=0.000) and
significantly higher levels of IL-10 (P=0.000). There were no significant differences in
the levels of the three antioxidant enzymes in preeclamptic women compared to women
with normal pregnancy. Women with mild preeclampsia (Diastolic blood pressure (DBP)
< 110) have slightly higher (not statistically significant) level of activity of erythrocyte
glutathione peroxidase, and lower activity levels of catalase and superoxide dismutase
compared to women with severe preeclampsia (DBP≥ 110). A significantly negative
correlation was observed between the activity of glutathione peroxidase and diastolic
blood pressure, r= -0.371, P = 0.02. In the three phospholipids fraction (PE, PC &
sphingomyelin), the mean level of EPA was significantly higher in women with
preeclampsia than the control group P = 0.012, 0.000, 0.000 respectively. The mean level
of DHA was significantly higher in women with preeclampsia than the control group, P=
0.04, 0.046, 0.000 respectively. The mean level of arachidonic acid was only significantly
higher in women with preeclampsia in the sphingomyelin fraction P=0.000. When
comparing women with mild and severe preeclampsia there was no statistically
significant difference in fatty acids profiles. The median level of α-tocopherol was
significantly higher in women with preeclampsia than the control group, P = 0.026. No
significant difference was noted between the two groups regarding retinol level.

**Conclusion:** The study findings do not support oxidative stress, but are in favor of
involvement of cytokines imbalance in the pathogenesis of preeclampsia.
The study aimed to investigate the role of cytokines Th1 and Th2 on the pathogenesis of the disease in two groups of patients: Group A (2007-2008) and Group B (2008-2009). The study was conducted to determine the oxidative stress (Oxidative stress) in the blood of patients with acute hemorrhagic disease.

The researchers measured the levels of interferon-γ (IFN-γ), interleukin-10 (IL-10), and interleukin-4 (IL-4) using ELISA. They also measured the activity of glutathione peroxidase, catalase, and superoxide dismutase.

The results showed that the levels of IFN-γ, IL-4, and IL-10 were significantly higher in Group A compared to Group B. The levels of these cytokines were highest in Group B, indicating a higher oxidative stress.

The researchers also measured the levels of AA, DHA, EPA, α-tocopherol, and retinol using gas chromatography and high-performance liquid chromatography (HPLC). They found that the levels of these nutrients were lower in patients with acute hemorrhagic disease compared to healthy individuals.

The study concluded that oxidative stress plays a significant role in the pathogenesis of acute hemorrhagic disease. The results also suggest that interventions to reduce oxidative stress may be beneficial in the management of this disease.
DBP 

Gas chromatography 

EPA, DHA, AA 

PC, PE 

Sphingomyelin 

Cytokines 

Oxidative stress
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2 - Khalid H. Bakheit¹, Kebreab Ghebremeskel², Gidon Zaiger², Mustafa I. Elbashir¹ and Ishag Adam¹. Erythrocyte antioxidant enzymes in Sudanese women with preeclampsia.

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Chapter one

Introduction & Literature review
Introduction and Literature Review

1.1 definition and incidence of preeclampsia

Pre-eclampsia is a potentially serious condition that occurs in 2–7% of all pregnancies [1], and can be life threatening for both the mother and child. It is defined as new onset, persistent hypertension after 20 weeks of gestation in association with significant proteinuria [2]. But this simple clinical definition gives little idea of the complexity of the disorder. This common disorder, which is more prevalent in first pregnancies, is associated with the highest maternal and fetal morbidity and mortality of all pregnancy complications, with 90% of the most serious outcomes occurring in developing countries [3]. Pre-eclampsia can present as asymptomatic hypertension and proteinuria at one end of the spectrum to multi-organ failure at the other. The precise origins of the disease remain enigmatic, but the placenta undoubtedly plays a role since delivery inevitably lead to rapid recovery. It is a complex disorder caused by a series of nutritional, environmental and genetic factors that lead to the creation of an imbalance between the free radicals nitric oxide (NO), superoxide (O$_2^-$) and peroxynitrate in the vascular endothelium [4]. Preeclampsia can occur at anytime after approximately 20 weeks of gestation, and because delivery is the only cure, this common complication of pregnancy is a major cause of premature delivery.
1.2 Criteria for diagnosis:

Diagnostic criteria for preeclampsia include new onset of elevated blood pressure and proteinuria after 20 weeks of gestation. Features such as edema and blood pressure elevation above the patient’s baseline no longer are diagnostic criteria [3, 4]. Severe preeclampsia is indicated by more substantial blood pressure elevations and a greater degree of proteinuria. Other features of severe preeclampsia include oliguria, cerebral or visual disturbances, and pulmonary edema or cyanosis [3, 4]. Diagnosis becomes less difficult if physicians understand where preeclampsia “fits” into the hypertensive disorders of pregnancy. These disorders include chronic hypertension, preeclampsia-eclampsia, preeclampsia superimposed on chronic hypertension, and gestational hypertension, [4]. Chronic hypertension is defined by elevated blood pressure that predates the pregnancy, is documented before 20 weeks of gestation, or is present 12 weeks after delivery [4]. In contrast, preeclampsia-eclampsia is defined by elevated blood pressure and proteinuria that occur after 20 weeks of gestation. Eclampsia, a severe complication of preeclampsia, is the new onset of seizures in a woman with preeclampsia. Eclamptic seizures are relatively rare and occur in less than 1 percent of women with preeclampsia [5].

1.2.1 Preeclampsia

Preeclampsia is diagnosed according to the criteria of the international society for the study of hypertension in pregnancy, previously normotensive women with two repeat (4 hours apart) diastolic blood pressure measurements of 90 mmHg or greater after the 20th
week of gestation, plus proteinuria of more than 300 mg/l in 24 hours as measured quantitatively or, ≥2+ protein by dipstick in two repeat measurements (4 h apart)

1.2.2 Severe preeclampsia

Blood pressure: 160 mm Hg or higher systolic or 110 mm Hg or higher diastolic on two occasions at least six hours apart in a woman at bed rest

Proteinuria: 5 g or more of protein in a 24-hour urine collection or 3+ or greater on urine dipstick testing of two random urine samples collected at least four hours apart Other features: oliguria (less than 500 mL of urine in 24 hours), cerebral or visual disturbances, pulmonary edema or cyanosis, epigastric or right upper quadrant pain, impaired liver function, thrombocytopenia, intrauterine growth restriction

1.3 Cytokines:

Although reductions in blood flow to the uteroplacental unit are known to result in cardiovascular and renal abnormalities consistent with the pathophysiological features of human preeclampsia, the physiological mechanisms linking placental ischemia with the abnormalities in the maternal circulation are unclear. [6,7]. Several lines of evidence support the hypothesis that the ischemic placenta contributes to endothelial cell activation/dysfunction of the maternal circulation by enhancing the synthesis of cytokines such as tumor necrosis factor-α [8]. Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They must be produced inside the body in response to an immune stimulus. They generally (although not always) act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to
cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effectors molecules. Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types (pleiotropy). Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines. Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can also act synergistically (two or more cytokines acting together) or antagonistically (cytokines causing opposing activities). Their short half life, low plasma concentrations, pleiotropy, and redundancy all complicated the isolation and characterization of cytokines [9]. Searches for new cytokines are now often conducted at the DNA level, identifying genes similar to known cytokine genes.

It is believed that the imbalance of the Th1/Th2 type responses, with a shift towards a Th1 response, may be involved in the etiology of preeclampsia [10], but the results on the Th1/Th2 in preeclampsia were inconsistent with each other. An elevated plasma level of Th2 type cytokines such as IL-4 was observed in preeclamptic patients [11]. No difference was found in Th1-cytokine production in peripheral blood and fetal cord blood between preeclamptic patients and normal pregnancies [12, 13]. In addition, CD8+ T (Tc) cells can differentiate into type 1 (Tc1) cells, producing mainly IFN-\(\gamma\), and type 2 (Tc2)
cells, producing mostly IL-4, IL-5, and IL-10. The Tc1/Tc2 balance also can modulate the type 1/type 2 immunity [14].

1.4 T helper cells (Th cells):

T helper (Th) cells can be divided according to the pattern of cytokines that they release into; Th1 cells, which release inflammatory cytokines such as interleukin-2 (IL-2) interferon gamma (IFN-γ) and tumor necrosis factor-β (TNF-β), induce cellular immunity, while Th2 cells, which release anti-inflammatory cytokines such as IL-4, IL-5, IL-6 and IL-10, induce antibody production [15]. Previous studies have suggested that in normal pregnant women, cytokines produced by Th2 cells predominate over those produced by Th1 cells, resulting in the maintenance of pregnancy [16-17].

1.5 Tumour necrosis factor alpha (TNF-α):

Is a multifunctional proinflammatory cytokine that is involved in the pathogenesis of a large number of autoimmune and inflammatory human diseases. The TNF-α gene is located in the chromosomal region 6p21.1–21.3, next to the major histocompatibility complex. Increased TNF-α expression in adipose tissue is associated with human obesity [19]. TNF-α act in a paracrine manner to interfere with insulin signalling by inhibiting autophosphorylation of the insulin receptor in fat and muscle cells [20]. It has been suggested to play an important role in insulin resistance and various components of the metabolic syndrome [19, 21]. Preeclampsia is a common and complex disease with a familial nature and a genetic background [22]. Impaired maternal immune response and different features of the metabolic syndrome, including insulin resistance, obesity, dyslipidemia and an increased risk of cardiovascular diseases are also characteristics of pre-eclampsia [23]. In addition, previous studies have demonstrated elevated circulating
concentrations of TNF-α in patients with severe preeclampsia and intrauterine growth restriction [24-27], and increased expression of the TNF-α gene in patients with preeclampsia [28, 29]. TNF-α has also been shown to affect early events in pregnancy, leading to failed trophoblast invasion and placentation [30].

1.6 Interferon gamma (IFN-γ):
IFN-γ may be considered a polypeptide hormone primarily because of its ability to transduce signals from one cell type to the other through diverse effects. The wide range of cellular responses to IFN-γ include proliferation, apoptosis, leukocyte-endothelial interactions, up-regulation of inducible nitric oxide synthase production in macrophages (Thereby increasing reactive oxygen species production), tryptophan metabolism, mediation of antigen presentation by class I and II MHC complexes, and regulation of numerous genes whose functions are yet to be identified [31, 32]. IFN-γ functions by binding to the receptor heterodimers; IFN-γ R1 and -2; IFN-γ R1 has a ligand-binding site, and IFN-γ R 2 facilitates signaling via Janus kinase-signal transducer and activator of transcription pathways [33, 34]. Recent genetic and immunological experiments have established that the proliferative or cytotoxic effect of IFN-γ is primarily determined by the relative density of IFN-γ R1 and -2 on the T cell surface [35-37]. Despite extensive immunological studies, very little is known about the placental regulation of expression of IFN-γ and its receptors in human pregnancy, except that IFN-γ R1 is localized in placenta throughout pregnancy [38].
1.7 Interleukin-10 (IL-10)

IL-10 is an important anti-inflammatory cytokine in pregnancy that promotes the termination of Th1 inflammatory rejection reactions against the fetal-placental unit. In a small number of preeclampsia cases, high levels of IL-10 are seen both in the placenta and in peripheral blood, which might be a compensatory response to elevated levels of IFN-γ, TNF-α, IL-2 and IL-12 [39-41]. On the other hand, IL-10 deficiency and an increase of TNF-α expression in the placenta and decidua are observed in preeclampsia compared to those with a normal pregnancy. This was interpreted as a modified immune balance consistent with inflammatory responses in preeclampsia [42]. This suggests that coupling of IL-10 deficiency and inflammatory signals at different stages of pregnancy may contribute to worse clinical conditions, including preeclampsia [43].

1.8 Interleukin-4 (IL-4):

Is one of type II cytokines (Th2 cytokines), which also include IL-5 and IL-10, their function is to down regulate proinflammatory immune responses mediated by type 1 cytokines. Elevated plasma level of Th2 type cytokines such as IL-4 was observed in preeclamptic patients [11]. Th1-type cytokines such as a-tumor necrosis factor (TNF-α), interferon gamma (IFN-γ) and interleukin 2 (IL-2) induce trophoblastic apoptosis, restrain differentiation and invasion of trophoblast, and thus may be involved in the incomplete invasion of trophoblast to spiral arteries and shadow implantation of placenta that are integral pathologies of preeclampsia [44]. On the other side, Th2 cytokines such as IL-4 and IL-10 stimulate the differentiation and proliferation of the trophoblast.
1.9 Basic immunology

The human immune system can be divided into innate and adaptive immunity. Innate immunity provides a rapid and first line of defense against invading microorganisms. The major cell types included in innate immunity are macrophages, natural killer (NK) cells and the granulocytes: mast cells, neutrophils, eosinophils and basophils.

These cells recognize microorganisms by their pattern-recognition receptors (PRRs) and they respond immediately by phagocytosis of microorganisms, eradication of infected cells, and cooperation with the adaptive immune response. The innate immune cells initiate immune responses and start to direct the adaptive immune responses, as well as aiding the adaptive immune response in the removal of pathogens targeted by the adaptive immune response. It takes 4–7 days before the initial adaptive immune response takes effect, and during this time the innate immune response has a critical role in controlling infections. Activated macrophages and NK cells secrete cytokines and chemokines, which initiate the inflammatory response and aid the destruction of phagocytosed particles. Furthermore, the macrophages have the capacity to present antigens from phagocytosed particles. The adaptive immunity reacts slower than the innate immunity, but has more long-lived and highly evolved antigen-specific protective responses, such as antibody production and cell-mediated immunity, Tosi (2005) [45].

The cells included in adaptive immunity are B- and T-lymphocytes, and these cells are also responsible for the immunological memory. B-lymphocytes are involved in the humoral defense against extracellular antigens by producing specialized antibodies upon stimulation; furthermore, these cells have the capacity to present antigens to the T-cells. A “helping” T-cell response is needed to induce B-cell proliferation and differentiation.
toward protein antigens. T lymphocytes, with the β-receptor, have a cell-mediated response where they respond to a foreign antigen presented on human leukocyte antigen (HLA). T-cells destroy intracellular pathogens by killing infected cells and by activating macrophages, as mediated by the Th1 subset. However, T-lymphocytes also have a central role in the destruction of extra cellular pathogens by activating B cells, and this is the specialized role of the second subset of CD4+ T-cells, called Th2 cells. Only a few antigens with special properties can activate naïve B lymphocytes on their own. Most antigens require an accompanying signal from helper T-cells before they can stimulate B-cells to proliferate and differentiate into cells secreting antibody. The T-lymphocytes expressing α, β-receptors are further divided into CD4+ T helper cells and CD8+ cytotoxic T-cells, depending on their type of antigen recognition and subsequent action. Cytotoxic T-cells mediate their function by “docking” onto a cell presenting foreign peptide antigen on HLA class I molecules, and induce apoptosis of that cell. T-helper cells direct the reaction of other cells of the innate and adaptive immune system more than they participate themselves in an immune reaction. They recognize antigens presented on HLA class II molecules on antigen-presenting cells (APC). The T-helper cells practice their effector functions on other immune cells by secreting cytokines. The signature cytokine of human Th1 cells is interferon gamma (IFN-γ) and lymphotoxin (LT), while Th2 cells are defined by their production of interleukin IL-4, IL-5 and IL-9. The cytokines produced by these T-cells determine the effector function as well as participate in the development and expansion of each subset. That is, the IFN-γ produced by the 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, the 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. 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. The main effector function of the Th1 cells is phagocyte-mediated and cytotoxic defense against infections, especially with intracellular microbes. The main effector function of the Th2 cells is IgE- and eosinophil/mast cell-mediated immune reactions, as well as inhibition of phagocytosis [46, 47]. Pro- and anti-inflammatory cytokines, just as for the Th1/Th2 concept, the balance between the effects of pro- and anti-inflammatory cytokines is thought to determine the outcome of the disease [46, 48]. 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) [49].

As reviewed by Dinarello (2000) [48], 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 NO synthase, since these are the enzymes that increase the synthesis of platelet-activating factor and leukotrienes, prostanoids 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.

1.10 Regulatory T-cells

Another cell type involved in the outcome of immune reactions is the regulatory T-cell. There are different types of regulatory T-cells identified up to now and more subsets are continuously being discovered; however, one type of regulatory T-cells (Treg) are the CD4+CD25+ self-reactive T-cells which suppress other, harmful, auto-reactive T-cells by cell–cell contact and/or by producing IL-10 and TGF-β [50, 51]. The suppression mediated by regulatory T-cells is essential for the induction and maintenance of tolerance to self-antigens [50, 51]. Furthermore, T-helper lymphocytes producing high amounts of TGF-β, IL-10 and IL-4 have been termed as type 3 cells (Th3) [47, 52]. These cells also display regulatory functions in that they actively suppress antigen-specific responses after re-challenge with antigens. The cytokine secretion in response to antigen-specific stimulation inhibits the development of Th1 responses, and is associated with low levels of antibody and a virtual absence of inflammatory T-cell responses [52]. Besides preventing autoimmune T-cell responses, Tregs are also believed to be involved in the suppression of other inflammatory responses, e.g., infection and allergy.

1.11 Immunology of normal pregnancy:

Among all the other changes that a woman’s body undergoes during pregnancy, her immune system also has to undergo adaptations, since the maternal immune system likely plays a part in the placentation process as well as in the maintenance of pregnancy. In the
late luteal phase of the menstrual cycle and early gestation, the decidua is infiltrated by large numbers of CD56-positive NK-cells, but their numbers decline as the pregnancy progresses. The number of macrophages and T-cells remains relatively constant throughout gestation. CD56 cells have been proposed as promoting trophoblast growth and invasion by secreting cytokines, especially leukemia inhibitory factor (LIF), in response to the HLA-G expressed on cytotrophoblastic cells. Furthermore, it has been shown that the expression of (LIF) is associated with Th2 responses [53]. At the adaptive level it is believed that the immune system during normal pregnancy is associated with a Th2 shift, as suggested by Wegmann et al. (1993) [16]. The idea of weakened cell-mediated immunity and increased humoral immunity is supported by clinical findings in pregnant women, where women with rheumatoid arthritis (RA), a cell-mediated autoimmune disorder, experience temporary remission of symptoms during pregnancy [16, 54, 55]. On the other hand, women with systemic lupus erythematosus (SLE), an autoantibody mediated disease, may experience worsened symptoms of the disease during pregnancy [16, 56]. Furthermore, Sacks et al. (1999) [57] has proposed that the alterations of adaptive immune responses during pregnancy are accompanied by increased innate immune responses. A majority of women experiencing unexplained recurrent spontaneous abortions (RSA) shows increased peripheral blood mononuclear cell (PBMC) production of IFN-γ and TGF-β in response to trophoblast antigens, while PBMCs from women not prone to RSA respond with IL-10, as reviewed by Raghupathy (1997) [58]. In addition, Piccinni et al. (1998) [53] showed that decidual cells from women with RSA had a lower production of IL-4, IL-10 and LIF in comparison with Decidual cells from normal pregnant women. This suggests that Th2 type immune
responses play a significant role in successful gestation and that Th1 type of immune responses are inconsistent with successful pregnancies [16, 53]. Furthermore, Wegmann et al. (1993) [16] also report a Th2 cytokine dominance in decidual cell culture supernatants from normal pregnancy.

Further laboratory findings of increased production of IL-4 and IL-10 and decreased production of IL-2 and IFN-\(\gamma\) from phytohemagglutinin (PHA, mitogen)-stimulated PBMCs supports the idea of a Th2 shift during normal pregnancy [59]. Reinhard et al. (1998) [60] also found increased levels of IL-4 and decreased levels of IFN-\(\gamma\) and IL-2 in T-lymphocytes from women with normal pregnancies compared to non-pregnant women. In addition, Ho et al. (2001) [61] detected higher spontaneous production of IL-10 than IFN-\(\gamma\) in PBMC cultures from normal human pregnancies. More specifically, the maternal immune system has been shown to have an increase of fetus-specific Th2 type responses, i.e., increased numbers of IL-4-secreting PBMCs in response to paternal antigen in normal human pregnancies as compared to non pregnant controls [62]. Taken together, these findings suggest that normal pregnancy requires a Th2/anti-inflammatory type of immunity, at least directed toward the fetus and placenta, while pregnancy complications such as RSA could be due to a skewed Th1/pro-inflammatory type of immunity [61, 62].

1.12 Immunology of preeclampsia:

In contrast to normal pregnancy, there are indications of increased inflammatory responses and also of an immune deviation toward Th1 in the established preeclamptic pregnancy. Roberts et al. (1989) [63] were one of the first to suggest that mediators released from the preeclamptic placenta are responsible for the endothelial damage seen
in preeclampsia. Subsequent to the damage, the injured endothelium initiates a dysfunctional cascade of coagulation, vasoconstriction and intravascular fluid redistribution that results in the clinical syndrome of preeclampsia.

Indeed, preeclampsia is associated with systemic maternal inflammation [64], which at the adaptive level has been suggested to be dominated by T-helper (Th) type 1 response, as reviewed in Saito and Sakai [18].

1.13 Th1/Th2 in preeclampsia

Results from studies designed to test the Th1/Th2 hypothesis in preeclampsia have not been consistent. Saito et al. (1999a) [10] measured the number of Th1 (CD4+IFN-γ) and Th2 (CD4+IL-4+) cells during normal pregnancy and preeclampsia, and found that the Th2 cytokines dominated during normal pregnancy while Th1 cytokines dominated during preeclampsia. In the same study, the PBMC production of IFN-γ and IL-4 was measured and the secreted levels were found to correlate with the number of Th1/Th2 cells. That is, normal pregnant women showed a significantly higher production of IL-4 and lower production of IFN-γ than women with preeclampsia. Darmochwal-Kolarz et al. (1999) [65] has also found increased production of IFN-γ in stimulated PBMCs. In contrast, Gratacos et al. (1998) [66] found that the serum levels of IL-4 and IL-10 did not differ between women with normal pregnancies and women with preeclampsia. In line with this, Henriques et al. (1998) [67] were able to detect IL-4 in placentae, but were unable to detect any differences between placentas from preeclamptic pregnancies and those from normal pregnancies, while Omu et al. (1995) [11] detected increased levels of IL-4 in serum from women with preeclampsia compared to normal pregnant women.
Furthermore, Daniel et al. (1998) [68] detected similar plasma levels of IFN-γ in between women with preeclampsia and women with normal pregnancies.

Pro- and anti-inflammatory balance during preeclampsia, likewise as for the studies of Th1 and Th2 balance, the reports on pro- and anti-inflammatory cytokines in preeclampsia differ from each other. The plasma levels of the pro-inflammatory cytokines IL-6 and TNF-α have been shown to be increased during preeclampsia when compared to normal pregnancy [8, 69], although no differences were seen for IL-1β and IL-10 [8]. In addition, Kupferminc et al. (1996) [70] found increased levels of IL-6 in plasma from women with preeclampsia compared to those with normal pregnancies. In contrast, Al-Othman et al. (2001) [71] found no differences in the levels of IL-6 in maternal serum from preeclamptic pregnancies compared to normal pregnancies. Furthermore, in studies on plasma levels of IL-10 and TGF-β1 Benian et al. (2002) [72] found increased levels of these markers during preeclampsia as compared to normal pregnancy. On studies of the serum levels of IL-12, Dudley et al. (1996) [73] found increased levels of the p40 subunit of IL-12 in women with preeclampsia, but no differences in the intact p70 dimer between women with preeclampsia and those with normal pregnancies. Although the levels of IL-12 p70 did not always reach detection levels, Daniel et al. (1998) [68] found increased levels of the IL-12 dimer in plasma from preeclamptic women compared to normal pregnant women. When measuring the stimulated cytokine production in PBMC cell culture supernatants, Darmochwal-Kolarz et al. (1999) [65] found increased levels of IL-2 and decreased levels of IL-10 in PBMCs from women with preeclampsia compared to women with normal pregnancies. Gratacos et al. (1998) [66] measured the serum levels of granulocyte-macrophage colony-
stimulating factor (GM-CSF) and found decreased levels during preeclampsia compared to normal pregnancy. Based on studies of murine pregnancies where this cytokine is found in high concentrations at the maternal–fetal interface and injection of high levels decreases fetal death in resorption prone animals, they suggested that GM-CSF may also be of importance in human pregnancy. Furthermore, Sacks et al. (1998) [74] found a pattern of increased cell surface markers and reactive oxygen species in leukocytes akin to that of sepsis in women with preeclampsia (Possible causes for the shifts in immune balance seen in preeclampsia). An earlier report has shown production of TNF-α in pure choriocarcinoma cell lines (Jar and JEG-3, equivalent to cytotrophoblastic cells) [75]. Furthermore, Wang and Walsh (1996) [76] found increased levels of TNF-α from preeclamptic placentas compared to placentas from normal pregnancies. These findings indicate that the placenta is involved in the production of TNF-α. The ischemia in the placenta during preeclampsia can also lead to the production and release of cytokines into the maternal circulation. As reviewed by Conrad and Benyo (1997) [8], it is highly possible that the ischemia leads to the production of TNF-α and IL-1. These cytokines are capable of producing endothelial cell activation and dysfunction. Apoptotic trophoblast and syncytiotrophoblastic microparticles are constantly shed from the placenta during pregnancy, with increased circulating levels during preeclampsia, as reviewed by Redmamm and Sargent (2003) [64]. Furthermore, it is possible that these trophoblastic cells might evoke an immune response in the mother. Curiously, Neale et al. (2003) [77], detected increased apoptosis of trophoblastic cell lines that were exposed to serum from preeclamptic women; this may in turn indicate a vicious circle involving the trophoblasts and general maternal systemic effects during preeclampsia, as detected by the influence
of their serum on the cell cultures. Furthermore, the excessive amounts of syncytiotrophoblastic microparticles might be the cause of the Th1 activation in preeclampsia (Redmann and Sargent 2003) [64]. These particles have been shown to inhibit endothelial cell function and to cause them to release proinflammatory factors. In addition, the monocytes can take up and process these particles with the possible result of increased production of the proinflammatory cytokines TNF-α and IL-12, the latter inducing Th1.

1.14 Preeclampsia and oxidative stress:

1.14.1 The role of the placenta:

The focus on the placental origin of pre-eclampsia led to the discovery that defective placentation is a likely predisposing factor in the evolution of the maternal syndrome. Placentation is the process by which the placental trophoblast invades the uterine wall; this occurs by cytotrophoblast invasion of the decidua and myometrium and, notably, of the maternal spiral arteries therein. This process has been found to be at fault in women with pre-eclampsia, as shown by histological examination of the placental bed, which has demonstrated partial failure of trophoblast invasion [78]. Because the invading trophoblast normally effects thinning of the vascular wall and thereby reduces uteroplacental vascular resistance, poor invasion of the spiral arteries will reduce blood flow to the placenta. Pregnant women at risk of pre-eclampsia often (but not always) demonstrate decreased blood flow in the uterine artery when assessed by analysis of the uterine artery Doppler waveform [79], which provides an indirect demonstration of the high resistance in the spiral arteries. It has long been hypothesized that poor placental blood flow sets up a chain of events, which culminates in the development of pre-eclampsia in a woman.
susceptible to the disease. This has given rise to the ‘2-stage theory of pre-eclampsia’; the first stage is reduced placental blood flow and the second is the subsequent development of the maternal syndrome [80]. Several studies suggest that placental lipid peroxides are increased [81-83] and placental antioxidant protective mechanisms decreased in pre-eclampsia [83, 84], indicating that the placenta may be the source for this imbalance in pro-oxidant/anti-oxidant activity. Whether placental hypoxia is the cause of this imbalance has still to be addressed, since it has been demonstrated that tissue hypoxia promotes lipid peroxidation in rats [85] and increases expression of xanthine oxidase, an enzyme which generates superoxide [86]. However, most of the literature describes increased reactive oxygen species generation after reperfusion of acutely ischaemic tissue [87, 88]. To date, a causative relationship between chronic placental hypoxia similar to that described in placenta in pre-eclamptic women and increased superoxide generation has not been established. Again, another possible scenario is that oxidative stress may be the result rather than the cause of pre-eclampsia, or that the process, once triggered by the ‘unknown’ causative factor, becomes amplified in a vicious circle. From early pregnancy the human placenta influences maternal homeostasis; it is rich in mitochondria and when fully developed consumes about 1% of the basal metabolic rate of the pregnant woman [89]. It is also highly vascular and is exposed to high maternal oxygen partial pressure. These characteristics explain, in part, the generation of superoxide, because about 5% of all electrons in the mitochondrial respiratory chain leak out of the mitochondria [90].
1.14.2 Oxidative stress

Oxidative stress in aerobic life can be defined as the imbalance between the generation of reactive oxygen species (ROS) and the rate of their consumption by antioxidants. Lipid peroxidation is a process that occurs normally at low levels in all cells and tissues. It involves conversion of unsaturated fatty acids to lipid hydroperoxides. This process can be initiated by free radicals, which are unstable molecules that possess an unpaired electron in their outer orbital. Following interaction between the lipid and a free radical, peroxidation chain becomes self-perpetuating, with the lipid hydroperoxide inducing the formation of more hydroperoxide [91]. The organism normally has anti-oxidative mechanisms that limit this process.

1.14.3 Reactive oxygen species:

There is increasing evidence to suggest endothelial cell damage and dysfunction in the pathogenesis of preeclampsia [63], although the actual cause of this endothelial damage is not well-known, neutrophils, through their ability to produce reactive oxygen species (ROS), have been implicated as likely candidates. [64]. Superoxide anions (O$_2^-$) have been shown to influence vascular tone either indirectly, by inactivating NO and reducing the release of prostacyclin, [92] or by directly contracting smooth muscle [93]. High concentrations of superoxide have been found to reorient the arachidonic acid pathway in cells toward the production of thromboxane A2, which is a potent stimulator of vasoconstriction and platelet aggregation [94]. The imbalance between prostacyclin and thromboxane A2 is well-documented in preeclampsia [95]. Furthermore, neutrophil-derived superoxide anions can damage vascular integrity and endothelial cell function [96]. In preeclampsia, investigators have demonstrated a significant increase in O$_2^-$.
production by neutrophils. [74, 97, 98]. Thus, it seems plausible that the increased ROS produced by neutrophils may be important in mediating endothelial damage in preeclampsia. Neutrophils synthesize NO in addition to ROS from L-arginine by the enzyme NO synthase (NOS) [99]. The NO released by neutrophils has been documented to prevent neutrophil adhesion to the vascular endothelium, [100] to control aggregation of neighboring platelets [101] and, in the absence of endothelium injury, to produce a vasodilatory effect [102]. NO also reacts with O$_2^-$ to yield the powerful peroxynitrite radical, which may alter vascular function [103]. In contrast, NO inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and scavenges free radicals [104, 105]. Thus, NO can either scavenge O$_2^-$ or be transformed by O$_2^-$ into highly reactive nitrogen intermediates. In addition, NO is metabolized by a variety of other pathways. One is oxidation to nitrate and nitrite, [106] and the other is reduction by S-nitrosoglutathione reductase to ammonia [107]. NO production in vivo is usually measured by the concentrations of nitrite and nitrate in plasma, serum, or urine. In preeclampsia, the data on nitrite levels in blood are controversial, with studies reporting reduced, [108], normal, [109], or elevated [110] nitrite levels. The circulating levels of nitrite and nitrate are affected by elimination, most of which occurs through the kidney, so renal changes in preeclampsia could affect circulating nitrite and nitrate levels. The generation of ROS from activated neutrophils has been shown to result in significant endothelial cell injury in a variety of disease states [111]. Several invitro studies have been performed to determine the mechanism of neutrophil-mediated cell injury [112, 113]. In those studies, H$_2$O$_2$ or the hydroxyl radical were implicated as the oxidant responsible for cell injury. Neutrophils from women with preeclampsia produced
significantly greater endothelial cell injury than did neutrophils produced in other diseases. This finding indicates that neutrophils from women with preeclampsia damage endothelial cells directly. Catalase, which catalyzes the conversion of H$_2$O$_2$ to oxygen and water, inhibited neutrophil-mediated endothelial cell injury in preeclampsia, suggesting that H$_2$O$_2$ may serve as a mechanism of neutrophil-mediated endothelial cell injury in preeclampsia. In contrast, SOD, which dismutates O$_2^-$ excess to H$_2$O$_2$, did not affect neutrophil-mediated endothelial cell injury. Two possible explanations should be considered. First, H$_2$O$_2$ is converted by endogenous catalase to water, and regulation of any of these endogenous enzyme systems may modulate H$_2$O$_2$ levels. Second, the decline in O$_2^-$ is expected to reduce the generation of ONOO$^-$, which has also been found to mediate cytotoxicity, and consequently to reduce endothelial cell injury mediated by ONOO$^-$. Under normal circumstances, the relatively high abundance of the SOD enzyme dismutates O$_2^-$ to H$_2$O$_2$; however, when NO is produced in large quantities, a significant amount of O$_2^-$ reacts with NO to produce ONOO$^-$ [114]. The production of ONOO$^-$ in neutrophils has also been found to mediate cytotoxicity [99]. Oxidative stress, resulting from the imbalance between increased reactive oxygen species (ROS) formation and defects in antioxidant defense mechanisms, has been implicated in the pathogenesis of many vascular disorders, including atherosclerosis, diabetes, and hypertension. Cells of the human body are continually attacked by ROS, which arise as natural by-products of normal cellular energy production or are generated in large amounts in special situations. In normal pregnancies, there is an increase in free radical production and lipoperoxidation towards the end of pregnancy when compared with non-pregnant women [115]. Others propose that endothelial dysfunction could originate from
oxidative stress in the placenta, for which there is strong evidence [116]. And which is likely to arise from hypoxia, or hypoxia/ reperfusion, both of which may occur because of failure of myometrial small artery remodeling. Indeed an elegant in vitro study of normal placenta has shown oxidative damage in syncytiotrophoblast after hypoxia/ reoxygenation [117]. The oxidative stress and microparticle hypotheses are not mutually exclusive as oxidative stress has been shown to increase apoptosis and thereby may lead to microparticle release [118]. Placental hypoxia and reoxygenation, through oxidative stress may also stimulate placental synthesis of cytokines e.g. TNF-α [119], and could lead to per oxidation of maternal plasma lipids during passage of maternal blood through the placenta and activation of maternal neutrophils; all of which could contribute to maternal endothelial cell activation. Recent invitro evidence has also suggested that the high concentrations of plasma endothelin-1 (ET-1) in women with pre-eclampsia could act as a trigger for oxidative stress in the placenta [120]. Oxidative stress was shown after invitro exposure to ET-1 in normal placental explants by increased levels of malondialdehyde (MDA), a marker of lipid peroxidation, and lowered expression of antioxidant defense molecules including glutathione and ascorbic acid. Another likely source of oxidative stress is increased superoxide generation, through activation of the enzyme NADPH oxidase; an increase in activity of this enzyme has been shown in placental tissue from women with early compared to late onset pre-eclampsia as assessed using luminometry (with lucigenin) to detect superoxide generation, and by employing pharmacological inhibitors of NADPH oxidase to delineate the contribution from the enzyme [121]. Others have shown upregulation of NADPH oxidase using immunohistochemical methods [122]. Angiotensin is a potent agonist of NADPH oxidase
and it has been proposed that NADPH oxidase activation could occur through synthesis of a stimulating antibody to the angiotensin receptor, which has been described by several groups in the plasma of women with pre-eclampsia [123]. In view of this literature they proposed that antioxidants might be effective in prevention of preeclampsia. They undertook a randomized placebo controlled trial of vitamin C (1 gm/day) and vitamin E (400 IU/day) in 2404 women at risk of pre-eclampsia on the basis of their clinical history. However, the incidence of pre-eclampsia was the same in both arms of the study [124], an observation repeated in another study from Australia in 2000 low risk women [125]. It is hoped that ongoing studies using a large biobank of plasma samples from participating women in the high-risk study will shed some light on the reasons why antioxidant, why prophylaxis was in effective.

1.14.4 Total antioxidant capacity:

Pregnancy is a physiological process, yet many diseases occur for the first time during pregnancy because of oxidative stress [126]. Pregnancy therefore tends to promote oxidative stress [127], as markers of oxidative stress are known to be raised in normal pregnancy [128]. Total antioxidant capacity represents the balance between oxidative stress (oxidants) and the neutralizing systems (antioxidants). The oxidants are mainly reactive oxygen species and their derivatives e.g. peroxynitrite anion, [129]. In contrast, homeostasis against the effects of reactive oxygen species and their derivatives is maintained by antioxidants such as catalase, super oxide dismutase, beta-carotene, vitamin C, vitamin E, glutathione peroxides, ceruloplasmin and transferin. These agents intercept, modify or destroy the reactive free radicals [130]. Antioxidants therefore
mitigate oxidative stress in pregnancy. Total antioxidant capacity is a measure of the neutralizing effects of antioxidants.

Problems arise when there is an imbalance between the prooxidants and antioxidants either because of excessive generation of pro-oxidants or because of reduced levels of the antioxidant systems, leading to inadequate reducing capacity of the antioxidants [131]. The consequence of this, is an increased plasma levels of pro-oxidant free radicals (e.g. hydroxyl radical (HO\(^{-}\)), superoxide anion radical (O\(_{2}^{-}\)), nitric oxide (NO), hydrogen peroxide (H\(_{2}\)O\(_{2}\)), hypochlorous acid (HOCI) and peroxynitrite anion (ONOO\(^{-}\)). These free radicals attack polyunsaturated fatty acids or cholesterol in membranes or lipoproteins (lipid hydroperoxidation). Lipid hydroperoxides function in normal physiology by regulating enzymes and redox sensitive genes [132], However this necessary physiology in normal pregnancy can become pathologic if lipid peroxidation becomes uncontrolled resulting in cellular dysfunction and damage and increase in vascular resistance, as in preeclampsia [133].
1.14.5 Body antioxidant defense mechanisms:

1. Antioxidant enzymes
2. Antioxidant vitamins
3. Other antioxidants

1.14.5.1 Antioxidant enzymes:

1.14.5.1.1 Glutathione peroxidase:

![Diagram of reaction catalyzed by glutathione peroxidase]

Glutathione peroxidase is a selenoenzyme responsible for elimination of reactive oxygen species. It requires two molecules of reduced glutathione to break hydroperoxides. It’s an important part of the antioxidant defense system. Today five isoforms are known; therefore it is called more like an enzyme family than a single enzyme. They are present in almost every cell of animals, but the tissue distribution of the isoforms shows high variation. There are several factors abrogating the activity of the enzyme. Some of these are internal, individual factors, resulting in significant variation in the enzyme activity of different organs, age groups and sex. Endocrine regulation can also control enzyme activity. However, environmental factors have also definite effect on enzyme action. Nutrition is one of the most essential factors as fat content and fatty acid composition of feed, or trace element intake as well as vitamin status of the animal play crucial role in normal enzyme activity. In addition to GPX1, there are five other known GPX enzymes:
GPX2–GPX6. Data from invitro activity assays suggest that all members use GSH to catalyze the reduction of hydrogen peroxide and lipid peroxides. Whereas GPX1 is the most abundant selenoperoxidase and is ubiquitously expressed in almost all tissues [134-137], GPX2 expression is most prominent in the gastrointestinal tract [138]. Expression of GPX3 is greatest in the kidney, although this enzyme is expressed in various tissues and is secreted into extracellular fluids as a glycoprotein [139, 140]. Different from other glutathione peroxidases, GPX4, or phospholipid hydroperoxide GPX, is not a tetramer, but rather a monomer, and is the only GPX enzyme that reduces phospholipid hydroperoxides [141]. In addition, GPX4 contains a mitochondrial isoform that mediates the apoptotic response to oxidative stress [142, 143], and has a peroxidase independent structural role after sperm maturation [144]. Recently, GPX6 was identified as a selenoprotein in the human genome by homology search. However, GPX6 from rodents and GPX5 from both humans and rodents do not contain Selenocysteine or Selenium [145].

1.14.5.1.2 Glutathione peroxidase in preeclampsia:

Glutathione levels and glutathione peroxidase enzyme activity were found to be higher in women with pre-eclampsia. [146]. Glutathione peroxidase, an enzyme that removes hydrogen peroxide and converts lipid hydroperoxides to less reactive alcohols, may be deficient in placental tissue from preeclamptic women. This is seen in conjunction with increased in vitro placental production of lipid hydroperoxides and thromboxane A2 (TXA2) [147]. TXA2 is a vasoconstrictive and pro-aggregatory prostaglandin normally counter regulated by prostacyclin (PGI2). Chemical inhibition of placental glutathione peroxidase resulted in increased production of lipid hydroperoxides and an increase in the
placental TXA2 to PGI2 output ratio [148]. Lipid hydroperoxides can inhibit PGI2 synthase enzyme activity and simultaneously stimulate the cyclooxygenase component of PGH synthase [149] whereas TXA2 synthase activity is unchanged or even stimulated. Since expression of the synthases is not altered in the uteroplacental unit [150], these effects of lipid hydroperoxides could be the source of the decreased placental PGI2 to TXA2 production ratio in preeclampsia. The altered prostaglandin ratio might provoke vasospasm with exacerbation of placental ischemia, increased cell damage, and increased lipid peroxidation (amplification of oxidative stress) [151].

1.14.5.1.3 Superoxide dismutase:

\[ 2 \text{O}_2^- + 2 \text{H}^+ \cdot \text{H}_2\text{O}_2 + \text{O}_2 \]

**Figure (1-2) reaction catalyzed by superoxide dismutase**

Superoxide Dismutase (SOD) catalyzes the reduction of superoxide anions to hydrogen peroxide; it’s present in virtually all cell types. It plays a critical role in the defence of cells against the toxic effects of oxygen radicals. SOD competes with nitric oxide (NO) for superoxide anion, which inactivates NO to form peroxynitrite. Therefore, by scavenging superoxide anions, SOD promotes the activity of NO.

The action of superoxide is normally limited by its low lipid solubility, its limited membrane transport, and also by its removal by SOD. SOD exists in several isoforms, including the cellular copper/zinc (cytosolic, 32 kD) and the manganese (mitochondrial, 80 kD) isoforms [152], and an extracellular isoform [153]. These isoforms show no sequence homology with each other. SOD activity has been described in the human placenta, and normally placental lipid peroxides decrease but SOD activity increases
throughout gestation [154, 155], presumably to prevent excessive superoxide accumulation and consequential deleterious effects in the placenta. In placentas of pre-eclamptic pregnancies, increased levels of lipid peroxides are seen [81], and decreased SOD activity has been described in patients with pregnancy-induced hypertension [156]. This decreased activity of SOD in pregnancy-induced hypertension may be associated with increased superoxide production and lipid peroxidation.

1.14.1.5.4 Catalase: is a common enzyme found in nearly all living organisms. Its functions include catalyzing the decomposition of hydrogen peroxide to water and oxygen. Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second. Catalase is a tetramer of four polypeptide chains; each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for catalase is approximately 7, while the optimum temperature varies by species.

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

**Figure (1-3) reaction catalyzed by catalase**
1.14.5.2 Non-enzymatic antioxidants:

The non-enzymatic antioxidant defense system includes ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH) and β-carotene (total antioxidant status). There is a balance between both the activity and intracellular levels of these antioxidants that are essential for the survival of organisms and their health [157-160].

1.14.5.2.1 Antioxidants vitamins:

It has been suggested that deficiency in antioxidant vitamins would be associated with the development of pre-eclampsia [161, 162]. Antioxidant vitamins have been reported to have an important function in regulating blood pressure [163]. Nitrous oxide (NO) is the most important endothelium dependent vasodilator and is highly susceptible to oxidative damage. The antioxidant vitamins C and E are able to inhibit formation of free radicals thereby inhibiting the oxidation of NO, and thus maintain the vasodilator status of blood vessels. Damage From free radicals has been implicated in many pathological conditions. It is believed that increased free radical activity arises from increased production of free radicals or deficiency in protective antioxidant systems(including antioxidant vitamins; vitamin A, E, C, and Beta-carotene) , which may lead to endothelial dysfunction in pregnancy induced hypertension(PIH) [164]. The unsaturated lipids and thiol containing proteins in cell membranes are susceptible to free radical attack. There are reports about increased free radical activity in PIH [165].But little is known about the part played by changes in specific antioxidants. Several lines of evidence indicate that adverse changes in structure and function of maternal vascular endothelium accounts for the vascular activity, activation of coagulation cascade and multi system damage in preeclampsia. Multiple circulating factors may provoke the spectrum of endothelial changes, including
lipoproteins [166]. Placental hypoxia or ischemia could result in release of products into maternal circulation which then initiates the maternal pathological changes. Lipid peroxidation products are candidate factors that may mediate disturbances of other maternal vascular endothelium.

1.14.5.2.1.1 Vitamin E:
The term vitamin E covers 8 different forms that are produced by plants alone: α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol [167]. The mechanism of vitamin E absorption is surprisingly unclear. All forms of vitamin E are taken up by intestinal cells and released into the circulation with chylomicrons. At this step there is probably no discrimination between the different forms.

The vitamins reach the liver via chylomicron remnants. In the liver, a specific protein, α-tocopherol transport protein (α–TTP), selectively sorts out α-tocopherol from all incoming tocopherols for incorporation into VLDL. The concentration of α-tocopherol in normal subjects is about 25µmol/L.

Vitamin E is a lipid soluble and chain breaking antioxidant. Vitamin E acts in vivo to prevent the formation of lipid peroxides and thus protect cell membranes. It scavenges radicals in membranes and lipoprotein particles and is central to the prevention of lipid peroxidation. In lipoproteins and cell membranes vitamin E traps peroxyl radicals, breaking the chain reaction of lipid peroxidation by minimizing the formation of secondary radicals [168].

Several studies found lower plasma Vitamin E in patients with PIH [169]. Ascorbate is the water soluble antioxidant vitamin, reacts rapidly with superoxide and peroxyl radicals
and even more rapidly with hydroxyl radicals to give semidehydroascorbate. Furthermore deficiency of Vitamin C causes impaired immune responses [170].

1.14.5.2.1.1 Vitamin E in human pregnancy:

During pregnancy, the blood α-tocopherol concentration increases in association with the increase in blood lipid concentration [171]. In abnormal pregnancies, blood α-tocopherol concentrations are lower than those in normal pregnancies at a corresponding gestational age [172]. It is assumed, though unproven, that vitamin E requirements increase during pregnancy. For this reason, vitamin supplements designed for pregnancy usually contain small doses (50 mg) of vitamin E, but adverse effects have not been observed with higher levels of supplementation. It is well established that there is no clear relation between maternal and fetal blood concentrations of vitamin E. Newborns have significantly lower plasma vitamin E concentrations than do their mothers [173]. However, when plasma vitamin E concentrations were standardized for phospholipids or total lipids, significant differences were not seen [174, 175]. Short-term supplementation of pregnant women before delivery significantly enhanced the vitamin E status of the mother only [176], suggesting that vitamin E does not pass efficiently through the placenta to the newborn circulation. What regulates the placental transfer remains unclear.

1.14.5.2.1.2 Vitamin E in preeclampsia:

Researchers investigating the role of vitamin E in the prevention of preeclampsia have focused on its antioxidants properties. In two studies in which the effect of vitamin E supplementation on women with established preeclampsia was examined, no benefit was observed [177, 178]. Importantly, however, the early provision of antioxidants like
vitamins E and C to women at high risk of developing preeclampsia has a marked clinical benefit. On the basis of an abnormal uterine artery as determined with 2-stage Doppler analysis or of a previous history of preeclampsia, Chappell et al. 1999 [179] identified 283 women who were at an increased risk of preeclampsia. The subjects who were supplemented with vitamins E (263 mg/d) and C (1000 mg/d) had an 8% incidence of preeclampsia, whereas those who received placebo had a 17% incidence of the disorder. Interestingly, vitamin usage was associated with better endothelial function and less placental dysfunction. These findings, along with the decreased concentrations of isoprostanes in the women who received vitamin supplements, provide a sound biochemical basis for understanding the improved clinical symptoms.

1.14.5.2.1.2 Vitamin A:

The term retinoids introduced in 1976 includes carotenoids, the natural forms of vitamin A as synthetic analogues, retinol, the vitamin A alcohol (which is derived from animal sources), retinal and retinoic acid, which are the main vitamin A active metabolites. The recommended adult intake of vitamin A (retinol), commonly found in liver, eggs, oily fish, fortified margarine, and dairy products, is 3000 to 5000 IU daily. Hepatotoxic effects, vision changes, hair and skin changes, and teratogenic effects can occur with intakes greater than 10,000 IU [180].

When taken orally, the body can absorb vitamin A much better than β-carotene. The bioavailability and conversion of β-carotene to vitamin A itself is very variable, being dependent on the dietary content of fat, bile salt concentration and the level of other vitamins in the body. β-carotene does not combine with specific carrier proteins and does not require receptors to perform its provitamin actions. Vitamin A, however, requires a
specific carrier serum protein (retinol-binding protein, RBP) and specific cellular receptors to perform its actions. Clinical and sub clinical infection as well as malnutrition affect RBP and receptor levels. Unlike β-carotene, vitamin A is highly toxic when taken in high doses and there is concern about its teratogenicity, although the level that could produce this in humans is still ill defined [181]. High doses of vitamin A are toxic because the body lacks efficient mechanisms to excrete or destroy excessive loads of it, this could cause catastrophic effects, particularly on cell membranes and possibly on the genome [182]. The antioxidant effect of vitamin A is largely attributed to β-carotene and other carotenoids. Therefore, while β-carotene can perform all the actions of vitamin A, the reverse is not true.

1.14.5.2.1.2.1 Vitamin A in pregnancy:

Placental development depends on a complex and incompletely understood interaction between foetal and maternal cells. Highly specific receptors for retinol binding protein – RBP- (essential for the action of retinol) are expressed in the human placenta [183]. The level of RBP in the placenta is higher than in any other tissue of the body, including the retina [184]. Placental β-carotene level is almost twice as great as those found in the liver [185]. The presence of these high and specific levels of both vitamin A and its precursors suggest that vitamin A is essential for the placenta, or foetus, or both. It is conceivable that vitamin A deficiency could lead to problems of implantation and placental development, which in turn could lead to problems of placental separation and postpartum or post-abortion haemorrhage, or placenta praevia and placental abruption. Indeed, Sharma et al 1986 [186] reported significantly lower levels in serum vitamin A and β-carotene when comparing cases with abruptio placentae to controls. However,
vitamin A deficiency may be the cause or effect of the abruption as a decrease in vitamin A can be part of an acute phase response and decreased β-carotene may be the result of increased consumption as an antioxidant to combat the oxidative stress of placental abruption. Other causes of obstetric haemorrhage include coagulopathy. Normal coagulation depends on the adequate function of platelets, healthy endothelium and harmony between coagulation factors. Vitamin A seems to have no effect on platelet aggregation and adhesion [187, 188]. Endothelium injury is the starting point that triggers abnormal aggregation of the coagulation factors and leads to coagulopathy. Vitamin A appears to increase the integrity and vitality of endothelial cells [189] and so could possibly prevent endothelial injury. After endothelium injury, coagulation factors start to accumulate, forming abnormal small clots that initiate a vicious cycle of disseminated intravascular coagulopathy. The increased consumption of coagulation factors leads to its exhaustion and profound haemorrhage. Vitamin A supplementation may have an effect on the clotting system through increasing thrombomodulin [190,191] decreasing antithrombin III [192] and increasing tissue plasminogen activator [193, 194]. This, together with the endothelial protective effect, suggests that vitamin A may be capable of preventing coagulopathy from the beginning or decrease its severity if it occurred.

1.14.5.2.1.2.2 Vitamin A and infection in pregnancy or the post-abortion or postpartum period:

Pregnancy-related infection (PRI) may result from colonization of retained products of conception (usually a part of the placenta), pre-existing infection or from non sterile invasive procedures. PRI has rarely been associated with vitamin A deficiency.

In the Vanderbilt cooperative study of infant and maternal nutrition of 1945, women who
experienced puerperal infection were found to have lower vitamin A levels starting from the second trimester and continuing through the postpartum examination [195]. Bacteriuria (suggestive of urinary tract infection) was observed in 80% of pregnant women presenting with clinical vitamin A deficiency [196]. Cases with vaginal candidasis showed a significant decrease in \( \beta \)-carotene concentration in exfoliated vaginal epithelial cells when compared to controls [197]. Pregnant women with night blindness were also twice as likely to have clinical genitourinary tract infections (lower abdominal pain, painful micturition or vaginal discharge) than controls without night blindness [198]. The relationship between infection and decreased vitamin A levels is, however, not that simple. Night-blind women in the study mentioned above were also more likely to be anaemic, undernourished, of lower cast and illiterate which could explain an element of the association [198]. The decreased vitamin A levels found in infection might be due to decreased absorption of dietary vitamin A (due to diarrhoea or the activity of intestinal pathogen) [199], decreased RBP production, [200] or increased loss of vitamin A in urine [201, 202]. In acute phase reaction, there is a transient decrease in the production of small molecular weight proteins, including RBP, and an increase in the permeability of renal epithelial cells. Decreased vitamin A might also be due to increased uptake of retinol by certain tissues [203]. PRI could be reduced through three main pathways: promotion of wound healing; increased resistance to infection; and immune enhancement if infection occurs. Vitamin A increases the production of TGF-b, which is involved in the promotion of wound healing [204], and decreases fibrosis through suppression of the fibroblasts. These two actions may increase the integrity of the mucosal surfaces of genitourinary tracts and promote better healing of episiotomy and perineal laceration,
thereby decreasing the chance of infection. Vitamin A augments natural resistance through increasing the naturally present antibodies, IgA, and promoting the function of neutrophils. Neutrophils are an important part of the first line of defense against infection as they engulf bacteria, viruses, parasites and virally infected cells. An alteration in all the components of neutrophil function has been demonstrated in vitamin A-deficient rats [205]. The progress of infection depends on three factors: (i) the virulence and dose of the pathogen; (ii) host immunity; and (iii) the usage of appropriate antimicrobial agents. It is unlikely that either vitamin A deficiency or supplementation could alter the pathogen but the expected effect will be brought about through enhancing different components of the immune system. Vitamin A helps the control of viral infection. Retinoids regulate cell growth and differentiation as well as apoptosis (programmed cell death seen in viral infection and tumour cells) [206]. Both the number and the activity of NK cells (which are involved in antiviral and antitumour immunity) are decreased in vitamin A deficiency [207]. Supplementation of vitamin A results in an increase of NK cells in children with acquired immunodeficiency syndrome (AIDS) [208]. The successful production of antibodies to compete with the bacteria depends on three types of cells: (i) antigen presenting cells (APC), which present the bacterial antigen to the immune-specific cells (lymphocytes); (ii) B lymphocytes, which are responsible for antibody production; and (iii) T lymphocytes, which regulate the immune response.

Retinol is required for growth and activation of B lymphocytes in humans [209, 210]. Transretinoic acid augments the production of two types of antibodies: IgM (antibody of the first immune response) and IgG (antibody of the secondary immune response) [211]. Retinol seems to be required for the activation of T lymphocytes [212]. In vitamin A
deficiency, antibody response is impaired for both T-cell dependent antigen [213] and T-
cell-independent antigen [214]. Vitamin A may decrease the incidence and severity of
RPI by promoting wound healing, increasing resistance to infection and immune
enhancement if infection occurs.

1.14.5.2.1.2.3 Vitamin A and hypertensive disorders of pregnancy:

Many observational studies found a significant decrease in vitamin A and β-carotene in
pre-eclamptic women when compared with normal pregnant women. This decrease was
found both in serum [215-218], as well as amniotic fluid [219]. The constant association
between decreased retinol and β-carotene levels with pre-eclampsia, however, does not
prove a causal relationship. Decreased retinol might be a part of an acute phase reaction.
Many studies found decreased serum proteins in toxaemia of pregnancy [215, 220, 221]
and retinol is carried on proteins, thus its decrease could be secondary to decreased
protein levels. Other studies correlated pre-eclampsia and eclampsia with increased
oxygen free radicals [222] and decreased antioxidant activity [223-227]. Thus, the
decreased β-carotene might be due to increased consumption as it is an antioxidant. These
observational studies are supported by an in vitro study that showed that β-carotene levels
present in normal pregnant women significantly inhibited peroxide-induced
vasoconstriction in a dose-dependent manner.

Lower levels of β-carotene, like those present in pre-eclampsia, were not able to achieve
the same effect [228]. A recent nested case-control study from Nepal compared 109
pregnant women with impending night blindness (xerophthalmia, XP) (cases) and
without XP (controls). Plasma levels of retinol (but not β-carotene) were significantly
decreased (P,0.001) while rates of pre-eclampsia/eclampsia were significantly increased in patients with imminent XP [198].

The effect on pre-eclampsia/eclampsia was not observed after XP had developed. Endothelial cell dysfunction is the common pathway in the pathogenesis of pre-eclampsia. β-carotene is a recognized antioxidant and free radical scavenger, and this beneficial effect is related to its provitamin activity. β-carotene and other carotenoids are much more effective antioxidants than vitamin A. Nitric oxide is a potent vasodilator substance that is believed to play a major role in increasing placental perfusion [229]. Oxidation of low density lipoprotein (LDL) decreases the release of nitric oxide. β-carotene is carried on LDL and protects it against oxidation and thus promotes nitric oxide vasodilatation. This effect has been demonstrated in animals [230], invitro [231] and in humans [232]. A randomized, double-blind placebo controlled trial of vitamins E and C (stronger antioxidants than β-carotene) supplementation in women at increased risk of pre-eclampsia showed a marked reduction in preeclampsia in the intervention group (adjusted odds ratio 0.39 [0.17±0.90]) [179]. The underlying pathology in pre-eclampsia appears to be insufficient placental implantation. This is thought to lead to a relatively hypoxic maternal-foetal interface, an increased turnover of trophoblast tissue, higher xanthine and hypoxanthine concentrations and higher levels of circulating cytokines and an increased production of uric acid and free radicals [233]. It is unlikely that β-carotene or other antioxidants can totally correct these multiple abnormalities. In established severe cases it is unlikely to restore normality except by delivery.
1.14.5.6 Omega-3 and omega-6 unsaturated fatty acids:

Some of the polyunsaturated fatty acids (PUFAs), omega-6 and omega-3 fatty acids, are called essential fatty acids because the body cannot synthesize them. Their contribution is 19% to 22% of energy intake from fats in the western diet. The rest is saturated and monosaturated fat [234]. PUFAs can be confusing because of the similarity of some of the names and the variety of names that refer to the same fatty acid. Omega-6 and omega-3 fatty acids are also called n-6 and n-3 fatty acids. These names refer to the chemical structures [235].

Figure (1-4) chemical structure of EPA

![Figure (1-4) chemical structure of EPA](image)

Figure (1-5) chemical structure of DHA

![Figure (1-5) chemical structure of DHA](image)

Figure (1-6) chemical structure of arachidonic acid

![Figure (1-6) chemical structure of arachidonic acid](image)
PUFAs are hydrocarbon chains with a methyl group at one end (called the omega end) and a carboxyl group at the other. “Omega-6” refers to the first double bond that occurs at the sixth carbon from the omega (methyl) end. It’s also noted as C18:2 omega-6 or C18:2n-6 (18 carbons, 2 double bonds with the first at the n-6 position) [235]. Omega-3 fatty acids have a double bond at the third carbon (n-3). Alpha-linolenic is an example of a short chain omega-3 fatty acid. Alpha linolenic acid is also called simply linolenic acid. Alpha-linoleic acid and gamma-linolenic acid are omega-6 fatty acids. It’s sometimes written as C18:3 omega-3 or C18:3n-3; 18 carbons, 3 double bonds with the first at the n-3 position [235]. Long chain omega-3 fatty acids include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA is C20:5 omega-3 and DHA is C22:6 omega-3. Linoleic acid is the predominant dietary omega-6 fatty acid; 84% to 89% of dietary PUFAs come from linoleic acid in meats, grains, and the seeds of most plants. Linoleic acid is metabolized to arachidonic acid. Arachidonic acid is converted to inflammatory prostaglandins and leukotrienes, which have been linked to cardiovascular and other diseases [234, 235]. Alpha-linolenic acid contributes about 9% to 11% of PUFAs in the U.S. diet. It is sometimes called plant or vegetable omega-3. Vegetable oils such as soy and canola are the primary sources, but flaxseed, walnuts, and other nuts also contain significant amounts of alpha-linolenic acid. The body can convert small amounts of alpha-linolenic acid to EPA, and minute amounts to DHA. EPA and DHA are also called marine omega-3s because they are found in fish. Fatty fish such as mackerel, herring, salmon, tuna, lake trout and halibut contain the highest concentrations [234, 235]. Despite the epidemiologic evidence of benefit, randomized trials don’t support supplementation
with fish oil to prevent pre-eclampsia, pregnancy induced hypertension, low birth weight, or intrauterine growth retardation. DHA or fish oil may increase length of gestation by several days, and reduce risk of recurrent preterm delivery in singleton pregnancies. Studies show a correlation between newborn DHA levels and newborn brain maturity and infant visual maturation. But despite these findings, maternal fish oil supplementation during pregnancy has not been shown to affect infant cognitive function. Fish oil supplementation during pregnancy through the first three months of breast feeding may very slightly benefit intelligence at age four.

Maternal supplementation with DHA during breastfeeding has also been shown to benefit psychomotor development at age 30 months. Some studies comparing DHA supplemented versus standard formula also suggest cognitive and visual benefits, particularly in preterm infants. Evidence from most cohort and case-control studies suggests omega-3s may protect against postpartum depression.

Habitual consumption of modest amounts of fish and other sea food has been associated with a reduction in the risk of heart disease [236]. Results from controlled metabolic studies have provided strong evidence for the role of fish oil (i.e., long chain omega-3 polyunsaturated fatty acids) played in reducing circulating triglycerides, decreasing platelet and leukocyte reactivity, attenuating imbalances between the potent eicosanoids such as prostacyclin and thromboxane in favor of vasodilatation, and reducing systolic and diastolic blood pressures [236, 237]. The beneficial effects of fish oil appear to be associated with the long chain polyunsaturated omega-3 fatty acids; eicosapentaenoic, docosapentaenoic and docosahexaenoic acids. Arachidonic acid, an omega-6 fatty acid,
has been shown to have opposite effects of omega-3 fatty acids on circulating lipids, platelet and leukocyte reactivity, as well as eicosanoid biosynthesis [237, 238].

1.14.5.6.1 Omega-3 and omega-6 fatty acids in preeclampsia:

Preeclampsia, one of the most common medical complications of pregnancy [239], is a leading cause of maternal mortality worldwide, as well as an important cause of premature delivery, fetal growth retardation, and perinatal mortality. Pregnancies complicated by preeclampsia are preceded by hyperlipidemia, particularly hypertriglyceridemia. Other pathologic changes associated with preeclampsia include maladaptation of spiral arteries of the placental bed, excessive lipid peroxidation, endothelial cell dysfunction, thromboxane/prostacyclin imbalance, elevations in proinflammatory cytokines, altered membrane fluidity, and elevations in plasma homocysteine concentrations [239-241].

Controlled metabolic studies provided evidence suggesting that manipulation of the dietary intake of omega-3 fatty acids, omega-6 fatty acids, and trans fatty acids may prevent or ameliorate several of these pathophysiological processes [236, 242]. Investigators have previously shown that derangements in omega-3 and omega-6 fatty acids in plasma phospholipids or erythrocytes are associated with preeclampsia and other adverse pregnancy outcomes [243-246].
GENERAL OBJECTIVES:

The general aim of this study was to investigate the role of cytokines imbalance and oxidative stress in the pathogenesis of preeclampsia in patients attending Khartoum Teaching Hospital.

SPECIFIC OBJECTIVES:

A. cytokines and pathogenesis of preeclampsia

1. To determine the levels of interferon gamma (IFN-γ), as Th1 cytokine and interleukin-4 (IL-4), interleukin-10 (IL-10), as Th2 cytokines in sera of women with preeclampsia and the control group.

2. To investigate the relationship of Th1/Th2 cytokines imbalance to the pathogenesis of preeclampsia.

B. oxidative stress and pathogenesis of preeclampsia

3. To determine the plasma levels of vitamin E (α-tocopherol) and vitamin A (retinol), in women with preeclampsia and the control group.

4. To determine the activity levels of erythrocyte anti-oxidant enzymes; glutathione peroxidase, catalase and superoxide dismutase in women with preeclampsia and the control group.

5. To determine the levels of red cell membrane omega-3, omega-6 fatty acids in three phospholipid fractions; phosphatidylcholine, phosphatidylethanolamine and sphingomyelins in women with preeclampsia and the control group.
Chapter two

Materials & methods
**Materials and methods**

**2.1 Study design:**

This is a case-control study conducted to study the role of cytokines imbalance and oxidative stress in the pathogenesis of preeclampsia in patients attending Khartoum Teaching Hospital.

**2.2 Study centre:**

Khartoum teaching hospital, Khartoum, Sudan

**2.3 Research ethics:**

Informed verbal consent was obtained from all patients and controls to participate in the study.

The study was approved by the board of medical research of the Faculty of Medicine, University of Khartoum.

**2.4 Study population:**

1. **Patients:**

A total number of 98 women with preeclampsia in the third trimester (33 for cytokines imbalance study, and 65 for evaluation of oxidative stress) were included in the study.

2. **Controls:**

A total number of 92 healthy pregnant women at the same gestational age as the patients in the third trimester (32 for cytokine study and 60 for study of oxidative stress) were included.

The study was conducted in two phases; the first for cytokine profiles; namely interferon gamma (IFN-γ) interleukin-4 (IL-4) and interleukin-10 (IL-10), was carried during the period of March through July 2007. Patients with preeclampsia (n=33) were approached to participate in the study. Volunteer women with normal pregnancy (n=32) at the same
gestational age were selected as a control group. Well-structured questionnaires were used to gather socio-demographic characteristics. Preeclampsia was diagnosed according to the criteria of the international society for the study of hypertension in pregnancy previously normotensive women with two repeat (4 h apart) diastolic blood pressure measurements of 90 mmHg or greater after the 20th week of gestation, plus proteinuria of more than 300 mg/l in 24 h as measured quantitatively or, >2+ protein by dipstick in two repeat measurements (4 h apart). Gestational age was calculated from the last menstrual period and confirmed by ultrasound in suspected cases.

2.5 Sample collection for cytokines:
Venous blood samples (5ml) were collected twice in plain tubes from 33 women with preeclampsia one at the time of hospital admission and seven days later during pregnancy, and once from 32 women with normal pregnancy at the same gestational age in the third trimester. The blood samples were centrifuged within 30 minutes from the time of collection at 2000 rpm, serum was aliquoted and stored at -20 degrees till the assay. All of these women were not in labour at presentations or seven days later.

2.6 Data collection:
Personal and clinical data were collected from patients and controls by a structured questionnaire
2.7 Cytokines measurement:

ELISA kits (eBioscience) were used to determine the levels of three cytokines; interferon gamma (IFN-\(\gamma\)), interleukin-4 (IL-4) and interleukin-10 (IL-10) in sera of preeclamptic women and women with normal pregnancy. All samples were run in duplicates and the mean value of optical density (OD) was calculated.

2.7.1 Interferon gamma (IFN-\(\gamma\)): sensitivity of the assay was 4 pg/ml. the standard curve range was 4-500 pg/ml.

2.7.1.1 Components:

1. Capture Antibody: Pretitrated, purified antibody, clone NIB42
2. Detection Antibody: Pretitrated, biotin conjugated antibody, clone 4S.B3
3. Standard: Recombinant cytokine for generating standard curve and calibrating samples
4. ELISA/ELISPOT Coating Buffer Powder: Reconstituted to 1L with dH2O.
5. Assay Diluent: 5X concentrated
6. Detection enzyme: pretitrated Avidin-HRP
7. Substrate Solution: Tetramethylbenzidine (TMB) Substrate Solution
8. ELISA plates (96 Well Plates).

2.7.1.2 Other materials needed:

1. Wash Buffer: 1 x PBS, 0.05% Tween20 (or eBioscience ELISA Wash Buffer Powder, cat 00-0400)
2. Stop Solution: 1M H3 PO4 or 2N H2SO4
3. ELISA plate reader (micro plate spectrophotometer)
4. ELISA plate washer
2.7.1.3 Storage:

Cytokine standard was stored at -80°C; other reagents were stored at 4 °C.

2.7.1.4 Experimental procedure: (was the same for the three cytokines)

1. The 96 well ELISA plate was coated with 100 µl/well of capture antibody in Coating Buffer. The plate was sealed and incubated overnight at 4°C.

2. Wells were aspirated and washed 5 times with >250 µl/well Wash Buffer. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes. Plate was blotted on absorbent paper to remove any residual buffer.

3. 1 part 5X concentrated Assay Diluent was diluted with 4 parts distilled water, 200 µl/well of 1X Assay Diluent was then added and the plate was incubated at room temperature for 1 hour.

4. Standards were diluted with 1X Assay Diluent, and 100 µl/well of standard was added to the appropriate wells. Two fold serial dilutions of the top standards were performed to make the standard curve, and then 100 µl/well of the samples were added to the appropriate wells. 5. The plate was sealed and incubated at room temperature for 2 hours (or overnight at 4°C for maximal sensitivity).

6. Aspiration/wash was done as in step 2 (repeated for a total of 5 washes).

7. 100 µl/well of detection antibody diluted in 1X Assay Diluent was added and the plate was sealed and incubated at room temperature for 1 hour.

8. Aspiration/wash done as in step 2 (Repeated for a total of 5 washes).

9. 100 µl/well of Avidin-HRP diluted in 1X Assay Diluent was added, the plate was sealed and incubated at room temperature for 30 minutes.
10. Aspiration and wash as in step 2 (in this wash step, the wells were soaked in Wash Buffer for 1 to 2 minutes prior to aspiration, repeated for a total of 7 washes).

11. 100 µl/well of Substrate Solution was added to each well. The plate was incubated at room temperature for 15 minutes.

12. 50 µl of Stop Solution was added to each well.

13. The plate was read at 450 nm with an ELISA reader, and the results for cytokines were printed in duplicates for each sample.

2.7.2 Interleukin-4 (IL-4):

Sensitivity of the assay was 2 pg/ml, and the standard curve range was 2 - 200 pg/ml.

2.7.2.1 Components:

1. Capture Antibody: Pre-titrated, purified antibody, clone 8D4-8

2. Detection Antibody: Pre-titrated, biotin-conjugated antibody, clone MP4-25D2

3. Standard: Recombinant cytokine for generating standard curve and calibrating samples

4. ELISA/ELISPOT Coating Buffer Powder: Reconstituted to 1L with distilled water.

5. Assay Diluent: 5X concentrated

6. Detection enzyme: pre-titrated Avidin-HRP

7. Substrate Solution: Tetramethylbenzidine (TMB) Substrate Solution

8. ELISA plates (96 Well Plate).

2.7.2.2 Other materials needed:

1. Wash Buffer: 1 x PBS, 0.05% Tween-20 (or eBioscience ELISA Wash Buffer Powder, cat 00-0400)

2. Stop Solution: 1M H3PO4 or 2N H2SO4

3. ELISA plates (96-well plate).

4. ELISA plate reader (microplate spectrophotometer)
5. ELISA plate washer.

2.7.2.3 Storage:
Cytokine standard was stored at -80°C; other reagents were stored at 4°C.

2.7.3 Interleukin-10:
The sensitivity of the assay was 2 pg/ml, the standard curve range was 2- 300 pg/ml

2.7.3.1 Components:
1. Capture Antibody: Pretitrated, purified antibody, clones NIB42, MQ117H12, 8D48, JES39D7
2. Detection Antibody: Pretitrated, biotin conjugated antibody, clones 4S.B3, Rabbit Polyclonal Antibody, MP425D2, JES312G8
3. Standard: Recombinant cytokine for generating standard curve and calibrating samples
4. ELISA/ELISPOT Coating Buffer Powder: Reconstituted to 1L with dH20 and filter (0.22 uM).
5. Assay Diluent: 5X concentrated
6. Detection enzyme: pretitrated AvidinHRP
7. Substrate Solution: Tetramethylbenzidine (TMB) Substrate Solution
8. 96 Well Plate

2.8 Oxidative stress:
The second phase was carried out during the period of March -June 2008 in Khartoum teaching hospital, to study the role of oxidative stress in the pathogenesis of preeclampsia, which was assessed by unsaturated fatty acid profile in three fractions of red cell membrane phospholipids, namely; phosphatidyl ethanolamine, phosphatidyl choline and sphyngomyelins, the level of plasma antioxidant vitamins, retinol and α-
tocopherol and three erythrocyte antioxidant enzymes, glutathione peroxidase, catalase and superoxide dismutase. After an informed consent 65 pregnant women in their third trimester with preeclampsia were approached to participate in the study. A well matched (for age, parity and gestational age) 60 women with normal pregnancy were selected as the controls. Cases were further divided into mild and severe preeclampsia according to the diastolic blood pressure of < 110, or $\geq$110 mmHg respectively. Those women with multiple pregnancies, medical disorders including; diabetes mellitus or inflammatory conditions were excluded.

2.8.1 Sample collection:
Venous blood samples (5ml) were collected from both groups into tubes containing EDTA, covered from direct light. Samples were transported to the lab in the department of biochemistry, Faculty of Medicine, Khartoum University in containers with ice bags, where they were centrifuged with in half an hour after collection at 3000 rpm. Plasma was separated and red blood cells were washed three times with 0.9% NaCl, both were flushed with oxygen free nitrogen and stored at -80 °C until analysis in London metropolitan University, Institute of brain chemistry and human nutrition (IBCHN). Erythrocyte glutathione peroxidase, catalase and superoxide dismutase were measured with ELISA, fatty acid profile with gas chromatography and plasma retinol and $\alpha$-tocopherol with high performance liquid chromatography (HPLC).
2.8.2 Erythrocyte antioxidant enzymes:

2.8.2.1. Sample preparation:

A. Red blood cell lysate:

To 100µL of red blood cells 400µL of ice cold HPLC grade water was added, this was then centrifuged at 10,000 X g for 15 minutes at 4°C. The supernatant was then collected into another tube and stored at -80 °C. All samples were assayed within the same week.

2.8.2.1.1 Sample dilution: 1:500 for catalase and super oxide dismutase, and 1:50 for glutathione peroxidase assay.

2.8.2.1.2 Enzymatic activity of red blood cell enzymes super oxide dismutase, catalase and glutathione peroxidase were assayed by ELISA kit (cayman chemical company U.S.A).

2.8.2.2. Assay range and precision:

A. For glutathione peroxidase:

The dynamic range of the assay is limited only by the accuracy of absorbance measurement. Most plate readers are linear to the absorbance of 1.2. Samples containing GPx activity between 50-344 nmol/min/ml can be assayed without further dilution or concentration. When a series of seventy-seven GPx measurement were performed on the same day, the intra assay coefficient of variation was 5.7% and 7.2% as interassay coefficient of variation on five different days.

B. For catalase:

The dynamic range of the assay is limited by the accuracy of the absorbance measurements. Most plate readers are linear to the absorbance of 1.2. Samples containing
catalase activity between 0.25-4 nmol/min/ml can be assayed without further dilution or concentration.

When a series of 45 catalase measurements were performed on the same day, the intra assay coefficient of variation was 3.8%. When performed on five different days under the same experimental conditions, the inter assay coefficient of variation was 9.9%.

C. For super oxide dismutase:

Under standard conditions of the assay, the dynamic range of the kit is 0.025-0.25 units/ml SOD. When a series of 60 SOD standard measurements were performed on the same day, the intra assay coefficient of variation was 3.2%. When performed on five different days under the same experimental conditions, the inter assay coefficient of variation was 3.7%.

2.8.2.3 Assay reagents:

1. Glutathione peroxidase:

1. Assay buffer (10 X): 50mM Tris-HCL, pH 7.6, containing 5 mM EDTA.
2. Sample buffer (10X): 50 mM Tris-HCL, pH 7.6, containing 5 mM EDTA and 1 mg/ml BSA.

2. Glutathione peroxidase control:

Solution of bovine glutathione peroxidase, it causes a decrease of 0.05 absorbance unit/min under the standard assay conditions.

4. Glutathione co substrate mixture:

Is a powder of NADPH, glutathione, and glutathione reductase.

5. Glutathione cumene hydroperoxide:

Is a solution of cumene hydroperoxide.
2. Catalase:

1. Assay buffer: 100 mM potassium phosphate, pH 7.0

2. Sample buffer: 25 mM potassium phosphate, pH 7.5, containing 1 mM EDTA and 0.1% BSA.


4. Catalase control: contain powder of bovine liver catalase.

5. Potassium hydroxide: contain potassium hydroxide pellets.

6. Methanol

7. Hydrogen peroxide: contain 8.8 M solution of hydrogen peroxide

8. Purpald (chromogen): contains a solution of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald) in 0.5 M hydrochloric acid.

9. Potassium periodate: contains a solution of potassium periodate in 0.5 M potassium hydroxide.

3. Super oxide dismutase:

1. Assay buffer (10 X):

50 mM Tris-HCl, PH 8.0, containing 0.1mM diethylenetriaminepentaacetic acid (DTPA) and 0.1 mM hypoxanthine.

2. Sample buffer (10X):

50 mM Tris-HCL, PH8.0.

3. Super oxide dismutase assay radical detector:

Contain a solution of tetrazolium salt.

4. Super oxide dismutase standard:

Contain a solution of bovine erythrocyte super oxide dismutase (Cu/Zn).
5. **Super oxide assay xanthine oxidase:**

Contain a solution of xanthine oxidase ELISA reader with selection of the appropriate filter (340 (GPX), 450 (CAT), 450 (SOD)) was used to determine the absorbance for each. The activity of each enzyme was then calculated by a specific equation.

### 2.8.3 **Fatty acid analysis**

**Stages of analysis of fatty acids:**

1. **Chemistry:**
   
   Extraction, thin layer chromatography and methylation.

2. **Gas chromatography:**
   
   Running samples on the GC and collecting the data.

3. **Analysis:**
   
   Processing the raw chromatography (get peak retention times, identify peaks, get areas and area percentages).

4. **Interpretation:**
   
   Looking at the computer reports critically to ensure that the results are accurate and realistic.

**A. Extraction of RBC:** 1 ml RBC was transferred into 15 ml methanol + BHT (Butylated hydroxy toluene) in a 100 ml extraction tube and shacked to suspend the RBC and prevent clumping. 30 ml chloroform + BHT was added and shacked. Flushed with oxygen free nitrogen (OFN), and Stored at 4 °C for 24 hours. The nitrogen nozzle was cleaned with chloroform or C/M between samples.
B. Partitioning:

1. Samples were filtered into separating funnel.
2. Extraction vessels were washed with 1 x 10 ml and 1 x 5 ml C/M (2+1 plus BHT) and each rinse was used to wash the filter paper.
3. Filter funnels were removed and 25% v/v of 0.85% saline was added (i.e. when using 45 + 10 + 5 ml = 60 ml C/M, add 60 / 4 ml = 15 ml saline).
4. Flushing with caps on for about 1 min by bubbling OFN through the liquids was done, the samples were then stored at 4 °C overnight.

Rotatory evaporation:

1. The separating funnels were allowed to stand at room temperature for 30 minutes (this stops condensation).
2. The lower organic layer was drained into a 100 ml or 250 ml round-bottomed flask.
3. The solvent was removed under reduced pressure in a water bath at 37°C using a rotary evaporator.
4. When dried, 1-2 ml methanol was added to rinse the inside of the flask, then rotavaped again to dry this off. This was done twice to draw off any residual water in the sample.
5. The dried whole lipid extract was removed into a 10 ml vial using 3 x 2 ml washes of C/M + BHT. (6ml).
6. The volume was reduced to about 1 ml under a stream of OFN at 37°C and stored at 4°C or -20°C until needed.
Thin layer chromatography (TLC):

Solvents

For phospholipid separations: C/M/M (chloroform, methanol, methyamine, 65:35:15 + BHT @ 100mg/l).

TLC Tanks

Lined with filter paper and containing about 150-200 ml of solvent, well-fitting lid. The tank was allowed to equilibrate for at least 30 minutes before use.

TLC plates

20 cm square plates coated with silica gel were used Application

Application

![Application](image)

Figure (2-1) application of samples in TLC plate
The samples were concentrated under a stream of OFN, and then applied to the plate using capillary tubes in a uniform line about 2 cm above the bottom of the plate, as narrow as possible.

**Running**

The plates were put in the tank without disturbing the equilibrium of the atmosphere inside. A good seal was ensured. When the solvent front has reached the line scored at the top of the plates, the plates were left for further 10 minutes, to improve the definition of the bands. (Phospholipid plates take about 90 minutes to run).

**Visualisation**

After the plates, were removed from the TLC tank, they were dried in a hot air stream (hair dryer) and sprayed (in fume cupboard) with a 0.1% solution of 2,7-dichlorofluoroscein in methanol until the plates were lightly and evenly coated. The bands were visualise under UV light, using glass goggles or a glass plate, for protection and marked with a soft pencil and compared with standard plates to identify the different bands.
Figure (2-2) visualization of lipid bands under UV light

**Scraping**

The appropriate bands were scraped onto filter paper using a blunt instrument (flat spatula) and transferred to a methylating tube.

**Methylation:**

**The methylating reagent**, 15% acetyl chloride in methanol, must be prepared freshly as follows:

Add, dropwise, 15 ml acetyl chloride to 100 ml dry methanol in a 500 ml conical flask while swirling the flask under a stream of cold water. Wear a facemask and a fully buttoned lab coat. Do not let the acetyl chloride boil in the methanol. Do not let water splash into the flask. Transfer the mixture to a stoppered bottle. BHT is not used during methylation as it appears to generate decomposition products.

1. For formation of fatty acids methylesters, 15% acetyl chloride in methanol was used. It
is also possible to use ethanol or propanol, which produce the less volatile and slower-running ethyl and propyl esters. The GC relative retention times (RRTs) are unchanged - that is the RRT of 18:1 ethyl ester to 18:0 ethyl ester is the same as for the corresponding methyl esters - they just run more slowly. This makes ethylation/propylation good for short chain FA, but not so good for the long chain polyunsaturated fatty acids.

2. To the bands scraped from the TLC plates, approximately 4 ml of the methylating reagent (above) was added.

3. Flushing thoroughly by bubbling OFN through the liquid, with cap held over neck of tube and Vortexing were done.

4. The level of the liquid in the tube was marked with a pen.

5. Methylation at 70 degrees for three hours. At 1 and 2 hours checking that the level has not decreased. If it has, change the cap (and the tube if the lip is cracked or chipped), and make up the volume with methanol and refill. Vortexing the tubes before putting them back in the oven was done.

**Extraction of methyl esters:**

1. The tubes then removed from the oven and let to cool to room temperature.

   To each tube 4 ml 5% saline were added and 2 ml petrol spirit + BHT, Capped and shaken well. This will form two layers.

2. The upper petrol layer was removed to a test-tube containing 2 ml 2% potassium bicarbonate (to neutralise any acid transferred).

3. Vortexing the petrol extract with the bicarbonate was done and the upper, petrol layer transferred to a test- tube containing 100-200 mg dried granular sodium sulphate, to remove any residual water.
4. The solution of fatty acid methyl esters in petrol was removed to a 3 ml vial.

5. The petrol was removed under a stream of OFN. The sample was taken up in 1 ml heptane + BHT, flushed with OFN and stored at -20 °C until ready for GC.

The individual fatty acids in the samples were identified by comparing them with standards of known retention time (time which is taken by the fatty acid sample to appear as a certain wave in the software after injection in the GC).

All the samples were loaded over night in patches; each patch consists of 30 samples in the sample plate with an auto sampler (Fisons A 2005).

**Specifications of the gas chromatography machine:**

MEGA 2 series, model EL 980 from Fisons S.P.A Company, Melano, Italy, equipped with flame ionized detector (FID), and soft-ware system from Hewlett-packard (Germany). The carrier gas or mobile phase was hydrogen which was produced from water with a hydrogen generator (2007 Whatman).

**Gas chromatography:**

**Sample preparation for gas chromatography:**

25 drops of the sample was transferred to the GC brown vial, dried under a steam of oxygen free nitrogen (OFN) at 37 °C. Then 3 drops of heptane +BHT was added to make the appropriate sample concentration for the run in the GC, the vial was then tightly capped and Loaded onto the auto sampler
Figure (2-3) gas chromatogram showing fatty acid peaks

2.8.4 Retinol and α-tocopherol:

Extraction of retinol, Vitamin E in Human Plasma:

1. 500μl of plasma was deproteinized with 500μl of 100% ethanol and vortexed for five minutes.
2. Then 1.5 ml of chloroform was added to extract the fat soluble vitamins, retinol and α-tocopherol in the plasma sample.
3. Again the mixture was vortexed for 5 minutes before centrifugation for further 8 minutes at 4000 rpm at 25 degrees.
4. The organic layer containing the vitamins was then removed and further extraction was done with 1ml of chloroform. (Repeated twice).
5. Evaporation of the vitamins extract to dryness was done under oxygen free nitrogen.

6. The dried extract was dissolved in 100µl of 98% methanol, and transferred into HPLC brown vials.

7. The HPLC vials containing the purified vitamins mixture were then stored at -20 degrees when not used in the same day.

2.9 HPLC system:

The HPLC system used for analysis of the vitamins retinol and α-tocopherol was a reverse phase system (Alligent 1100 series) in which 98% HPLC grade methanol (Fisher scientific UK) was used as the mobile phase, in addition to 2% deionized water. The flow rate of the mobile phase was 1ml/minute.

The purities of vitamin standards which were used were 99% for retinol, 97% for α-tocopherol (Sigma Aldrich).

2.9.1 Column specifications:

The column was made of C 18 Hypersil gold, 250 mm in length and with a diameter of 4.6 mm (Therma scientific).

2.9.2 Detector: The system uses a photodiode-array detector, which identified the vitamins in the following wave lengths; 293nm for α-tocopherol, 326 nm for retinol.

System soft-ware: The soft ware used in the HPLC system was from Hewlett-packard (Germany).

2.9.3 Auto sampler:

The system contained auto sampler with its plate for sample loading (Agillent 1100 series).
2.10 Statistics

Data was entered in computer using SPSS for windows and double checked before analysis. The mean (SD) of the basic characteristics were compared between the two groups using student t-test. Data were checked for normality, in normally distributed data student t-test and ANOVA were used to compare two and more groups respectively, if the data were not normally distributed; Mann-Whitney U test was used to determine the significance of difference between the variables. Correlations between continuous variables were assessed by the Spearman rank test. $P< 0.05$ was regarded as significant.
Chapter Three

Results
**Results:**

This is a case-control study comprising 98 Sudanese women with preeclampsia, and 92 healthy pregnant women, both in the third trimester. The study aimed to evaluate the role of cytokines imbalance and oxidative stress in the pathogenesis of preeclampsia.

**3.1 Cytokines profiles:**

Table (3.1), shows the clinical characteristics of patients with preeclampsia and the control group. The two groups were well matched in their age, gravidity, gestational age and weight.

Data for cytokines was not normally distributed, the median and interquartile ranges were used and Mann-Whitney U test was used to compare the significance of difference between the variables.

Table (3.2) shows that the levels of IFN-γ and IL-4 were slightly -not statistically significant- higher in the women with preeclampsia. IL-10 was significantly higher in the women with preeclampsia; Women with preeclampsia had significantly lower levels of IFN-γ and IL-4 and significantly higher levels of IL-10 7 days later in comparison with the presenting levels, table (3.3).

**Correlation between the three cytokines at presentation:**

Positive correlation was observed between IL-4 and IFN-γ $r=0.495$, $P<0.007$, also between IL-4 and IL-10 $r=0.445$, $P<0.009$. IL-10 and IFN-γ were not positively correlated $r=0.094$, $P<0.602$. 
Table (3-1) Clinical characteristics of patients with preeclampsia and the control group

<table>
<thead>
<tr>
<th>variables</th>
<th>Women with preeclampsia (n=33)</th>
<th>Control (n=32)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>29.5(5.7)</td>
<td>29.5(5.90)</td>
<td>0.9</td>
</tr>
<tr>
<td>gravidity</td>
<td>2.1(2.1)</td>
<td>1.8(1.9)</td>
<td>0.3</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>35.2(2.8)</td>
<td>35.6(2.9)</td>
<td>0.5</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>59.7(7.4)</td>
<td>57.6(6.8)</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Table (3.2): The median and interquartile of cytokines in cases and control group

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Case (n=33)</th>
<th>Control (n=32)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>210 (142.40-287.06)</td>
<td>125.38 (85.67-184.95)</td>
<td>0.437</td>
</tr>
<tr>
<td>IL-4</td>
<td>10.3 (3.33-18.35)</td>
<td>9.69 (3.69-14.63)</td>
<td>0.780</td>
</tr>
<tr>
<td>IL-10</td>
<td>8.6 (2.39-16.66)</td>
<td>6.99 (3.39-16.84)</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Table (3.3) the median and interquartile of cytokines in patients with pre-eclampsia at presentation and day seven.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>At presentation</th>
<th>Day seven</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>210.48 (142.40-287.06)</td>
<td>136.73 (81.42-192.04)</td>
<td>0.035</td>
</tr>
<tr>
<td>IL-4</td>
<td>10.36 (3.33-18.35)</td>
<td>9.99 (2.15-15.36)</td>
<td>0.000</td>
</tr>
<tr>
<td>IL-10</td>
<td>8.61 (2.93-16.66)</td>
<td>10.63 (6.18-25.41)</td>
<td>0.000</td>
</tr>
</tbody>
</table>
3.2 antioxidant enzymes:

The two groups were well matched and there was no statistical difference concerning age, parity, gestational age and weight, table (3.2.1).

Data for antioxidant enzymes was not normally distributed, the median and interquartile ranges were used and Mann-Whitney U test was used to compare the significance of difference between the variables.

The activity levels of the three enzymes (glutathione peroxidase, catalase and superoxide dismutase) were slightly (not statistically significant) higher in preeclamptic women compared to the control group, table (3.2.2)

This was also true- the slightly higher levels of erythrocyte glutathione peroxidase and slightly lower levels of the other two enzymes catalase and superoxide dismutase -when the women with mild (diastolic blood pressure < 110 mmHg) and severe preeclampsia (diastolic blood pressure ≥ 110 mmHg) were compared, table (3.2.3)

A significantly negative correlation was observed between the activity of glutathione peroxidase and diastolic blood pressure r= -0.371, P = 0.02. There was no significant correlation between catalase (r= - 0.033, P = 0.840) or superoxide dismutase r = 0.067, P = 0.676 and diastolic blood pressure.
Table (3.2.1): Clinical characteristics of preeclamptic and normal pregnant women

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Preeclamptic women</th>
<th>Control women</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>28.98 ( 5.64)</td>
<td>27.88 (5.28)</td>
<td>0.2</td>
</tr>
<tr>
<td>Gestational age,</td>
<td>33.74 (2.83)</td>
<td>34.37 (3.04)</td>
<td>0.234</td>
</tr>
<tr>
<td>Parity</td>
<td>2.4(1.93)</td>
<td>2.23 (1.42)</td>
<td>0.581</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>67.86 (12.10)</td>
<td>69 (13.34)</td>
<td>0.531</td>
</tr>
</tbody>
</table>
Table (3.2.2): Median (25-75 quartiles) levels of activities of erythrocyte glutathione peroxidase, catalase, and superoxide dismutase in women with preeclampsia and the control group

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Case</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>416 (340-450)</td>
<td>359(307.5-432)</td>
<td>0.106</td>
</tr>
<tr>
<td>Catalase</td>
<td>62.2 (37.2-80.0)</td>
<td>52.6(45.7-59.8)</td>
<td>0.276</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>2824(2271-2987)</td>
<td>2550 (2309-2729)</td>
<td>0.104</td>
</tr>
</tbody>
</table>
Table (3.2.3): Median (25-75 quartiles) levels of activities of erythrocyte glutathione peroxidase, catalase, and superoxide dismutase in women with mild and severe preeclampsia

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mild preeclampsia</th>
<th>Severe preeclampsia</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxidase</td>
<td>426(340-512)</td>
<td>399(334.5-436.5)</td>
<td>0.2</td>
</tr>
<tr>
<td>Catalase</td>
<td>59.2(31.4-83.3)</td>
<td>62.8(37.5-78.3)</td>
<td>0.8</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>2706(2238-2941.7)</td>
<td>2873(3209-3031)</td>
<td>0.6</td>
</tr>
</tbody>
</table>
3.3: Results of omega-3 (ecosapentaenoic acid –EPA-, docosahexaenoic acid –DHA-) and omega-6 (arachidonic acid –AA-) fatty acids profiles in three fractions of red blood cell membrane phospholipids, namely phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelins (SM) in Sudanese women with preeclampsia (mild and severe) and the control group:

The two groups were well matched and there was no statistical difference concerning age, parity, gestational age and weight, table (3.3.1).

The fatty acid data was normally distributed in the PE fraction, and the T-test was used.

1. Phosphatidylethanolamine fraction (PE):
   a. The mean level of EPA was significantly higher in women with preeclampsia (n = 65) than the control group (n = 60), P = 0.012, but when the level was compared between women with severe preeclampsia (DBP > or = 110 mmHg), (n=25) and mild preeclampsia (DBP<110 mmHg), (n=40) it was slightly higher although not statistically significant in women with severe preeclampsia, P = 0.688.
   b. The mean level of DHA was significantly higher in women with preeclampsia (n=65) than the control group (n=60), P= 0.04, but was not significantly different when cases with mild and severe preeclampsia were compared, P = 0.489.
   c. The mean level of arachidonic acid (AA) was slightly higher in the control group, but the difference was not statistically significant, P = 0.308. When cases of mild and severe preeclampsia were compared, the mean level although higher in cases with severe preeclampsia, was not statistically significant, P = 0.484.

Tables: (3.3.2), (3.3.3).
Table (3.3.1): Clinical characteristics of preeclamptic and normal pregnant women

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Preeclamptic women</th>
<th>Control women</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>28.98 (5.64)</td>
<td>27.88 (5.28)</td>
<td>0.2</td>
</tr>
<tr>
<td>Gestational age,</td>
<td>33.74 (2.83)</td>
<td>34.37 (3.04)</td>
<td>0.234</td>
</tr>
<tr>
<td>Parity</td>
<td>2.4 (1.93)</td>
<td>2.23 (1.42)</td>
<td>0.581</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>67.86 (12.10)</td>
<td>69 (13.34)</td>
<td>0.531</td>
</tr>
</tbody>
</table>
Table (3.3.2): Mean (SD) levels of EPA, DHA and AA in women with preeclampsia and the control group in the PE fraction.

<table>
<thead>
<tr>
<th>Variable</th>
<th>case</th>
<th>control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total fatty</td>
<td>acids</td>
<td></td>
</tr>
<tr>
<td>Ecosapentaenoic</td>
<td>0.1437(0.0381)</td>
<td>0.1275(0.0322)</td>
<td>0.012</td>
</tr>
<tr>
<td>acid (EPA)</td>
<td>n=65</td>
<td>n=59</td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic</td>
<td>5.1372(0.9087)</td>
<td>4.0848(0.8780)</td>
<td>0.04</td>
</tr>
<tr>
<td>acid (DHA)</td>
<td>n=65</td>
<td>n=60</td>
<td></td>
</tr>
<tr>
<td>Arachidonic</td>
<td>20.2205(1.4532)</td>
<td>20.4637(1.1655)</td>
<td>0.308</td>
</tr>
<tr>
<td>acid (AA)</td>
<td>n=65</td>
<td>n=60</td>
<td></td>
</tr>
</tbody>
</table>
Table: (3.3.3) Mean (SD) levels of omega-3 and omega-6 fatty acids in phosphatidylethanolamine (PE) in mild and severe preeclampsia:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mild preeclampsia</th>
<th>Severe preeclampsia</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total fatty</td>
<td>DBP&lt;110 mmHg</td>
<td>DBP&gt;=110</td>
<td></td>
</tr>
<tr>
<td>acids</td>
<td>n=40</td>
<td>n=25</td>
<td></td>
</tr>
<tr>
<td>Ecosapentaenoic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acid (EPA)</td>
<td>0.1422(0.0371)</td>
<td>0.1461(0.0404)</td>
<td>0.688</td>
</tr>
<tr>
<td>Docosahexaenoic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acid (DHA)</td>
<td>5.1994(1.0498)</td>
<td>5.0376(0.6283)</td>
<td>0.489</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(AA)</td>
<td>20.1197(1.4056)</td>
<td>20.3817(1.5416)</td>
<td>0.484</td>
</tr>
</tbody>
</table>
2. Phosphatidylcholine fraction (PC):

The fatty acid data was also normally distributed in the PC fraction, and the T-test was used.

a. The mean level of EPA was significantly higher in women with preeclampsia (n = 62) than the control group (n = 52), P = 0.000, but in the group of women with preeclampsia, the difference was not statistically significant when women with mild and severe preeclampsia were compared, P = 0.615, although the level was slightly higher in women with severe preeclampsia.

b. The mean level of DHA was significantly higher in women with preeclampsia (n=64) than the control group (n=60), P= 0.046, but was not significantly different when cases with mild (n=40) and severe (n=24) preeclampsia were compared, P = 0.973.

c. The mean level of arachidonic acid (AA) was slightly higher in the preeclampsia group (n=64), than the control (n=60) but the difference was not statistically significant, P = 0.671. When cases of mild (n=40) and severe (n=24) preeclampsia were compared, the mean level although higher in cases with mild preeclampsia, was not statistically significant, P = 0.469.

Tables: (3.3.4), (3.3.5).
Table (3.3.4): Mean (SD) levels of EPA, DHA and AA in phosphatidylcholine (PC) among women with preeclampsia and the control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case</th>
<th>control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total fatty</td>
<td>acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecosapentaenoic acid (EPA)</td>
<td>0.0937(0.0352)</td>
<td>0.0663(0.0296)</td>
<td>0.000</td>
</tr>
<tr>
<td>n=62</td>
<td>n=52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>1.367(0.4160)</td>
<td>1.234(0.3140)</td>
<td>0.046</td>
</tr>
<tr>
<td>n=64</td>
<td>n=60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid (AA)</td>
<td>8.1780(1.5310)</td>
<td>8.0760(1.0890)</td>
<td>0.671</td>
</tr>
<tr>
<td>n=64</td>
<td>n=60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table (3.3.5): Mean (SD) levels of omega-3 and omega-6 fatty acids in phosphatidylcholine (PC) in mild and severe preeclampsia:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mild preeclampsia (DBP&lt;110 mmHg)</th>
<th>Severe preeclampsia (DBP&gt;=110)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecosapentaenoic</td>
<td>0.0919(0.0330)</td>
<td>0.0966(0.0389)</td>
<td>0.615</td>
</tr>
<tr>
<td>acid (EPA)</td>
<td>n=38</td>
<td>n=24</td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic</td>
<td>1.3660(0.4194)</td>
<td>1.3696(0.4185)</td>
<td>0.973</td>
</tr>
<tr>
<td>acid (DHA)</td>
<td>n=40</td>
<td>n=24</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>8.2865(1.5555)</td>
<td>7.9971(1.5055)</td>
<td>0.469</td>
</tr>
<tr>
<td>(AA)</td>
<td>n=40</td>
<td>n=24</td>
<td></td>
</tr>
</tbody>
</table>
3. Sphingomyelin fraction (SM):

Fatty acid data including EPA, DHA and AA was not normally distributed, but was converted to normally distributed data by taking log10 for each of them and the T-test was used to compare the mean level between the women with preeclampsia and the control group, and mild and severe preeclampsia.

a. The mean level of EPA was significantly higher in women with preeclampsia (n = 62) than the control group (n = 59), P = 0.000, but in the group of women with preeclampsia, the difference was not statistically significant when women with mild (38) and severe (24) preeclampsia were compared, P = 0.138, although the level was slightly higher in women with severe preeclampsia.

b. The mean level of DHA was significantly higher in women with preeclampsia (n=65) than the control group (n=60), P= 0.000, but was not statistically significantly different when cases with mild (n=40) and severe (n=24) preeclampsia were compared, P = 0.158.

c. The mean level of arachidonic acid (AA) was significantly higher in the preeclampsia group (n=65), than the control (n=60), P = 0.000. When cases of mild (n=40) and severe (n=24) preeclampsia were compared, the mean level although higher in cases with severe preeclampsia, was not statistically significant, P = 0.475.

Tables: (3.3.6), (3.3.7).
Table (3.3.6): Mean (SD) levels of EPA, DHA and AA in sphingomyelin fraction among women with preeclampsia and the control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>case</th>
<th>control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecosapentaenoic acid (EPA)</td>
<td>0.8503(0.1483)</td>
<td>0.3548(0.1398)</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>n=62</td>
<td>n=59</td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>0.0912(0.0353)</td>
<td>0.0356(0.0238)</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>n=65</td>
<td>n=60</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid (AA)</td>
<td>0.8421(0.1223)</td>
<td>0.2720(0.1226)</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>n=65</td>
<td>n=60</td>
<td></td>
</tr>
</tbody>
</table>
Table (3.3.7): Mean (SD) levels of omega-3 and omega-6 fatty acids in sphyngomyelin fraction (SM) in mild and severe preeclampsia.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mild preeclampsia</th>
<th>Severe preeclampsia</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total fatty</td>
<td>DBP&lt;110 mmHg</td>
<td>DBP&gt;=110</td>
<td></td>
</tr>
<tr>
<td>acids</td>
<td>n=38</td>
<td>n=24</td>
<td></td>
</tr>
<tr>
<td>Ecosapentaenoic acid (EPA)</td>
<td>0.8281(0.1696)</td>
<td>0.8856(0.0996)</td>
<td>0.138</td>
</tr>
<tr>
<td></td>
<td>n=38</td>
<td>n=24</td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>0.1401(0.0292)</td>
<td>0.0129(0.0127)</td>
<td>0.158</td>
</tr>
<tr>
<td></td>
<td>n=40</td>
<td>n=24</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid (AA)</td>
<td>0.8335(0.1159)</td>
<td>0.8560(0.1332)</td>
<td>0.475</td>
</tr>
<tr>
<td></td>
<td>n=40</td>
<td>n=24</td>
<td></td>
</tr>
</tbody>
</table>
3.4: Plasma antioxidant vitamins; retinol and α-tocopherol in plasma of women with preeclampsia and the control group, and in women with mild and severe preeclampsia:

Data was not normally distributed, and hence quartiles (25th, 75th) and the median levels of each of the vitamins were used, Mann-Whitney U test was used to determine the significance of difference between the variables.

1. The median level of retinol was higher in the control group (n=58) than in plasma of women with preeclampsia (64), but the difference was not statistically significant, P = 0.297.

When the level of retinol was compared between women with mild and severe preeclampsia, it was slightly higher in women with mild preeclampsia, but the difference was not statistically significant, P = 0.984.

2. The median level of α-tocopherol was significantly higher in women with preeclampsia than the control group, P = 0.026.

When the level of α-tocopherol was compared between women with mild and severe preeclampsia, it was slightly higher in those with mild preeclampsia, but the difference was not statistically significant, P = 0.618.

Tables (3.4.1), (3.4.2).
Table (3.4.1): The median (interquartile range) of plasma retinol and α-tocopherol in women with preeclampsia and the control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Women with preeclampsia</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=64</td>
<td>n=58</td>
<td></td>
</tr>
<tr>
<td>Retinol</td>
<td>21.60 (15.750-34.025)</td>
<td>26.70 (16.225-35.265)</td>
<td>0.297</td>
</tr>
<tr>
<td>µgm/dl</td>
<td>34.025</td>
<td>35.265</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>8.60 (5.70-11.05)</td>
<td>6.70 (3.45-9.40)</td>
<td>0.026</td>
</tr>
</tbody>
</table>
Table (3.4.2): The median (interquartile range) of plasma retinol and α-tocopherol in women with mild and severe preeclampsia.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Women with mild preeclampsia</th>
<th>Women with severe preeclampsia</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol µgm/dl</td>
<td>21.80 (15.70-32.20)</td>
<td>21.50 (14.65-38.50)</td>
<td>0.984</td>
</tr>
<tr>
<td>α-tocopherol mg/dl</td>
<td>8.60 (6.825-10.875)</td>
<td>8.20 (2.75-11.25)</td>
<td>0.618</td>
</tr>
</tbody>
</table>
Chapter Four

Discussion and conclusion
Discussion

Pregnancy is a physiological state accompanied by a high metabolic demand and elevated requirements for tissue oxygen [247] and causes an increase of ROS production [248]. Moreover, the placenta is a major source of oxidative stress because of its enrichment with PUFA [249]. Falkay et al. [250] suggested that the increase in the lipid peroxide levels was due to the increased prostaglandin synthesis in the placenta. Lipid peroxidation is enhanced in the second trimester and tapers off later in gestation and decrease after delivery [251]. Monitoring of the oxidative stress in pregnant women is important to enable an understanding of the relationship between oxidative stress and pregnancy outcome [252]. Placental oxidative stress was suggested to play a role in the pathogenesis of pre-eclampsia [253, 254] and fetal growth retardation [254, 255]. On the other hand, the placenta is a source of antioxidative enzymes to control placental lipid peroxidation during healthy pregnancy. Placental production of lipid peroxides decreases as normal gestation advances, most likely because of an increase in the activity of superoxide dismutase and catalase [256]. Placental antioxidant defense is considered sufficient to control lipid peroxidation in healthy pregnancy [249].

This study was conducted to evaluate the role of cytokine (Th1/Th2) imbalance by studying serum interleukin 4 and 10 (IL-4 and IL-10) as examples of Th2 anti-inflammatory cytokines and interferon gamma (IFN-γ) as an example of Th1 inflammatory cytokine and oxidative stress which was assessed by unsaturated fatty acid (omega-3 and omega-6) profile in three fractions of red cell membrane phospholipids, namely; phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelins, the level of plasma antioxidant vitamins, retinol and \( \alpha \)-tocopherol and three erythrocyte
antioxidant enzymes, glutathione peroxidase, catalase and superoxide dismutase, in the pathogenesis of preeclampsia in pregnant Sudanese women in the third trimester compared to well matched pregnant controls.

4.1 **Cytokines profile in Sudanese women with preeclampsia**

Our finding of increased level of IFN-γ in the pre-eclamptic group at presentation was consistent with that of Alexander E. OMU etal [257] who has reported increased Th1 cytokines in pre-eclampsia. But the rise was not statistically significant when compared to the control group, \( P<0.305 \). This was further correlated with the pattern of Th1 cytokines Production by decidual lymphocytes and peripheral mononuclear cells in patients with pre-eclampsia, as reported by Jonsson et. al 2005 [258]. At day seven (selected because it would be possible to catch patients in hospital at this period) the level of IFN-γ was significantly reduced \( P< 0.035 \). We have found a slightly increased level of IL-4 in pre-eclamptic women compared to the control group at presentation but the rise was not statistically significant \( P<0.531 \), contrary to what had been reported by Alexander E. OMU etal [257] who have demonstrated that pregnancy is associated with modifications of TNF-α and IL-4 which are secreted by CD4+ T lymphocytes, and there is significantly higher increase of IL-4 (from 12-24 weeks) of gestation in normal pregnancy compared to pre-eclamptic pregnancy, this may be because our patients and the control group were selected in the third trimester of pregnancy, where the immune response may be different from the early and mid pregnancy; which is either due to gestational age difference or different pattern of steroid hormone release . At day seven the level of IL-4 was significantly reduced in the pre-eclamptic group \( P< 0.035 \).
It has been suggested that the production of IL-4 in pregnancy may in fact have an important role in human gestation; it acts as a regulator of pro-inflammatory cytokines to prevent untimely delivery and modulate the role of Th1 cytokines in the pathophysiology of pre-eclampsia [259]. We also found a statistically significant rise in the level of IL-10 in the preeclamptic group at presentation (P< 0.002) when compared to the control group, and at day seven in the pre-eclamptic group (P< 0.000). This was not correlated with the report of Orange S et al.,2003 [260] who reported low expression of Th2 cytokines IL-10 and IL-5, our finding raises the possibility of a protective role of IL-10 in pre-eclampsia, because it antagonizes the effects of Th1 cytokines.

Pr-eclampsia is associated with both local and systemic changes in type1/ type2 cytokine balance compared to normal pregnancy. Decidual and peripheral blood mononuclear cells from patients with pre-eclampsia are generally primed to synthesize high levels of the Th1 cytokines, [261]. On the other hand, they exhibit low expression of Th2 cytokines IL-10, and IL-5. These variations may be due to the unknown aetiology and pathophysiology of pre-eclampsia, and this raises a big question to be answered by further research in this field, whether these cytokines play an aetiologicical role or their pattern of production is sequelae to other pathology.

4.2 Antioxidant enzymes

The main results of the study were; no significant differences in the levels of the three antioxidant enzymes; glutathione peroxidase, catalase and superoxide dismutase between women with preeclampsia (n=37) and controls (n=38), in between women with severe (n=17) and mild (n=19) preeclampsia. However, a significantly negative correlation was observed between the activity of glutathione peroxidase and diastolic blood pressure.
There was no significant correlation between catalase or superoxide dismutase and diastolic blood pressure. Similarly it has been found that preeclamptic women compared with women with normal pregnancy, have similar erythrocyte glutathione content, total plasma antioxidant capacity. According to these results, the authors concluded that, oxidative stress might not be a pathogenetically relevant process causally contributing to the disease [262]. In contrast, recently Patil and colleagues (2007) [263] reported that, enzymatic antioxidants namely superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were reduced significantly in preeclamptic women as compared to normal pregnant and non-pregnant controls. Furthermore, Bayahan, et al.,[264] reported that antioxidant enzyme activities (superoxide dismutase and glutathione levels) in erythrocytes were significantly decreased in women with preeclampsia and eclampsia compared with the controls. However, the later group [264] observed that, groups of preeclampsia and eclampsia had similar values of catalase activities as the controls and there were no correlations between antioxidant enzyme observed activities and systolic-diastolic blood pressure of pregnant women with preeclampsia and eclampsia.

Anyway, oxidative stress has been proposed as a mechanism that contributes to endothelial dysfunction in preeclampsia. Lipid peroxidation is considered as a main feature of oxidative stress which may change membrane integrity and/or functions. It can be assessed in several ways such as estimation of levels of end products of peroxidation or antioxidants like enzymes, vitamins and minerals [265]. Arguably, the most important event during normal placental development is establishment of an effective maternal circulation, a process that is linked with the physiological conversion of the spiral arteries from highly tortuous and thick-walled vessels to flaccid sinusoidal conduits of low
Failure of spiral artery remodeling in the placental bed of pregnancies affected by preeclampsia was first demonstrated by Brosens et al [267] and later associated with a partial failure of placental trophoblast invasion [268]. These observations are fundamental to the current theory of preeclampsia. More recently, it was shown in normal pregnancy that at 10 to 12 weeks’ gestation, the onset of maternal blood flow in the placenta results in a local increase in oxygen tension and parallel elevation in expression and activity of several antioxidant enzymes [269]. It was hypothesized that a putative diminution of the antioxidant response to this oxygenation stimulus could result in oxidative stress that may lead to trophoblast degeneration and possibly contribute to impairment of trophoblast invasion and diminished remodeling of the spiral arteries [269]. A defective response to an oxidant stimulus could therefore be one of the earliest events in preeclampsia. Whatever the cause of impaired trophoblast invasion, the resultant inadequacy of placental perfusion is likely to result in oxidative stress by the following potential mechanisms. Maintenance of the muscular coat of the spiral artery may lead to intermittent placental perfusion, because the spiral arteries would retain susceptibility to maternal humoral and neuronal constrictor influences [270]. Together with frequent thrombotic occlusion followed by clot dissolution, this may lead to a repeated hypoxia/reoxygenation insult in the affected placenta throughout pregnancy. Hypoxia/reoxygenation is a potent stimulus to the activation of xanthine oxidase, an important source of superoxide generation, which is abundantly expressed in cytotrophoblast, syncytiotrophoblast, and villous stromal cells [271]. As might be anticipated, placental tissue from women with preeclampsia demonstrates enhanced expression and activity of this enzyme [271]. Thus xanthine oxidase is likely to play a
fundamental role in free radical-induced tissue damage in the human placenta. In support of this, Hung et al have shown that in vitro hypoxia/reoxygenation in normal third trimester placenta leads to free radical-induced tissue damage as evidenced by nitrotyrosine staining in trophoblast and activation of apoptotic pathways, both of which were preventable by addition of a free radical scavenger [118].

Their study also supports the suggestion that as a result of underperfusion, aponecrotic processes could lead to deportation of syncytiotrophoblast microvesicles into the maternal circulation. These microparticles, normally present in the circulation in pregnancy, have been shown to increase in preeclampsia and have been directly linked to activation of maternal neutrophils, which in turn may contribute to activation of the vascular endothelium [64].

The source of the lipid peroxides in preeclampsia is unknown, but it has been suggested that poorly perfused placental tissue may evoke the free radical production which can be the cause of generalized lipid peroxidation [272]. The root of the controversy may lie in the fact that definitive evidence for the involvement of free radicals in disease processes is hampered by the lack of comparative methods and, therefore, the difficulty of comparing published data to assess absolute levels of oxidative stress in vivo. Small and heterogeneous patient groups might also have been leading reasons for disagreement. It seems that variation in the activity of antioxidant enzymes in these different reports may be the result of genetic variability between different populations, and further studies are needed to investigate this in more depth and to explore the genetic background of preeclampsia too.
4.3 Erythrocyte omega-3 and omega-6 fatty acids profiles:

The main findings regarding omega-3 and omega-6 fatty acids profiles in the three phospholipids fractions of erythrocyte membrane; phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) and phyangomyelins, were;

In the PE fraction we found significantly higher level of omega-3 fatty acids (EPA and DHA) in women with preeclampsia and insignificant difference in their level when cases with mild and severe preeclampsia were compared. There was no statistically significant difference in the level of arachidonic acid (omega-6 fatty acid), neither between the cases and controls, nor among women with mild and severe preeclampsia.

In the PC fraction there was significantly higher level of omega-3 fatty acids (EPA and DHA) in women with preeclampsia and insignificant difference in their level when women with mild and severe preeclampsia were compared. There was no statistically significant difference in the level of arachidonic acid (omega-6 fatty acid), between women with preeclampsia and the controls, and among women with mild and severe preeclampsia.

In the sphyngomyelin fraction there was significantly higher level of omega-3 (EPA and DHA) and omega-6 fatty acids in women with preeclampsia and insignificant difference in their levels when women with mild and severe preeclampsia were compared.

Contrary to our findings, investigators have previously shown that derangements in omega-3 and omega-6 fatty acids in plasma phospholipids or erythrocytes are associated with preeclampsia and other adverse pregnancy outcomes [243-246]. Similarly, Williams et al. 1995 [245] reported in line with our current findings in PC and sphyngomyelin
fractions concerning omega-6 fatty acids, in their study of predominately white women in Seattle (Washington, USA) that low erythrocyte levels of omega-3 fatty acids and high levels of omega-6 fatty acids, particularly arachidonic acid, were associated with an increased risk of preeclampsia.

K.Mahomed et. al 2006 [273] in their report on association between maternal erythrocyte omega-3, omega-6 and trans fatty acids and risk of preeclampsia, found women in the highest quartile group for total omega-3 fatty acids compared with women in the lowest quartile experienced a 14 % reduction in risk of preeclampsia. They found little support for the hypothesized inverse association between omega-3 fatty acids and preeclampsia risk in their study population. There was some evidence suggestive of a weak positive association between increasing levels of arachidonic acid and risk of preeclampsia. In our study in the PE fraction, the level of arachidonic acid was slightly higher in the control group, but in PC and SM fractions, the level was slightly higher in women with preeclampsia although not statistically significant, which can not be considered as a strongly associated finding. We did not even find significant difference in the level of arachidonic acids, EPA or DHA between women with mild and severe preeclampsia. The raised level of omega-3 fatty acids (EPA and DHA) in the three phospholipids fractions in women with preeclampsia in our study does not support oxidative stress as an aetiological factor in preeclampsia, but the raised level of arachidonic acid in the sphingomyelin fraction in women with preeclampsia is in line with the findings of K.Mahomed et. al 2006 [273], who relate omega-6 fatty acid to the risk of preeclampsia.
4.4 Antioxidant vitamins:

The main finding regarding antioxidant vitamins was that, the median level of retinol was higher in the control group compared to women with preeclampsia, and in women with mild preeclampsia than those with severe preeclampsia, although the difference was not statistically significant. The median level of α-tocoferol was significantly higher in women with preeclampsia than the control group, \( P = 0.02 \), and insignificantly higher in women with mild than those with severe preeclampsia. In contrary to our finding, Burton, 1994, [169] reported lower plasma Vitamin E in patients with pregnancy induced hypertension (PIH). Also no benefit was observed when vitamin E was supplemented in two studies to women with established preeclampsia [177, 178]. Chappell et al. 1999, [179] reported 8% incidence of preeclampsia in women with increased risk of preeclampsia that were supplemented with vitamin E, compared to 17% incidence in the placebo group.

Our finding of higher plasma retinol level in the control group although insignificant is in line with a previous report of many observational studies that found a significant decrease in vitamin A and β-carotene in pre-eclamptic women when compared with normal pregnant women. This decrease was found both in serum [215-218] as well as amniotic fluid [219].
Conclusion

Preeclampsia is one of the life threatening complications during pregnancy in Sudan. Our results support the hypothesis of cytokine imbalance as a pathogenic factor for preeclampsia. The significantly raised levels of IL-10 in women with preeclampsia suggest its role in pathogenesis of preeclampsia as it is an anti-inflammatory Th2 cytokine that is raised as a compensatory factor for the raised Th1 cytokines. Regarding oxidative stress, there was no statistical difference between the level of antioxidant enzymes; glutathione peroxidase, catalase and superoxide dismutase in erythrocyte membranes in preeclamptic and the control groups. But the negative correlation between the diastolic blood pressure and the level of glutathione peroxidase may point to the involvement of oxidative stress in the severity of preeclampsia. There was no reduction in the level of omega-3 fatty acids in women with preeclampsia compared to the control group; this finding does not support oxidative stress as an aetiological factor for preeclampsia. The level of omega-6 fatty acid (arachidonic acid) was only reduced in preeclamptic patients in the PE fraction; this may not also support oxidative stress. The insignificantly raised level of the natural antioxidant vitamin retinol in the control group and the significantly higher level of the other antioxidant vitamin α-tocopherol in preeclamptic women are also not in favor of oxidative stress as an aetiological factor for preeclampsia.
Recommendations

It is good to encourage pregnant ladies to increase the intake of foods rich in omega-3 fatty acids especially docosahexaenoic acid (DHA), because we realized that its level in our study group is low compared to European values from samples analyzed by other students at the IBCHN laboratory. This fatty acid is important for brain development during pregnancy [274].

Establishment of large multicentre research plans in the future for the in depth study of the life threatening, yet unknown preeclampsia.
References


23- Kilpatrick DC. HLA-dependent TNF secretory response may provide an immunogenetic link between pre-eclampsia and type 1 diabetes mellitus. Dis Markers. 1996; 13: 43–47.


41- Murphy S, Fast L, Sharma S. IL-10, uterine NK cells, inflammation, and pregnancy.


64- Redman CW, Sargent IL. Pre-eclampsia, the placenta and the maternal systemic inflammatory response, a review. Placenta. 2003; 24:21–27.


67- Henriques C.U, Rice G.E, Wong M.H, Bendtzen K. Immunolocalisation of interleukin-4 and interleukin-4 receptor in placenta and fetal membranes in


133- Cindrova-Davies T. Gabor Than Award Lecture 2008: Pre-eclampsia - From Placental Oxidative Stress to Maternal Endothelial Dysfunction. Placenta. 2008, ONLINE.


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238- Kestin A, Clifton P, Belling GB and Nestel PJ. N-3 fatty acids of marine origin lower systolic blood pressure and triglycerides but raise LDL cholesterol compared to n-3 and n-6 fatty acids from plants. Am J Clin Nutr. 1990; 51: 1028-34.


Cytokines Profiles in Sudanese Women with Preeclampsia

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Faculty of Medicine, University of Khartoum, Sudan

Background. Cytokine imbalance in preeclampsia may be one of the etiological factors for preeclampsia. Objectives: The study was conducted to investigate interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-10 (IL-10) in preeclampsia. Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of these three pro-inflammatory cytokines in sera from 33 Sudanese women with preeclampsia (at presentation and 7 days later) and 32 women with normal pregnancy as a control group. Results. The levels of IFN-γ and IL-4 were slightly— not statistically significant— higher in the women with preeclampsia. IL-10 was significantly higher in the women with preeclampsia. Women with preeclampsia had significantly lower levels of IFN-γ and IL-4 and significantly higher levels of IL-10 7 days later in comparison with the presenting levels. Conclusion. Thus, the significantly raised levels of IL-10 in women with preeclampsia suggest its role in pathogenesis of preeclampsia, and further research is needed.

Keywords: Cytokines, Preeclampsia, Pathogenesis, Sudan.
Appendix

Questionnaire

Date..............................................................Serial No. ............D0...........D7..........

Name.........................................................Age........Residence.........................

Occupation............................................Education...........................................

Tribe...............................Contact No...........................................................

Gravidity..................Parity.........Gestational age...................................

Weight...................BP....................Hb....................................................gm/dl

Urine albumin +1 (                 ) ..................+2 (                 ) ..................... > +2 (               )

Antihypertensive treatment..........................................................

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

Steroids.................................................................

Urea...............................................................creatinine........................................

Uric acid.................................AST...............................ALT.................................