ANGIOGENIC FACTORS AND OXIDATIVE STRESS BIOMARKERS IN GESTATIONAL HYPERTENSION AND PREECLAMPSIA

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Molecular Medicine, KNUST. This work has not been submitted for any other degree and the contents of thesis are mine.

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ABSTRACT

Hypertensive disorders of pregnancy are multisystemic disorders whose etiology is still unknown. An Imbalance in angiogenic factors and oxidative stress (OS) biomarkers levels has been implicated. It is against this background that this study prospectively evaluated angiogenic factors and oxidative biomarkers and the role they play in the pathogenesis of gestational hypertension (GH) and preeclampsia (PE).

This prospective study recruited 235 pregnant women at 20-40 week gestation from the Obstetrics and Gynaecology (O&G) department of the Komfo Anokye Teaching Hospital (KATH). Finally 150 (50 GH, 50 PE and 50 normal pregnant) gave informed consent and their course of pregnancy were followed until delivery. Serum levels of placental growth factor (PLGF), soluble fms-like tyrosine kinase 1 (sFlt-1) and 8-epi-prostaglandin F2alpha (8-epi-PGF2a) levels were estimated by ELISA and total antioxidant capacity (T-AOC) was measured spectrophotometrically.

Median levels of sFlt-1, 8-epi-PGF2a and sFlt-1/PLGF ratio were significantly elevated while PLGF, T-AOC and PLGF/sFlt-1 ratio were significantly reduced in GH and PE compared to normal pregnant (NP) controls ($p<0.05$) at baseline. Conversely, levels of sFlt-1, 8-epi-PGF2a and sFlt-1/PLGF significantly decreased while PLGF and T-AOC were significantly increased after 48 hours delivery in all studied participant ($p<0.05$). Levels of sFlt-1, 8-epi-PGF2a and sFlt-1/PLGF tend to peak in the third trimester of pregnancy specifically at 32-36week gestation. Advanced maternal age (35-40 year) pregnant women were significantly associated with angiogenic and oxidative stress imbalance compared to age group 18-24 year ($p<0.05$). Increased proportion of adverse maternal and fetal outcomes such as preterm delivery, emergency caesarean section, placental previa, placental abruption, stillbirth, intrauterine growth restriction (IUGR), intrauterine fetal death (IUFD), and postpartum hemorrhage (PPH), low birth weight (LBW), fetal distress, birth asphyxia and Apgar score below 7 after 5 minutes were significantly associated with preeclamptic pregnancies compared to normal control groups ($p<0.05$). A significant positive correlation (PIGF vs T-AOC; and sFlt-1 vs 8-epi-PGF2a) and a negative correlation (PLGF vs sFlt-1, sFlt-1 vs T-AOC, PLGF vs 8-epi-PGF2a, and T-AOC vs 8-epi-PGF2a) was observed among studied groups after adjusting for maternal age, BMI, gestational age and parity ($p<0.05$). Angiogenic profile and oxidative stress biomarkers were significantly associated with systolic and diastolic blood pressure, gestational BMI and adverse pregnancy outcomes such as IUGR, placental abruption, stillbirth, IUFD, and PPH after adjusting for maternal age and pregestational BMI ($p<0.05$). The best area under the curve obtained on analysis using ROC curve indicated that ratio of PLGF/sFlt-1 followed by sFlt-1/PLGF ratio and PLGF could be used as predictive markers for early onset third trimester pregnancy in PE.

GH and thus PE create an imbalance in the levels of angiogenic factors and oxidative biomarkers as depicted by elevated levels anti-angiogenic factors and pro-oxidants and a reduced concentration of pro-angiogenic factors and antioxidants. Pharmacologic remedies of exogenous pro-angiogenic molecules, antioxidant supplements and inhibiting the action of anti-angiogenic molecules could provide inventive approaches to the management of GH and PE and potentially alleviate the adverse complications suffered by these patients.

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ABBREVIATIONS

8-epi-PGF2α	8-epi-prostaglandin F2alpha
ACOG	America society of Obstetricians and Gynaecologist
ANT	Antenatal
APGAR	Appearance, pulse, grimace, activity, respiration.
APH	Antepartum haemorrhage
AT1-AA	angiotensin receptor type 1 receptor agonistic antibody
BMI	Body mass Index
eNOS	endothelial nitric oxide synthase
FRAP	ferric reducing ability of plasma
GH	Gestational hypertension
GHS	Ghana Health Service
HELLP	haemolysis, elevated liver enzymes and low platelet count
HUVECS	human umbilical vein endothelial cells
IUFD	Intrauterine fetal death
IUGR	Intrauterine growth restriction
KATH	Komfo Anokye Teaching Hospital
LBW	Low birth weight
MDA	Malondialdehyde
MED	Maternal endothelial dysfunction
NADP) (H)	Nicotinamide Adenine Dinucleotide Phosphate
OS	Oxidative Stress
PE	Preeclampsia

PIH	Pregnancy Induced Hypertension
PLGF	Placental growth factor
PPH	Postpartum haemorrhage
PPROM	Premature prerupture of membrane
PT	Postpartum
ROS	Reactive oxygen species
sEng	soluble Endoglin
sFlt-1	soluble Foetal-like tyrosine kinase
SGA	Small for gestational age
T-AOC	Total antioxidant capacity
TGF	Transforming growth factor
VEGFR-1	Vascular endothelial growth factor receptor 1
WC	Waist circumference
WHR	Waist to hip ratio
WHO	World Health Organisation

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Across the globe, 536,000 women die each year, 800 women die every day and one woman dies every minute as a result of Pregnancy and Childbirth (WHO, 2004). Approximately, 99% of all maternal mortality occurs in low resource settings and the poorest communities (Patton *et al.*, 2009) of which Ghana is no exception. It is reported that 80% of these maternal deaths are caused by sepsis, haemorrhage, complications of unsafe abortions, hypertension disorders of pregnancy and obstructed labour (Allen *et al.*, 2004b; Conde-Agudelo *et al.*, 2004) of which 40% are due to antepartum and postpartum haemorrhage (PPH) (Osei-Nketiah, 2001). The incidence of gestational hypertension (GH), preeclampsia (PE), and pregnancy induced hypertension (PIH) among Ghanaian pregnant women in 2007 were reported as 8.01%, 9.03% and 17.04% respectively (Owiredu, 2008b; Ahenkorah, 2009).

Preeclampsia, the most complicated amongst these disorders is enigmatic due to its multisystemic effect. It is characterized by hypertension and proteinuria noticeable after the 20th week of gestation. The development of new arterial hypertension in pregnancy with the absence of proteinuria has been termed "Gestational hypertension" due to the fact that it occurs during gestation. The aetiology of PE is not understood though it has been linked with a widespread maternal endothelial dysfunction originating from an insufficient cytotrophoblast invasion and thus hypoxic placenta (Maynard *et al.*, 2003). Various factors such as uteroplacental blood flow, genetic, endothelial damage, endothelial integrity, neutrophils, oxidative stress, inflammatory response, alterations in the renin-

angiotensin-aldosterone axis and coagulation system have been shown to interact with the pathogenesis of preeclampsia (Wang *et al.*, 2009).

The process of angiogenesis and oxidative stress (OS) is essential in normal pregnancy but can risk placental development when they are outside normal range. Production of anti-angiogenic factors has been shown to be upregulated in the pathogenesis of PE (Lam *et al.*, 2005; Wang *et al.*, 2009). Imbalance between pro-angiogenic and anti-angiogenic factors is a remarkable basis for maternal endothelial dysfunction (MED) (Levine *et al.*, 2004; Petla *et al.*, 2013). Increased soluble foetal-like tyrosine kinase (sFlt-1), a remarkable antiangiogenic factor and decreased placental growth factor (PLGF) levels have been observed in PE compared to normal pregnancy (Levine *et al.*, 2006). Nulliparous women destined to deliver small for gestational age (SGA) neonates are reported to have high levels of foetal-like tyrosine kinase (sFlt-1) throughout pregnancy compared to those with normal pregnancy (Romero *et al.*, 2008a).

Inflammation is associated with increased reactive oxygen species (ROS) production leading to an imbalance in the generation and removal of free radical species (superoxide, hydroxyl radical etc.) which results in OS (Da Silva *et al.*, 2010). Although the generation of free radicals is a normal physiological process in pregnancy, increased production has adverse effects on DNA, lipids and proteins (Tiwari *et al.*, 2010a). A recent study compared the oxidative stress levels or antioxidant capacities of women with normal pregnancies and those with established pregnancy complications near the time of delivery and observed differential changes in OS markers during different modes of delivery (Chen *et al.*, 2012b). Palm *et al.*, (2012) observed that levels of angiogenesis, OS and inflammation fluctuate throughout normal pregnancy and after delivery.

Though medical intervention and various management strategies are in place to ameliorate hypertension in pregnancy, increased morbidity and mortality are still

high. Till date, the only cure for PE is delivery of the placenta. The controversies in the definition and classification of hypertensive disorders of pregnancy coupled with discrepancy in understanding the pathogenesis of the condition has led to research dilemma. In order to comprehend the dynamics of hypertension in pregnancy, the role of maternal angiogenesis and oxidative stress in endothelial dysfunction needs a thorough investigation. Understanding the link between these markers and the conditions would help in early diagnosis and a subsequent reduction in maternal and fetal mortalities and morbidities.

1.2 PROBLEM STATEMENT

The mechanism of how some pregnancies lead to adverse maternal outcomes is not fully understood though many reviews relate this to endothelial dysfunction, advanced maternal age and an imbalance in angiogenic factors and OS (Suplee *et al.*, 2007; Huang *et al.*, 2008). Diagnosis of PE is mainly based on blood pressure measurement and urine protein analysis. These measurements however, have a low sensitivity and specificity with respect to prediction of the course of the disease or maternal and perinatal outcomes.

Although hypertensive disorders of pregnancy especially pre-eclampsia seem to be a clearly defined disease, the dynamics of the clinical course as well as its clinical presentation vary immensely. The characterization for normal pregnancy varies and the actual developmental stage where normal turns to be abnormal remains questionable. At present there is no conclusive evidence on the actual gestational age where PE and GH pregnant women experience the peak in angiogenic factors and oxidative stress imbalance.

Again, due to the multiorgan effect caused by PE, public health still face a pharmaceutical burden with regards to manufacturing the appropriate medicinal intervention. The ideal strategies in the care of the women with preeclampsia have not been fully clarified, leaving physicians and other healthcare professionals with incomplete data to guide their clinical decision making. This is because

preeclampsia is a progressive disorder of which delivery is the only promising approach needed to halt the progression to the benefit of the mother and fetus. Nevertheless, the need for premature delivery has adverse consequences on neonatal outcomes which are not limited to the most premature infants.

Hypertensive pregnant women still face severe adverse complications during the course of the pregnancy until delivery although early diagnosis helps reduce maternal and fetal morbidity and mortality. The Ghana Health Service (GHS) and Ministry of Health, recommend multivitamins (antioxidants) and iron-based medicines as part of the management plan. Despite these interventions, some mothers still face a lot of adverse pregnancy complications which may be suggestive of suboptimal medicinal dose to meet the oxidative stress demand, wrong duration of administration, and other antagonist factors in diet which may interfere with therapeutic effects. Another major problem is the unclear mechanism involved in the pathogenesis of GH and PE. Could pregnancy alone lead to these complications and other adverse outcomes or is partly attributed to imbalance in angiogenic factors and OS biomarkers? This study evaluated the role angiogenic factors and oxidative stress biomarkers play in the pathogenesis of GH and PE.

1.3 AIMS/OBJECTIVES

This study seeks to evaluate baseline and postpartum levels of maternal angiogenic factors and oxidative stress biomarkers in normal pregnant women, newly diagnosed gestational hypertensive and preeclamptic women and also assess subsequent pregnancy outcome.

1.3.1 **Specific Objectives are to:**

1. Determine changes in pro-angiogenic (PIGF) and anti-angiogenic factors (sFlt-1) and the ratio of sFlt-1/PIGF and PIGF/sFlt-1 at baseline sampling and 48hrs postpartum
2. Estimate oxidative stress using serum 8-epi-PGF2α and total antioxidant capacity (T-AOC) at baseline sampling and 48hrs postpartum
3. Identify the intrapartum and postpartum adverse pregnancy outcomes
4. Assess the correlation between angiogenic factors, OS markers and adverse maternal outcome.
5. Assess the effect of BP, BMI, Gestational age, parity, maternal age on angiogenic and oxidative stress markers
6. Evaluate the diagnostic accuracy of the angiogenic factors and Oxidative biomarkers in PE

1.4 RATIONALE OF THE STUDY

Previous cohort studies have explored the changes of angiogenic factors and oxidative stress markers in normal pregnant (NP) women but little or no attention was given to women presenting with PE and GH. Moreover, there is no data on the combination of angiogenic factors and oxidative stress biomarkers in normal pregnancy, GH and PE women. Again, most of these studies analyzed few and non-specific markers and did not adjust for confounding factors such as maternal age, pregestation body mass index, parity etc. Evaluation of maternal serum angiogenic factors and oxidative stress using sensitive and specific markers may not only help in the early diagnosis but also provide information on the high risk population so as to potentially alleviate the adverse complications suffered by the

mother and neonates. Many others published data also focused on estimating the concentrations of biochemical markers at the point of diagnosis of the condition, but have not looked at its association in the profile on the clinical onset of the disease thus, whether it's involved in the pathogenesis of a condition and/or precedes its clinical expression. The ability of this study to assess the changes overtime of these markers in pregnancy will not only elucidate the debate on the actual effect and stage of significant imbalance but further provide baseline data on the course of angiogenesis and oxidative stress among Ghanaian women. It is against this background that this study sought to evaluate angiogenic factors and oxidative biomarkers and the role they play in the pathogenesis of GH and thus PE. This study thus recruited normal pregnant women and newly diagnosed GH and PE and measured various levels of maternal angiogenic and oxidative stress markers, followed their courses of pregnancy to postpartum, and evaluated the association between the levels of angiogenic factors and oxidative stress biomarkers.

1.5 HYPOTHESES

1. Preeclampsia and gestational hypertension are associated with significant increases in both anti-angiogenic factor (sFlt-1) and prooxidant (8-epi-PGF2α) and a correspondingly reduced level of pro-angiogenic factor (PLGF) and antioxidant (T-AOC) compared to normotensive pregnant women.
2. Postpartum levels of both anti-angiogenic factor (sFlt-1) and prooxidant (8-epi-PGF2α) and a correspondingly reduced levels of pro-angiogenic factor (PLGF) and antioxidant (T-AOC) compared to normotensive pregnant women

Chapter 2

LITERATURE REVIEW

2.1 HYPERTENSIVE DISORDERS OF PREGNANCY

Hypertensive disorders are common in pregnancy and form one of the lethal triad, coupled with hemorrhage and infection, which result in maternal and child morbidity and mortality worldwide. Hypertension is generally defined as systolic blood pressure of at least 140 mmHg and or diastolic blood pressure of at least 90 mm Hg on two separate readings at least 6hr apart (Mustafa *et al.*, 2012).

2.2 CLASSIFICATION OF HYPERTENSIVE DISORDER OF PREGNANCY

For decades, the diagnostic criteria and definition for hypertensive disorders of pregnancy continue to create arguments due to the different systems used by major working groups and international societies (WHO, 2004). The advancement of science has led to combination of terminologies that has come out with accepted definitions. One of these definitions were suggested by the international society for the study of hypertension (also known as the international definition) and other by the US National High Blood Pressure Education Program Working Group (2000) (also known as the America society of Obstetricians and Gynaecologist (ACOG) definition. Later a modified version of the international definition (known as the Oxford definition) emerged. The High Blood Pressure Education Program Working Group (2000) classified hypertensive disorder of pregnancy as gestational hypertension, preeclampsia, eclampsia, chronic hypertension and preeclampsia superimposed on preexisting hypertension (Mustafa *et al.*, 2012).

2.2.1 *Gestational Hypertension*

GH is onset of elevated blood pressure (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg), which develops after 20 weeks of gestation in a previously normotensive woman, in the absence of significant proteinuria. It occurs in 6% of all pregnancies and the condition is expected to return to normal within three (3) months after delivery (Brown and Buddie, 1995). Approximately 15–45% will eventually develop preeclampsia (Barton *et al.*, 2001; Brown *et al.*, 2007). About 8.01% of new case of this condition occurs among Ghanaian pregnant women (Owiredu, 2008b; Ahenkorah, 2009).

The current use of ''2+'' and 0.3g/l on urinary dipstick testing and quantitative proteinuria method respectively as the cutoff for proteinuria in preeclampsia (Brown and Buddie, 1995; Saudan *et al.*, 1998) was due to the increased rate of false-positive with ''1+'' proteinuria (Davey and MacGillivray, 1988; Sibai *et al.*, 2000). Accordingly during classification, pregnant women who presented with ''1+'', trace and negative proteinuria on dipstick or 24-hour urinary protein measurement are also considered as having gestational hypertension. Pregnant women with gestational hypertension and proteinuria (1+) are at increased risk for developing preeclampsia and other severe form of maternal disease (Saudan *et al.*, 1998).

2.2.1 *Preeclampsia*

Preeclampsia is a multisystemic condition that occurs with pregnancy, and it's characterized by hypertension and proteinuria first detected after 20weeks of gestation (Cnossen *et al.*, 2006). It is a common complication of pregnancy affecting about 5 to 10% of all pregnancies. Although, complications of preeclampsia have been recognized for centuries, the aetiology of the disease is unknown. Recent studies have shown that this disorder appears to originate in placenta and is characterized by widespread maternal endothelial dysfunction (Petla *et al.*, 2013).Till date, the only cure for the disease is delivery of placenta. Though

possible causes and mechanisms linking preeclampsia remains unknown, immunological, maternal, genetic factors and placenta have been implicated (Petla *et al.*, 2013). It can also be defined as a rise in blood pressure of more than 25-30 mmHg systolic and/or 15 mmHg diastolic from preconception or first trimester of pregnancy (Seligman, 1987).

There is conflicting argument in the definition for mild preeclampsia though the Australasian Society for the study of Hypertension in pregnancy (1993) system classified preeclampsia as mild or severe. This has led to a controversy between gestational hypertension and mild preeclampsia, and for that matter the revised system of classification by the Australasian Society for the Study of Hypertension in Pregnancy, 1993 does not stratify preeclampsia (Brown *et al.*, 2007). Preeclampsia is a condition that precedes eclampsia, a Greek word which means sudden flashing (Mutter and Karumanchi, 2008). Preeclampsia occurring at <34 week gestation is termed as "early onset preeclampsia" while late onset preeclampsia which occurs after 34 week of gestation.

Endothelial dysfunction is common in both onset and it's responsible for the proteinuria and hypertension symptoms. Failure to control these symptoms would result in foetal prematurity and premature delivery (Mustafa *et al.*, 2012). Pregnancies complicated with preeclampsia have a high incidence of preterm delivery (Backes *et al.*, 2011), placental abruption, intrauterine growth restriction (IUGR), low birth weight, caesarian delivery, elevated liver function test, subcapsular liver hematoma, seizures, renal insufficiency, thrombocytopenia, and disseminated intravascular coagulation (Seely and Solomon, 2003). Nulliparous women are at higher risk than multiparous women although both may be at predisposed to chronic hypertension later in life.

The criteria for diagnosis of severe preeclampsia are based on sustained systolic blood pressure ≥160 mm of Hg, sustained diastolic blood pressure of ≥110 mm of

Hg, pulmonary edema, oliguria <500 ml/24 hours, persistent headaches or scotoma, thrombocytopenia <100,000/mm3, persistent right upper quadrant pain or epigastric pain, and intrauterine growth restriction <10th percentile (Mustafa *et al.*, 2012). Different management plan are required for patients with severe preeclampsia as chances for maternal and fetal complications are much higher in such cases. The ultimate treatment procedure in severe preeclampsia is prompt delivery and the timing of delivery is based on both maternal and fetal indications (Mustafa *et al.*, 2012). If gestational age is below 34 weeks, expectant management, when possible should be attempted with the goal of improving neonatal outcome without neglecting the safety of the mother (Mustafa *et al.*, 2012).

2.3 PATHOGENESIS AND PATHOPHYSIOLOGY OF PREGNANCY INDUCED HYPERTENSION

Pregnancy induced hypertension especially preeclampsia is a multisystemic disorder due to the fact that it affects essential organs such as the liver, kidney, brain and blood systems and is also associated with generalised vasospasm (George and Granger, 2010).

The early era of this millennium has observed major improvement in our understanding of the pathophysiology of pregnancy induced hypertension. Preeclampsia also known as the "disease of theories," has been associated with numerous mysterious patterns in its pathophysiology and history as a whole. The molecular pathogenesis of preeclampsia is beginning to be threadbare with a key discovery about the modifications in placental antiangiogenic factors. These anti-angiogenic markers, such as sFlt1 and soluble endoglin (sEng), neutrophil associated gelatinase lipocalin (NGAL) produce systemic endothelial dysfunction, subsequent to hypertension, proteinuria, and the other systemic manifestations of preeclampsia (Maynard *et al.*, 2003; Venkatesha *et al.*, 2006).

The role of angiogenic proteins in early placental vascular development and trophoblasts invasion have received little research attention, and the molecular basis for placental dysregulation of these pathogenic factors is still not known (Venkatesha *et al.*, 2006). Various factors such as the syncytiotrophoblast debris, immune maladaption, renin-aldosterone-angiotensin II axis, platelet aggregation, excessive oxidative stress, and genetic susceptibility plays an important role in the pathogenesis of preeclampsia (National High Blood Pressure Education Group, 2000). Placental hypoxia is an indispensable upregulator of preeclampsia. Its effects initiate tissue oxidative stress, increase placental apoptosis and necrosis leading to endothelial dysfunctions and subsequent increase inflammatory response (Maynard *et al.*, 2003; Romero *et al.*, 2008a; Petla *et al.*, 2013).

Previous studies (Granger *et al.*, 2002; Gilbert *et al.*, 2008) have also indicated that decreased formation of vasodilators such as nitric oxide and prostacyclin are associated with gestational hypertension and preeclampsia. A decreased bioavailability of the endothelial nitric oxide (NO) synthase (eNOS), cofactor tetrahydrobiopterin (BH4) and an increased reactive oxygen species (superoxide and peroxynitrite) production could exacerbate to maternal endothelial dysfunction in rats preeclampsia and those with numerous characteristics of gestational hypertension (Mitchell *et al.*, 2008).

The occurrence of preeclamptic condition has been classified into two stages (George and Granger, 2010). The first stage is asymptomatic and it characterized by placental insufficiency and impaired trophoblasts invasion of the maternal spiral arterioles occurring in the first trimester while the second stage is symptomatic and involves the development of hypertension, proteinuria, and renal impairment. Subjects with second stage preeclampsia are at increased risk for the HELLP syndrome (haemolysis, elevated liver function enzymes and low platelets count), eclampsia, and multiple organ damage (Haram *et al.*, 2009).

2.3.1 *Role of the Placenta*

The placenta plays a central role in the pathogenesis of pregnancy induced hypertension especially in preeclamptic conditions. Several studies have linked its origin to placenta rather than the fetus because the disease seems to only remit after placental delivery (Maynard and Karumanchi, 2011). In situation of hydatidiform mole, presence of a fetus is not necessary for the development of this condition (Maynard *et al.*, 2005). Other complication such as postpartum eclampsia has been linked with retained placental fragments, with rapid improvement after uterine curettage (Matsuo *et al.*, 2007). Placental hypoperfusion and ischemia are pathological evidence of severe preeclampsia.

Pathologic examination of placentae from preeclamptic pregnancies generally reveals acute atherosis, a lesion of diffuse vascular obstruction that includes fibrin deposition, intimal thickening, necrosis, sclerotic narrowing of arteries and arterioles, and endothelial damage. Placental infarcts with typical reduced endovascular invasion by cytotrophoblast and impaired remodeling of the uterine spiral arterioles are also common (Salafia *et al.*, 1995). An abnormal uterine artery Doppler ultrasound, consistent with decreased uteroplacental perfusion, predate the clinical onset of preeclampsia (North *et al.*, 1994). The severity of clinical disease appears to correlate with massive gross placental pathology though these findings are not consistent with some studies (Salafia *et al.*, 1995).

2.3.1.1 Abnormal Placentation

In normal placental pregnancies, cytotrophoblast from fetal origin invades maternal spiral arterioles of the decidua and myometrium thereby transforming them from small caliber high resistance vessels to high caliber conduit vessels (Figure 2.1). The process of invasion begins at the end of first trimester (10-12 week) and ends by 18 to 20 week of gestation which is capable of differentiating the cytotrophoblast from epithelial phenotype to an endothelial phenotype, a process known as pseudovasculogenesis (Petla *et al.*, 2013).

These physiological modifications is upregulated by a considerable number of transcription factors, alpha v beta-3 integrins, growth factors and cytokines like vascular endothelial-cadherin (Zhou *et al.*, 1997). This result in decreases peripheral blood vessel resistance and increases placental perfusion thus sustain the growing foetus by providing essential nutrients and oxygen (Zhou *et al.*, 1997). In pre-eclampsia, there is failure of cytotrophoblasts invasion into myometrial segments of the arteries, decreased transformation of epithelial phenotype into endothelial phenotype, defective uteroplacental circulation and subsequently placental underperfusion (Figure 2.1) (Maynard and Karumanchi, 2011).

In preeclampsia, the intervillous space of placenta becomes ischemic / hypoxic which consequently activate tissue oxidative stress and increase placental apoptosis and necrosis finally leading to endothelial dysfunction (Agarwal and Karumanchi, 2011). Histological evidence indicated that preeclamptic women have considerable defective cytotrophoblast invasion (Brosens *et al.*, 1977). Toxic metabolites from a hypoxic placenta released to the maternal circulation may aggravate generalized syndrome of preeclampsia (Roberts and Redman, 1993). Placental insufficiency is also seen in cases of foetal growth restriction devoid of preeclampsia. The evidence of normal birth weight after preeclamptic pregnancies is suggestive of normal primary placentation (Zhou *et al.*, 1997).

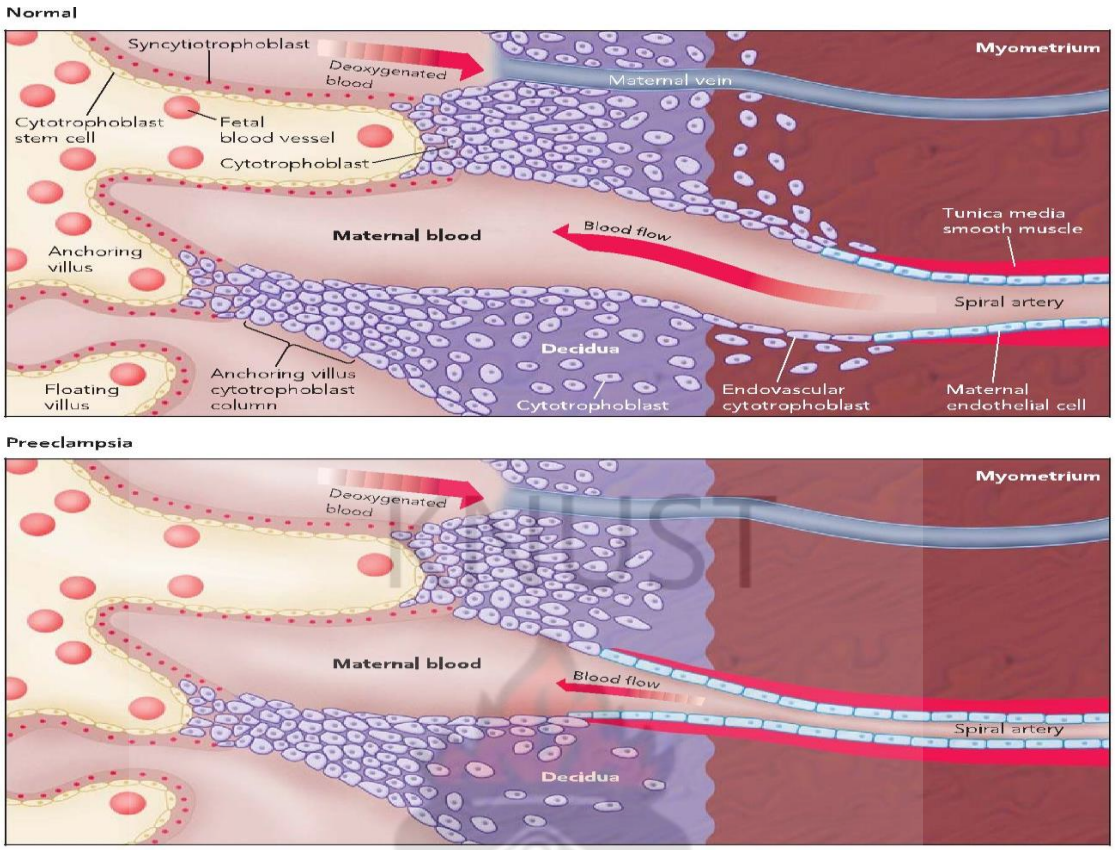

Figure 2. 1 Abnormal placentation in preeclampsia

In normal placental development, invasive cytotrophoblasts of fetal origin invade the maternal spiral arteries, transforming them from small-caliber resistance vessels to high-caliber capacitance vessels capable of providing placental perfusion adequate to sustain the growing fetus. During the process of vascular invasion, the cytotrophoblasts differentiate from an epithelial phenotype to an endothelial phenotype, a process referred to as "pseudovasculogenesis" or "vascular mimicry" (top). In preeclampsia, cytotrophoblasts fail to adopt an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow, and they remain small caliber, resistance vessels (bottom) source : (Lam *et al.*, 2005)

2.3.2 *Involvement of Circulating Factors*

Previous in-vitro and in-vivo studies conducted by several researchers have extensively linked the involvement of circulating factor (s) in the pathogenesis of preeclampsia and gestational hypertension. These circulating factors originate

from the placenta and thus target and stimulate widespread endothelial cell activation. Preeclamptics have been associated with cultured human umbilical vein endothelial cells (HUVECS) cytotoxicity compared with sera from healthy control (Rodgers *et al.*, 1988). The cytotoxic effect of HUVECS in the serum of preeclamptics before delivery was greater than serum obtained after delivery and so suggest that the these factor (s) are mostly linked to conception than pregestation or postpartum (Rodgers *et al.*, 1988).

Circulating factor (s) are not likely to induce gross morphological injury and cell death, as proposed earlier but induce an alteration in endothelial function (Tsukimori *et al.*, 1992). The role of these circulating factor (s) is its ability to pass liberally into the maternal circulation, modify prostacyclin and nitric oxide production, amplify cellular permeability and probably cell turnover, and promote a functional change in the response to endothelium-dependent agonists (Myers *et al.*, 2005). Circulating factors such as angiogenic factors, oxidative stress, genetic factors, immunological factors, and maternal factors play an important role in the pathogenesis of preeclampsia and gestational hypertension.

2.3.3 *The Role of Circulating Angiogenic Factors*

One of the most important processes that contribute to placental vascular system development is angiogenesis. Angiogenesis is the development of new vessels from pre-existing blood vessels (Palm, 2012). Circulating angiogenic factors play an important role in the pathogenesis of preeclampsia and gestational hypertension as postulated by many authors in the past decade. Increased expression of soluble endoglin (sEng) and fms-like tyrosine kinase (sFlt1), coupled with decreased placental growth factor (PLGF) and vascular endothelial growth factor (VEGF) signaling were the first anomalies observed (Levine and Karumanchi, 2005).

2.3.3.1. Vascular Endothelial Growth Factor (s) (VEGF)

Among the various angiogenic factors expressed by the placenta, VEGF plays a very key role in the development of placental vascular system (Agarwal and Karumanchi, 2011). VEGFs are a family of structurally related dimeric glycoproteins whose members comprises VEGF-A, VEGF-B, VEGF-C, VEGF-D and homologous PLGF. The biological function of VEGF is to promote vascular permeability and sustenance, migration and differentiation of endothelial cells by interacting with VEGFR-2 (also known as Kinase Domain Region), high affinity receptor tyrosine kinases Flt-1 (VEGFR-1) on the placental endothelial cells (Romero *et al.*, 2008a).

Similar to circulating levels found in-vivo, VEGF makes use of an in-vitro effect which is specific to vascular endothelial cells (Levine *et al.*, 2004). It has been documented that the increased expression of VEGF was associated with trophoblasts cells and placental tissue cultured in hypoxic conditions (Trollmann *et al.*, 2003). In addition, VEGF production may increase in response to vascular endothelial cell injury. As endothelial cell damage is common in preeclampsia it implies that the increased levels of VEGF detected in preeclampsia may participate in the pathogenesis of vascular damage (Tsurumi *et al.*, 1997).

VEGF is commonly expressed by glomerular podocytes, and thus are present on glomerular endothelial cells (Levine and Karumanchi, 2005). Glomerular endothelial damage coupled with proteinuria has been observed in Anti-VEGF therapies animals (Sugimoto *et al.*, 2003). Anti-angiogenesis cancer trials with anti-VEGF antibodies have led to proteinuria, hypertension, and loss of glomerular endothelial fenestrae in women with preeclampsia (Eremina *et al.*, 2008).

2.3.3.2 Placental growth factors (PLGF)

Placental Growth Factor (PLGF) is a potent angiogenic factor produced from the placental trophoblasts, primarily in the syncytiotrophoblast, which expresses itself

in several different isoforms. Along with VEGF, PLGF plays a significant role in angiogenesis and placental vasculature especially during embryogenesis (Hertig and Liere, 2010). Unlike VEGF which binds to both VEGFR-1 and VEGFR-2, PLGF binds to only VEGFR-1 (Torry *et al.*, 2003). The splice variant of VEGFR-1, also known as soluble Foetal-like tyrosine kinase receptor-1 (sFlt-1), easily neutralizes PLGF, and thus decreases its level in serum of women who developed pre-eclampsia (Hertig and Liere, 2010; Agarwal and Karumanchi, 2011)

2.3.3.3 sFlt-1: A Circulating Antagonist to VEGF and PIGF

Many researchers has agreed on the finding that pregnant women may produce a soluble variant of vascular endothelial growth factor receptor, a kinase which has been designated as sFlt-1 (Agarwal and Karumanchi, 2011). It's a potent anti-angiogenic factor which consists of an extracellular ligand binding domain of Flt-1, but lacks the transmembrane and intracellular signaling domain. It exert its activity freely in circulation by interfering with the agonistic functions of both VEGF and PLGF in placental development (Wang *et al.*, 2009). Circulating levels of sFlt1 are changed several weeks before the clinical onset of disease and are linked with severity of the disease (Levine *et al.*, 2004). However, levels become normalize several days postpartum, corresponding with improvement in proteinuria and hypertension. More recent clinical findings supports the idea that higher levels of both circulating and placental levels of this soluble receptor blocker are shown in women with preeclampsia than in women with uncomplicated pregnancy (Lapaire *et al.*, 2010). Numerous findings on the key role of Flt-1 in the pathophysiology of preeclampsia has been gathered from studies undertaken in a baboon model of hypertension in pregnancy induced by uteroplacental ischemia (Makris *et al.*, 2007). The central role of impaired VEGF signaling in the development of preeclampsia is the specific elevation in the serum levels of sFlt-1 5 weeks before the onset of hypertension and proteinuria (Agarwal and Karumanchi, 2011) . This

elevation in the kinase is also associated with the lower activity of PIGF and VEGF. A study used restricted uterine arteries and observed that an association between the onset of hypertension, renal dysfunction and the increase level of the kinase existed (Agarwal and Karumanchi, 2011).

A second splice form of sFlt-1 is expressed in cytotrophoblasts, which differs in its c-terminus also appears to be upregulated in preeclampsia. Levels of sFlt-1 have proven to be usefulness in predicting early onset of preeclampsia due to increased levels associated with first trimester pregnancy (Baumann *et al.*, 2008).

Lower urinary protein levels, stabilized blood pressure, and permitted prolongation of pregnancy in a small group of women with early onset of preeclampsia was observed in selectively removing soluble Fms-like tyrosine kinase, using an extracorporeal adsorption technique (Valdiviezo *et al.*, 2012). This observation indicted that this protein kinase has a significant role in the pathogenesis and pathophysiology of preeclampsia. Women with preeclampsia have elevated circulating levels of placental debris which have been shown to be associated with sFlt-1 in the maternal circulation and thus may be an additional source of circulating sFlt-1 in preeclampsia (Redman and Sargent, 2007).

2.3.3.4 Soluble Endoglin: A Circulating Antagonist to Transforming Growth Factor-B.

Aside vascular endothelial growth factor-receptor which is a potent antagonist blocking the action of these growth factors another factor, soluble endoglin (sEng/CD 105), has also been found to be upregulated in hypertensive pregnancies especially preeclampsia (Grill *et al.*, 2009). sEng is a transmembrane glycoprotein with two splice variants, endoglin S and endoglin L, a cell surface co-receptor for transforming growth factor (TGFβ1, TGFβ3), which modulates TGF β signaling in angiogenesis and regulates vascular tone (Grill *et al.*, 2009).

It is highly expressed on the cell membrane of syncytiotrophoblast and endothelial cells. It does not only act as a potent agonist for sFlt-1 action but also interfere with the binding of TGFβ1 to its receptor, and thus reduced the production of nitric oxide (NO) leading to vasoconstriction and abnormal capillary formation by endothelial cells (Levine *et al.*, 2006). Endothelial abnormality on account of sEng action results in disseminated intravascular coagulation and the other hematologic derangement seen in patients with severe preeclampsia (Gu *et al.*, 2009).

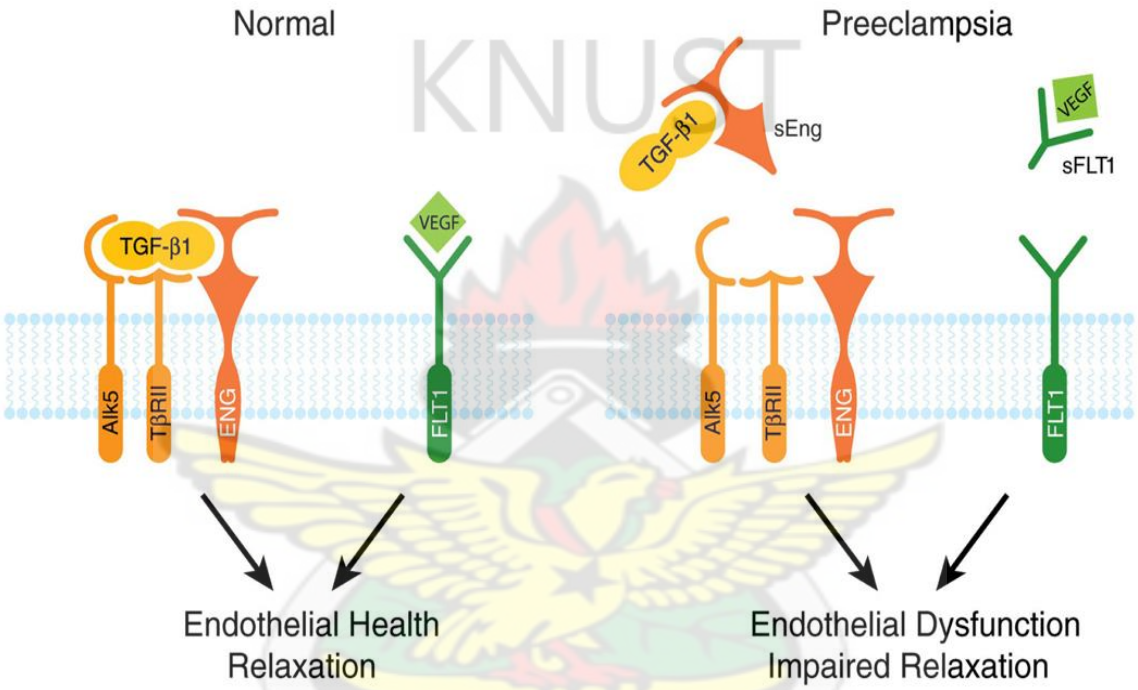

Figure 2. 2 sFlt1 and sEng Causes Endothelial Dysfunction by Antagonizing VEGF and TGF-β1 signaling

There is mounting evidence that VEGF and TGF-β1 are required to maintain endothelial health in several tissues including the kidney and perhaps the placenta. During normal pregnancy, vascular homeostasis is maintained by physiological levels of VEGF and TGF-β1 signaling in the vasculature. In preeclampsia, excess placental secretion of sFlt1 and sEng (two endogenous circulating anti-angiogenic proteins) inhibits VEGF and TGF-β1 signaling respectively in the vasculature. This results in endothelial cell dysfunction, including decreased prostacyclin, nitric oxide production and release of procoagulant proteins. Figure source: (Agarwal and Karumanchi, 2011)

2.3.4 The Role of Oxidative Stress

The process of oxidative stress is not totally harmful in pregnancy because they exert a fundamental regulatory function during pregnancy (Palm, 2012). At the onset of pregnancy, when a certain grade of inflammatory change is required to the trophoblasts invasion of maternal tissue, nitric oxide and reactive oxygen species are activated to initiate the remodeling of uterine extracellular matrix (Biondi *et al.*, 2005). In normal pregnancy oxidative stress plays a pivot role in placental development through stimulation of cytotrophoblast proliferation, differentiation and migration (Toescu *et al.*, 2002). Increase in oxygen tension (P02) by more than 50mmHg may stimulate hypoxia in placenta. Increased placental hypoxia may expose the placenta to subsequent generation of reactive oxygen species (ROS) which are chelated by increased levels of antioxidants implicated in normal pregnancy (Roberts and Hubel, 2004).

Normal pregnancy is associated with high metabolic demand and elevated requirement for tissue oxygen which results in increased oxidative stress and antioxidant (Tiwari *et al.*, 2010b). Level of oxidative stress is an important factor in embryogenesis, as well as in pregnancy and normal birth. An imbalance between the prooxidants and antioxidants either due to excessive generation of pro-oxidants or reduced levels of the antioxidant systems leading to inadequate reducing capacity of the antioxidants and thus results in OS (Stark, 2001). The result of this, is an elevated plasma levels of pro-oxidant free radicals and reactive oxygen species (ROS) such hydroxyl radical (HO.), superoxide anion radical (O2–), nitric oxide (NO.), hydrogen perioxide (H_20_2), hypochlorous acid, (HOCl) and peroxynitrite anion (ONOO–) (Hubel, 1999). These free radicals attack polyunsaturated fatty acids or cholesterol in membranes or lipoproteins (lipid hydroperoxidation). Although the generation of free radicals is a normal physiological process, increased production of free radicals has adverse effects on DNA, lipids and proteins (Tiwari *et al.*, 2010a).

Under conditions of oxygen deficiency in tissues, stimulation of neovascularization process and induced angiogenesis occurs in pregnancy, which itself is the result of hypoxia (Agarwal *et al.*, 2005). Impaired trophoblasts invasion of maternal spiral arteries leads to impaired placental perfusion and a subsequent placental hypoxia or ischemia in the intervillous space, which increase expression of xanthine oxidase and Nicotinamide Adenine Dinucleotide Phosphate (NADP) (H) oxidase and resultant increased free radicals such superoxide production, which are predominantly expressed in cytotrophoblast, syncytiotrophoblast, and villous stromal cells (Many et al., 2000; Agarwal et al., 2012). This leads to numerous cell damage like cell death by necrosis or apoptosis, lipid peroxidation, nucleic acids injury, inhibition of protein synthesis and subsequent endothelial dysfunction in maternal placenta, brain, liver and kidney (Agarwal *et al.*, 2005; Redman and Sargent, 2005). These observations support the hypothesis that placental underperfusion generates oxidative stress and leads to general endothelial dysfunction in preeclampsia and gestational hypertension (Roberts and Hubel, 2004; Hung et al., 2010). Enzyme activities and mRNA expression of the placental antioxidant enzymes such as superoxide dismutase and glutathione peroxidase were significantly decreased in placentas from women with pregnancies complicated by preeclampsia in contrast to those from normotensive pregnant women (Hubel, 1999; Richter et al., 2012).

Placental anomalies and uteroplacental ischemia/hypoxia may induce the shedding of placental microparticles into the maternal circulation, and these particles may lead to vascular damage and increased inflammatory response (Hung *et al.*, 2001; Hung and Burton, 2006). The shedding of placental debris has also been proposed as an undeniable cause of free radical production and in vitro evidence indicates that syncytiotrophoblast microvesicles may culminate in activation of maternal neutrophils (Raijmakers *et al.*, 2004). Local activation of maternal neutrophils may occur during passage of maternal blood through the

placenta. Release of cytokine synthesized from activated neutrophils and lipid peroxides into the maternal circulation could result to maternal endothelial cell activation, subsequent leukocyte adhesion, and later lead to neutrophil activation (Raijmakers *et al.*, 2004) (Figure 2.3). Women with preeclampsia generate isolated neutrophils which stimulate the synthesis of superoxide on activation with either receptor-mediated N-formyl-methionylleucyl-phenylalanine or non-receptor-mediated phorbol 12-myristate 13-acetate stimuli than those of normotensive pregnant women (Tsukimori *et al.*, 1993; Lee *et al.*, 2003), and this is mediated by NAD(P)H oxidase.

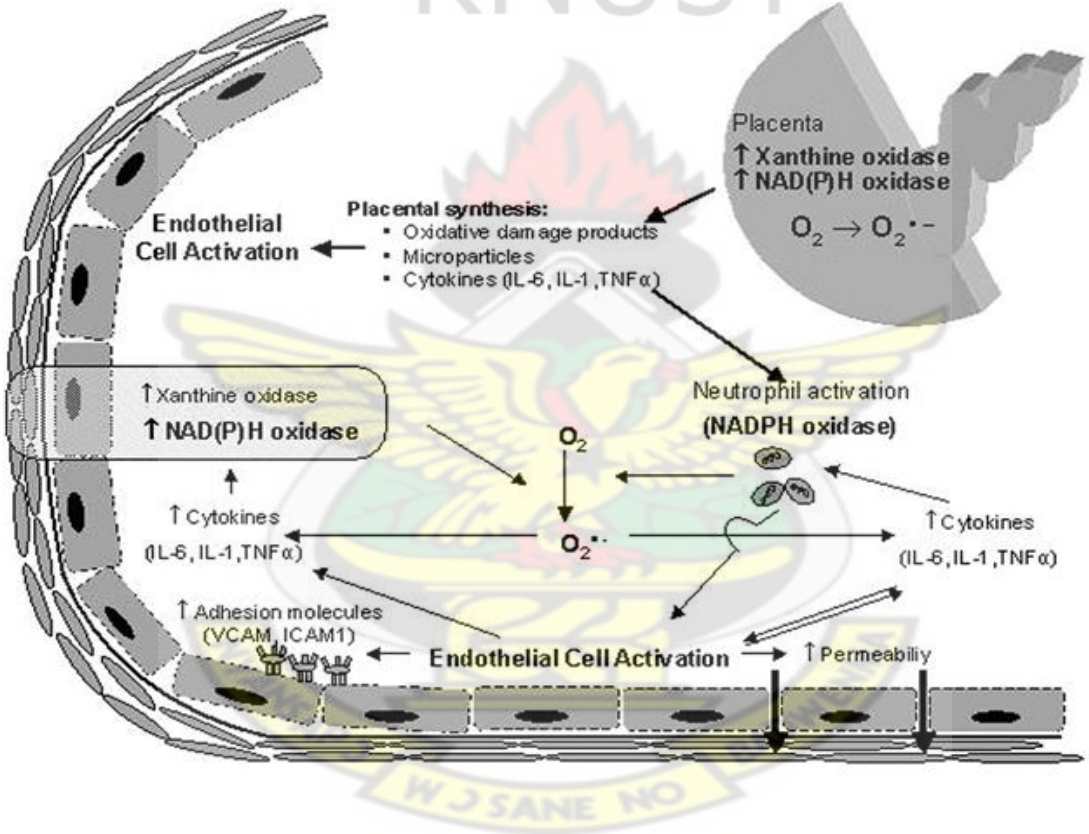

Figure 2. 3 Proposed association between placental oxidative stress and maternal vascular dysfunction in preeclampsia

It is hypothesized that free radical generation through xanthine oxidase or NAD(P) H oxidase in the placenta leads indirectly to maternal neutrophil activation. In the maternal circulation, a vicious circle of maternal endothelial and neutrophil activation may result in sustained NAD(P) H oxidase activity and release of superoxide. Source: (Raijmakers *et al.*, 2004)

2.3.4.1 Lipid Peroxidation

The formation of lipid peroxide occurs when free radicals such as OH* and NO attack polyunsaturated fatty acid or cholesterol in plasma lipid membranes (Hubel et al., 1989). Lipids are a key target of free radical attack, which induces lipid peroxidation.

Free radical induced lipid peroxidation represents an oxidative damage seizing cell membranes, lipoproteins and other molecules containing lipids in conditions of oxidative stress existence. This leads to changes in membrane biological characteristics, such as fluid degree change, resulting in an inactivation of membrane receptors and enzymes which impair normal cell function (Gubaljevia and Aceauaievia, 2013). Several pathological changes observed in the diseased condition are due to the amplification in lipid peroxide formation by the placenta in preeclampsia. Lipid peroxides formed at a primary site may build up in lipoproteins and be transported throughout the circulation due to their higher half-life (Hubel *et al.*, 1989). Lipid peroxides have in addition been noted to stimulate smooth muscle contractions in an array of isolated arterial preparations. Evaluating various products of lipid peroxidation is imperative to assess oxidative stress or lipid membrane damage.

F2-Isoprostanes belong to a family of prostaglandin derivatives that are mainly formed by peroxidation of arachidonic acid catalysed by free radicals. It possesses biological effects in vivo, including vasoconstriction, endothelium derangement and platelet activation (Patrono and FitzGerald, 1997). These effects may possibly be mediated by interaction with the thromboxane A2 (TXA2) receptor.

Normal pregnancy is associated with oxidative stress and lipid peroxidation compared with non-pregnant women and this become worsen in hypertensive disorder (Agarwal *et al.*, 2005). The increased production of free radical and a

resulting lipid peroxidation presupposes that there is an unevenly rise in placental generation of lipid peroxides in preeclampsia.

2.3.4.2 Peroxynitrite

Another potential marker of oxidative stress is peroxynitrate, produced by the vasorelaxant nitric oxide (NO) reacting with superoxide anions. Peroxynitrite is considered as a marker of "nitrative stress" which, leads to oxidative stress, is observed preeclamptic placenta and diabetes in relationship with placental dysfunction. Under, oxidative stress condition NO from the endothelial cells is also known to react with superoxide anions, producing peroxynitrite that may damage vascular function (Roggensack *et al.*, 1999).

2.3.5 Endothelium Involvement

Preeclampsia is associated with a loss of endothelial cell integrity and a resultant increase in vascular permeability (Hayman *et al.*, 1999). There are several data relating to circulating endothelial cell markers and the development of preeclampsia. Women with preeclampsia are associated with increased levels of fibronectin, factor VIII antigen, von Willebrand factor, tissue plasminogen activator, and plasminogen activator inhibitor-1 than normal pregnancies (Powe *et al.*, 2011). Several studies (Taylor *et al.*, 1990; Schiff *et al.*, 1992) have also reported elevated levels of circulating endothelin-1 in preeclampsia which, is an indication of increased activated endothelial cells synthesis. Despite different cell types and synthesis, circulating endothelial cells are produced in the vascular endothelium.

An important component of the coagulation system is an intact endothelium. More sensitive markers of coagulation dysfunction which include an altered factor VIII–related antigen to coagulation activity ratio (Redman *et al.*, 1977), a reduction in the platelet count and an increase in the levels of plasma β-thromboglobulin are present in a high proportion among women with preeclampsia (Macey *et al.*, 2010). Endothelium-dependent relaxation of resistance in arteries plays crucial role in

vascular endothelial cell development in normal pregnancies but become impaired in preeclampsia. The vascular endothelial cell appears to be the target of the disease process in preeclampsia. Clinical manifestations of preeclampsia can be accounted for by activation of vascular endothelial cell. Yet, the exact nature of the activation and subsequent function of the endothelial cell in vivo has not been fully explored (McCarthy *et al.*, 1994). Although preeclampsia appears to originate in the placenta, the most important affected tissue is the maternal endothelium. Failure of maternal remodeling and a subsequent imbalance in angiogenic factors are undeniable contributing factors to a widespread endothelial dysfunction, and clinical manifestations of preeclampsia (Mustafa *et al.*, 2012). Maternal endothelial dysfunction is a widespread characteristic feature of preeclampsia, but whether it is a cause or consequence of the disorder, is still undefined (Bdolah *et al.*, 2005). The evidence of endothelial dysfunction has been found as the focal point of preeclampsia. Endothelial "activation" and dysfunction are reflected in the inappropriate vasoconstriction and its tendency toward a hypercoagulable state and the widespread microvascular thrombi (Taylor *et al.*, 2009). Some investigators proposed that preeclampsia represents a pregnancy induced inflammatory response leading to maternal endothelial cell dysfunction (Taylor *et al.*, 2009; Weiss *et al.*, 2009).

An altered synthesis and release of endothelial cell products were supportive evidence of the manifestation of endothelial dysfunction (Taylor *et al.*, 2009). Among the various compounds, prostanoids and nitric oxide act on the endothelium surface. Nitric oxide (NO) synthesis is increased in women undergoing physiologic pregnancy, but its production is impaired in preeclamptic women. Nitric oxide synthase (NOS), is the enzyme that produces nitric oxide (NO) from arginine, through the endothelial endothelin B receptor (Jeyabalan *et al.*, 2003). In an animal model, nitric oxide synthase inhibition produced a presentation similar to preeclampsia (Venuto and Lindheimer, 2009). The possible

imbalance between vasodilating and vasoconstricting prostaglandins may play a role in endothelial dysfunction. Physiological pregnancies tend to be associated with increased synthesis of the vasorelaxant prostacyclin, whereas pregnancies complicated by high blood pressure and proteinuria produce more of the vasoconstrictor endothelin, thromboxane A2 (Mustafa *et al.*, 2012). In preeclampsia, derangement of endothelial-derived vasoactive factors is believed to result in the predominance of substances that are vasoconstrictors (endothelin, thromboxane A2) over vasodilators (NO, prostacyclin) (Jeyabalan *et al.*, 2003). Whether these particular compounds play a primary role in the pathogenesis or are only a part of the progression of the pathophysiology is unclear.

2.4 MATERNAL ANGIOGENIC FACTORS IN GESTATIONAL HYPERTENSION AND PREECLAMPSIA

Several studies have evaluated the levels of angiogenic factors in hypertensive disorders of pregnancy such as preeclampsia

2.4.1 Levels of VEGF in gestational hypertension and preeclamptic pregnant women

VEGF has been demonstrated in serum of women with hypertensive disorders of pregnancy due to ability to stimulate an increase in vascular permeability through the protein kinase C pathway (Spyridopoulos *et al.*, 2002). Reports from several studies (Levine and Karumanchi, 2005; Romero *et al.*, 2008b; Lapaire *et al.*, 2010) have observed a mild decreased levels of VEGF in gestational hypertension with severe decrease in preeclamptic subjects. Others also reported that levels of VEGF were significantly decreased in normotensive pregnant women than in non-pregnant women and was further reduced in preeclampsia (Hertig and Liere, 2010). Development of the endothelial dysfunction characteristic of the maternal syndrome in preeclampsia was due to decreased production of VEGF by

circulating T and natural killer cells (Molvarec *et al.*, 2010a). Some studies (Baker *et al.*, 1995; Sharkey *et al.*, 1996) indicated that preeclampsia is associated with an increased VEGF while others observed a reduced level (Levine and Karumanchi, 2005; Romero *et al.*, 2008b; Lapaire *et al.*, 2010). The discrepancy was explained by the fact that certain binding proteins interfere with quantification of VEGF (Baker *et al.*, 1995). Immunohistochemical staining techniques for VEGF in preeclamptic women was found to be increased (Akercan *et al.*, 2008) though the expression of VEGF mRNA is reported low in pregnancies complicated by preeclampsia (Lyall *et al.*, 1997).

Some studies (Grill *et al.*, 2009; Maynard and Karumanchi, 2011) found serum VEGF as a promising marker in the prediction of pre-eclampsia, while some (Lam *et al.*, 2005) could not detect its levels because the circulating levels of VEGF were as low as <30 pg/ml, which is below the detection limit of most available ELISA kits. The latter finding suggest that such limitation can be overcome if a sensitive, highly reliable and single digit picogram concentration ELISA Kits is used.

2.4.2 Levels of PLGF in gestational hypertension and preeclamptic pregnant women

Maternal serum levels of PLGF in both early and late onset pre-eclampsia is reduced due to increased sFlt-1 concentration (Hertig and Liere, 2010). The reduction in PLGF levels during the 5th week and 9-11th week before the onset of preeclampsia is an important observation that necessitates investigation of the predictive accuracy of PLGF (Lam *et al.*, 2005; Romero *et al.*, 2008b). PLGF expression is heterogeneous in uncomplicated pregnancies during the first trimester and levels tend to increase from 15 weeks of the second trimester pregnancy to 27-32 weeks and decrease thereafter to week 40 in normal pregnancy (Palm, 2012). Again levels of PIGF have been shown to fluctuate throughout

normal pregnancy and postpartum (Palm, 2012). Women who are destined to develop preeclampsia mostly show lower PLGF at 15-18 week of gestation than those who do not develop the condition (Romero *et al.*, 2008b). A mean of 137 pg/mL serum PLGF concentrations in women with preeclampsia compared to 669 pg/mL levels in normal pregnancies have been reported (Levine *et al.,* 2004).

The combined effect of PLGF and sFlt-1 (PLGF: sFlt-1 ratio) has been suggested as one of the best methods for predicting pre-eclampsia before the onset of the disease than using the individual markers (Hertig and Liere, 2010). Verlohren *et al.,* (2012) observed that a shift in the PLGF: sFlt-1 ratio showed additional valuable information on the status and progression of the preeclampsia and thus should be implemented in the diagnostic algorithm (Verlohren *et al.*, 2012).

2.4.3 Levels of sFlt-1 in gestational hypertension and preeclamptic pregnant women

Previous authors (Levine *et al.*, 2004; Petla *et al.*, 2013) have consistently reported elevated levels of serum sFlt-1 in women presenting with preeclampsia compared with normal pregnancies. sFlt-1 levels were as high as 4382 pg/ml in women with pre-eclampsia compared to 1643 pg/ml in control group suggesting that higher than normal levels of sFlt-1 are predictive of pre-eclampsia (Levine *et al.*, 2004). Similar findings were reported in other studies (Lam *et al.*, 2005; Maynard and Karumanchi, 2011). Interestingly, this higher level in serum sFlt-1 may be detected up to 5 weeks before the clinical onset of clinical symptoms. Unfortunately, there are reports of poor predictive value and lack of specificity of sFlt-1 in the early onset of pregnancy (Agarwal and Karumanchi, 2011).

In the last decade, a few variants of sFlt-1, like sFlt1-14 have been discovered and increased levels are associated with preeclampsia, thus, questioning the specificity of sFlt-1 in predicting preeclampsia (Agarwal and Karumanchi, 2011).

Chinese women in the first trimester have reported normal levels of sFlt-1 levels but PLGF levels increased in PE patients (Wa Law *et al.*, 2011).

The mean serum levels of sFlt-1/PIGF ratio are increased in early onset preeclampsia at less than 34 weeks of gestation when compared with the women who do not developed preeclampsia from 22 weeks of gestation onwards (Moore Simas *et al.*, 2007). Conversely, there was a considerable overlap in the sFlt-1 and PLGF levels between patients who will go on to have normal pregnancies and those destined to develop preeclampsia (Romero *et al.*, 2008b). Substantial differences in the methods of various studies and concentrations of sFlt-1 in a systematic review published in 2007 have also been reported (Widmer *et al.*, 2007).

2.5 MATERNAL OXIDATIVE STRESS MARKERS IN GESTATIONAL HYPERTENSION AND PREECLAMPSIA

Oxidative stress (OS) generally describes a pathological state where a cell antioxidative defence is inadequate to completely inactivate reactive oxygen species (ROS), which results in an increased pro-oxidants (increase release of free radical) and decreased endogenous antioxidant defence (Betteridge, 2000; Gupta *et al.*, 2005). The pathogenesis of GH and thus PE though not yet elucidated, hypoxic placenta resulting from a shallow trophoblasts invasion has been suggested as primary cause (Owiredu, 2008b; Palm, 2012).

Like PE and GH, pregnancy itself may induce oxidative stress (Raijmakers *et al.*, 2004), and may contribute to widespread endothelial dysfunction and other clinical manifestation of preeclampsia (Bdolah *et al.*, 2005). Although substantial evidence supporting an increase in products of oxidative stress in women with preeclampsia, have been shown both in the placenta (Staff *et al.*, 2003) and in maternal peripheral blood cells (Harsem, 2008), as well as in maternal circulation

(Roberts and Hubel, 2004), there is still some disagreements with regards to the results for latter section (Barden *et al.*, 1996; Morris *et al.*, 1998). In preeclamptic pregnancies, the impaired trophoblasts invasion of maternal spiral arteries, reduced placental perfusion and the disordered release of spiral artery plugs, may worsen placental hypoxic feature of pregnancy, to the degree that may cause OS and antioxidant disproportions (Roberts and Hubel, 2004; Hung *et al.*, 2010).

2.5.1 Lipid peroxidation biomarkers in gestational hypertension and preeclamptic pregnant women

In general, the combination of prooxidant (such as elevated lipid peroxidation markers) and decreased antioxidant capacity gives a strong indication of oxidative stress (Raijmakers *et al.*, 2005). Malondialdehyde (MDA), a major metabolite of lipid peroxide breakdown is among of the earliest biomarkers of lipid peroxidation found to be increased in the plasma of women presenting with preeclampsia (Hubel *et al.*, 1989; Owiredu, 2008b; Ahenkorah, 2009). Several other studies have also found an evidence for lipid peroxidation, using a variety of appropriate assays comprising F2-Isoprostane (mostly 8-epiprostaglandin-F2α) (Palm *et al.*, 2009; Chen *et al.*, 2012b; Gubaljevia and Aceauaievia, 2013), 4-hidroxynonenal (HNE), 2-propenal (acrolein), thiobarbituric reactive substance, α, β-unsaturated reactive aldehydes and conjugated dienes. Women with preeclampsia are shown to report about two (2) fold increase in the level of 8-epiprostaglandin-F2α (8-epiPGF2α) compared to normal pregnant women (Walsh *et al.*, 2000).

2.5.2 Antioxidant status in gestational hypertension and preeclamptic pregnant women

Aside the evaluation of oxidative damage, some authors have determined antioxidant capacity in the maternal circulation by measuring total antioxidant capacity (T-AOC), the concentration of specific antioxidants, or the activity of

antioxidant enzymes (Raijmakers *et al.*, 2005). Pregnancy tends to promote oxidative stress as markers of oxidative stress are known to be raised in normal pregnancy (Nwagha and Ejezie, 2005). Total antioxidant capacity (TAO-C) characterizes the balance between oxidative stress (oxidants) and the neutralizing systems (antioxidants). The oxidants are mainly ROS and their derivatives such as peroxynitrite anion, superoxide anion (Halliwell, 1997; Halliwell and Guttertidge, 1999). Conversely, homeostasis against the effects of ROS and their derivatives is maintained by antioxidants such as catalase, superoxide dismutase, beta-carotene, vitamin C, vitamin E, glutathione peroxides, ceruloplasmin and selenium. These agents intercept, modify or chelate the reactive free radicals (Halliwell and Guttertidge, 1999) and thus alleviate oxidative stress in pregnancy. The consequences of reduced antioxidant systems in pregnancy results in reduced placental efficiency and calcification (Idogun *et al.*, 2008) fetal malformation, pregnancy complications such as preeclampsia and eclampsia as well as aggravate respiratory distress syndrome (Hu and Cassano, 2000). Studies show that vitamins C and E given antenatally in high dosages during pregnancy significantly reduced the incidence of preeclampsia (Chappell *et al.*, 1999). The tendency to hypertension in pregnancy has been substantially reduced by adding trace amounts of selenium to the diet (Lu, 1990). Placentas from preeclamptic produce less antioxidant capacity and have greater quantities of superoxide than normal placenta (Richter *et al.*, 2012). Previous reports indicates that both the water soluble antioxidant (such as ascorbic acid) and the lipid-soluble antioxidants (alpha tocopherol and beta-carotene) levels are decreased in the serum of women with preeclampsia compared to those of normal pregnancies (Raijmakers *et al.*, 2004; Palm, 2012). This hypothesize that antioxidant or micronutrients may be overutilized in preeclampsia to balance free radical–mediated cell disturbances, resulting in a reduction in antioxidant plasma levels in this pathology (Mistry and Williams, 2011). A large randomized controlled trial did not show any significant effect of

antioxidants vitamin C and vitamin E on the risk of developing preeclampsia (Rumbold *et al.*, 2006; Villar *et al.*, 2009; Roberts *et al.*, 2010).

2.6 ADVERSE PREGNANCY OUTCOMES IN GESTATIONAL HYPERTENSION AND PREECLAMPSIA

Pregnancy and thus hypertensive pregnancies are associated with adverse pregnancy outcomes such as stillbirth, IUGR, SGA infants, abruptio placental, intrauterine fetal death (IUFD), postpartum and antepartum haemorrhage and preterm births etc. The mechanisms underlying these outcomes are not fully understood though some studies attribute it to a widespread endothelial dysfunction. Pregnancies complicated by IUGR is more likely to be associated with preeclampsia than gestational hypertension (Aucott *et al.*, 2004). Preeclampsia is reported as a significant risk factor in the development of IUGR and the most common cause of IUGR in neonates. Although IUGR is associated with severe preeclampsia, it has not been extensively explored in pregnancies complicated by mild preeclampsia and gestational hypertension (Resnik, 2002). A study by Ødegård *et al.,* showed that pregnancies complicated by severe preeclampsia had infant birth weights 12% lower than expected, while pregnancies with mild preeclampsia showed no statistically significant difference in weight gain from expected normal weight (Ødegård *et al.*, 2000).

Previous guideline emphasize that neonates from preeclamptic women are delivered preterm when there is evidences of IUGR (Resnik, 2002). Increased prevalence of IUGR in preeclamptic women have been attributed to decreased uteroplacental blood flow and ischemia originating from defective cytotrophoblast invasion and endothelial dysfunction (Levine *et al.*, 2004). Levels of angiogenic factors are observed to be altered in pregnancies complicated with preeclampsia

and IUGR (Maynard *et al.*, 2003; Schlembach *et al.*, 2007). Stillbirth, a complication of hypertensive pregnancy is the pivot cause of fetal loss in the late-preterm infant (Wen *et al.*, 2004). The rate of stillbirth is approximately 3 per 1000 live births beyond 28 weeks gestation though greater than 90% of fetal deaths occur in the first 20 weeks of gestation. Preeclampsia and or gestational hypertension are significantly associated with stillbirth than normal pregnancy though the odds of stillbirth among preeclamptic women with twin pregnancies (Allen *et al.*, 2004a) is markedly high.

Women with hypertension in pregnancy were 1.6 times (95% CI 1.5–1.6) more likely to have a live birth with SGA and 1.4 (95% CI 1.1–1.8) times more likely to have a stillbirth as compared with normotensive women. Adjusted analyses indicated that women with GH were 1.5 times (95% CI 1.4–1.6) while those with PE were 3.2 times (95% CI 2.8–3.6) more likely to have infants with SGA (Allen *et al.*, 2004a). Reports suggest that the risk of intrauterine fetal demise (IUFD) increases significantly beginning at approximately 36 weeks of gestation (MacDorman *et al.*, 2007). Despite the increased relative risk between PE and the development of SGA infants, women with preterm PE are more likely to deliver SGA than those with term pregnancies (Groom *et al.*, 2007). In cases of mild PE, the risk of IUFD is over 50% less than pregnancies with severe preeclampsia. Severe PE is association with significant risk factor for IUFD with estimated stillbirth rate of 21 per 1000 (Simpson, 2002). There is the need for obstetricians to balance the small but important risks factors of IUFD, with the benefits of pregnancy prolongation and potential for continued in utero maturation, mostly in pregnancies less than 37 weeks gestation. Risk of placental abruption is significantly associated with preeclamptic pregnancies than normotensive pregnancies. Workdone by Ananth *et al.,* showed that placental abruption is associated with 1.73 times increased risk of developing preeclampsia (Ananth *et al.*, 1999).

2.7 BIOMARKERS IN PREDICTION AND DIAGNOSING OF PREECLAMPSIA

Diagnosis of preeclampsia with several markers might help to predict early onset and prevent subsequent adverse pregnancy outcome. Several studies have demonstrated the use of sFlt-1 and PLGF in predicting second trimester pregnancy (Erez *et al.*, 2008; Kusanovic *et al.*, 2009).

Evaluation of the sFlt-1: PLGF ratio in the maternal serum has been suggested as more reliable marker of overall preeclampsia risk than using the individual markers alone (Levine and Karumanchi, 2005; De Vivo *et al.*, 2008). However, assessing the diagnostic accuracy of both sFlt-1: PLGF and PLGF: sFlt-1 ratio in preeclampsia have received little or no attention although both have been studied separately by previous studies. Kusanovic *et al.,* (2009) investigated the PLGF: sEng ratio and observed that ratio had a predictive performance with a sensitivity of 100%, a specificity of 98–99 % and likelihood ratios for a positive test of up to 90%.

The use of a ratio of sFlt-1 and PLGF, in this case the PLGF: sFlt-1 ratio, proved to be a reliable tool for the second trimester prediction of preeclampsia in that impressive study. The additional measurement of the sFlt-1: PLGF ratio has been shown to improve the sensitivity and specificity of Doppler measurement in predicting preeclampsia. Studies on the usefulness of the sFlt-1: PLGF ratio as an early prediction marker in the first trimester has yielded inconsistent results. Thadhani *et al.,* (2004) indicated that low PLGF levels were associated with a risk of developing preeclampsia or delivering a small for gestational age (SGA) infant in the first trimester however, levels of sFlt-1 was not statistically significant different from women not developing preeclampsia in the first trimester (Thadhani *et al.*, 2004). In a previous study where maternal serum levels of sFlt-1, sEng and PLGF in women with normal pregnancies and late-onset preeclampsia were assessed, the

serum concentrations of PLGF and the sFlt-1/PLGF ratio in cases did not differ from controls (Baumann *et al.*, 2008).

Placental protein 13 (PP13) levels gradually increases in normal pregnancy but reduced drastically in women who developed pre-eclampsia during the first trimester (Carty *et al.*, 2008). Increased levels of PP13 were found in pre-eclampsia, IUGR and preterm delivery during second and third trimesters, thus, signifying that the measure of serum PP13 during the first trimester can be useful for early prediction of risk of preeclampsia (Grill *et al.*, 2009). A recent study by D'Anna *et al.*, (2009), showed that the levels of pregnancy associated plasma protein-A (PAPP-A) were significantly reduced in the early stage preeclampsia while the levels in the late onset preeclampsia did not differ from the normal pregnancies (D'Anna *et al.*, 2010). This queries the diagnostic accuracy of first trimester PAPP-A as a useful predictive marker in late onset preeclampsia and thus larger trials are required to confirm these preliminary predictions (Bersinger *et al.*, 2002).

In view of endothelial injury, high blood pressure and kidney impairment characteristics in preeclampsia, a recent study established that the serum levels of neutrophil gelatinase associated lipocalin (NGAL) increased at the end of the second trimester in women destined to develop preeclampsia compared to the healthy control (D'Anna *et al.*, 2010). The NGAL serum values and their positive relationship with blood pressure (the systolic and diastolic blood pressure) and with proteinuria, makes NGAL a reliable biomarker for early prediction of pre-eclampsia (D'Anna *et al.*, 2010).

Serum levels of human Placental lactogen (hPL), a marker of syncytiotrophoblast differentiation are significantly decreased in hypertensive pregnancy with least concentration in preeclamptic pregnancies (Ahenkorah, 2009; Owiredu *et al.*, 2012). An increased level of hPL is observed throughout gestation and is associated with placental syncytiotrophoblast mass. Other conditions such as aborting molar

pregnancy, choriocarcinoma and placental insufficiency are associated with reduced hPL levels. Markedly elevated levels of hPL are associated with multiple pregnancies, placental tumour, diabetes, and Rhesus incompatibility.

The other suggested markers for the prediction or detection of preeclampsia are inhibin A, P-selectin, activin A, pentraxin, cell-free fetal DNA, beta-HCG (Mustafa *et al.*, 2012).

2.8 FACTORS ASSOCIATED WITH GESTATIONAL HYPERTENSION AND PREECLAMPSIA

The epidemiology of preeclampsia and other hypertensive disorders reflect a wide range of risk factors as well as the complexity and heterogeneity of the disease. Risk factors can be classified into pregnancy-specific characteristics and maternal preexisting features. These risk factors for preeclampsia and gestational hypertension may be pregnancy related factors (parity, new paternity, limited sperm exposure, multifetal gestation, hydatidiform mole, the use of barrier contraceptives, young maternal age) or preexisting maternal factors such as advanced age, obesity, pregestational diabetes, dietary salt intake, chronic hypertension, renal disease (Duckitt and Harrington, 2005).

2.8.1 *Parity*

The strong relationship between parity and PE and GH was documented over 300 years ago by Mauriceau, who showed that nulliparous are at far greater risk of convulsions than multiparas.'' Pregnancies complicated by preeclampsia are associated with 25-30% of nulliparous pregnant women than in multiparous women. Assessing putative risk factors among Ghanaian women with pregnancy induced hypertension indicated that nulliparity was an independent risk factors for gestational hypertensive pregnancies but not women with preeclampsia (Ahenkorah, 2009; Owiredu *et al.*, 2012)

GH has been undeniably reported to be associated with 1.6- to 2-fold increase risk in nulliparous women than pregnancies from multiparous women, nevertheless the association is less significant than that seen in PE (Campbell and Macgillivray, 1999). Workdone by Campbell & MacGillivray, (1999) in Scottish among over 130,000 pregnancies indicated that the relative risk (RR) of GH in nulliparous women compared to multiparas was 1.98 (95% CI 1.94–2.03) and 1.85 (95% CI 1.55–2.21) in singleton pregnancies and in twin pregnancies respectively. Further confirmation to this finding was reported by Eras and colleagues who found nulliparous women to be at increased risk [odds ratio (OR) 2.29 (95% CI 1.65-3.20)] of non-proteinuric hypertension compared to multiparous (Eras *et al.*, 2000). Several others have corroborated this observation and others have revealed that the association between nulliparity and gestational hypertension was weak among white and not associated with risk among blacks (Misra and Kiely, 1997). However, PE was significantly associated with increased risk among whites (OR=2.86; 95% CI [0.94-8.73]) and blacks (OR=2.94; 95% CI [0.94-9.18]) (Misra and Kiely, 1997). Actually PE is commonly considered as being a clinical condition of first pregnancies (Misra and Kiely, 1997) than GH and its occurrence is more common in nulliparous than multiparous women (Miranda *et al.*, 2011). However the shielding effect of multiparity, is lost with change of partner (Saftlas *et al.*, 2003)

2.8.2 *Maternal Age*

Some epidemiological studies have considered the link between maternal age and the risk of GH and PE. Nevertheless, there have been varying reports on the effects of maternal age on PE. Several studies did not find age as a confounding factor (Eskenazi *et al.*, 1992; Anorlu *et al.*, 2005). Sibai, (2009) reported that younger women aged 21 years and below are at increased risk of preeclampsia (Sibai, 2009) while others found an increased risk of preeclampsia with women who are 35 years or older (Conde-Agudelo and Belizán, 2000; Ahenkorah, 2009)

Several studies also reported that advanced maternal age is associated with an increased risk of GH (Lamminpää *et al.*, 2012; Khalil *et al.*, 2013). Young and advanced maternal aged (35 years and over) are mostly associated with greater risk of adverse perinatal outcomes, such as low birth weight (LBW), small-for-gestational age, preterm birth (PTB), and perinatal or infant mortality and these are largely associated with PE and GH (Kenny *et al.*, 2013). Several studies show a higher incidence of preeclampsia among advanced older women, independent of parity; however, most of them did not control for preexisting medical conditions (Duckitt and Harrington, 2005; Wallis *et al.*, 2008). After adjusting for baseline differences, women aged 40 years or older had almost 2-fold increased odds of developing preeclampsia with an risk ratio of 1.68 [95%CI 1.23–2.29] and 1.96 [95%CI 1.34–2.87] for primiparas and multiparas respectively (Jahromi and Husseini, 2008)

2.8.3 *Obesity*

Elevated body mass index (BMI) is mostly risk factor for GH and PE pregnancies. The risk of preeclampsia increases progressively with increasing BMI even within the normal reference range. Obesity increases the risk of PE by approximately 2-3 fold (Bodnar *et al.*, 2005). Essentially, it is not only the risk of early and severe forms of preeclampsia but also the risk of late or mild forms of preeclampsia is increased, which are associated with increased perinatal morbidity and mortality (Bodnar *et al.*, 2007). Preeclampsia has been reported to occur mostly in women with high prepregnancy BMI Several studies have demonstrated the association between preeclampsia risk and obesity in varying populations across the globe (Mahomed *et al.*, 1998; Hauger *et al.*, 2008). Fortner et al., (2009) proposed that extreme maternal weight gain is associated with increased risk of preeclampsia, though confounding factor like an increase in fluid retention may be implicated (Fortner *et al.*, 2009). Aside BMI several anthropometric indices such as ponderal index (PI), thigh circumference/head circumference ratio (THR), waist-to-hip

ratio (WHR), waist-to-height ratio, waist circumference, hip circumference, mid-arm circumference/head circumference (MAC/OFC), weight/head circumference (W/OFC), and weight/length (W/L) have been used to assess body fat composition in hypertensive disorders of pregnancy (Olinto *et al.*, 2004; Snijder *et al.*, 2004; Ahenkorah, 2009). It has also been observed that women in whom GH and PE develop, enter into pregnancy either overweight or obese and also demonstrate, during pregnancy, some risk factors characterizing atherosclerosis, such as metabolic syndrome (Owiredu, 2008a; Ahenkorah, 2009) and endothelial dysfunction (Wolf *et al.*, 2001). Duckitt and Harrington, (2005) have also reported that increasing BMI was associated with 1.7-3 folds increased odds of developing gestational hypertension (Duckitt and Harrington, 2005). Increased WHR have been established as a significant predictor of preeclampsia, irrespective of overall adiposity (Yamamoto *et al.*, 2001). Waist circumference (WC) and hip circumference (HC) are also associated with obesity-related adverse pregnancy outcome in hypertensive disorders (Wendland *et al.*, 2007). Waist-to-height ratio (WHtR) is another anthropometric index of abdominal adiposity marker; it is a better predictor of metabolic and cardiovascular risk than BMI, WC and WHR (Lu *et al.*, 2011).

2.9 TREATMENT AND PREVENTION OF GESTATIONAL HYPERTENSION AND PREECLAMPSIA

Preeclampsia is one of the leading and controversial form of hypertensive disorders of pregnancy account for 5-7% of all pregnancies. Due to the unknown etiology coupled with the multisystemic effects of preeclampsia, developing an appropriate medicinal intervention has been the dilemma of most researchers and clinicians across the globe. Till date the only cure for this condition is by delivering of the placental tissue because it has been observed that if parts of the placenta are retained after delivery clinical symptoms can persist (Redman and Sargent, 2007). Despite the dilemma in developing an appropriate medicinal intervention several

approaches to managing gestational hypertension and preeclampsia have been made.

Treatment of hypertensive symptoms with antihypertensive drugs such as hydralazine, nefidipine and aldomet tablet have been shown to promote vasodilation but no positive effects on mother and neonate outcome have been documented so far (Wood and Sibai, 1996). Sibai and colleagues observed in randomised controlled trial that treatment of hypertension with glucocorticoids enhance fetal lung maturation and thereby reduce fetal mortality and morbidity. However, the therapeutic nature of this drug have showed no positive effect in the management of preeclampsia (Sibai *et al.*, 1994). Administering aspirin as a prophylactic has been shown to reduce the development of preeclampsia by 19%, however the exact groups of pregnant women to which this therapy is beneficial is unknown (Knight *et al.*, 2000). The use of magnesium sulphate (MgSO4) in preeclamptic women reduce the maternal mortality and the risk of eclampsia by 50% in a randomised control trial (Altman *et al.*, 2002). However, MgSO4 treatment tends to only prevent seizure but does not have a positive effect on the associated complication of seizure.

Antioxidant supplementation of using vitamins E and C have been shown to reduce the rate of preeclampsia in a high-risk population (Chappell *et al.*, 2000). Other study showed that consumption of vitamin E and C by pregnant women increases the enzymatic activity of antioxidant and reduce the development of preeclampsia (Maynard *et al.*, 2003). However, this effect was not enough to improve neonatal birth weight as vitamin-treated group had increased rate (28%) of low birth weight than the non-vitamin treated group (24%) in a randomized, multi-center trial (Poston *et al.*, 2006). From their study the vitamin-treated groups were at increased risk of developing gestational hypertension and that resulted in an increased need for antihypertension treatment. Administering the antioxidants to gestational hypertensive and preeclamptic pregnant women in a randomised trial was too late to chelate the oxidative stress posed by the condition. It was

therefore postulated that earlier antioxidant prophylactic intervention might have a positive effect and thus throw more light on the need to evaluate oxidative stress biomarkers in both maternal and fetal outcome (Poston *et al.*, 2006). Supplementation of using calcium have also shown in previous studies to be effective against preeclampsia especially in developing countries where nutrition is inadequate (Villar *et al.*, 2006a).

In a recent study by Ephraim *et al.,* (2014) observed that preeclamptic and pregnancy induced hypertensive women were hypocalcaemic and suggested that nutritional supplementation of calcium may be effective against the condition. An observational studies have also proposed a potential role for omega-3-fatty acids in decreasing the incidence of preeclampsia. However is therapeutic effect though have been elucidated data is not enough (Jensen, 2006). Preeclamptic women have been shown to be associated with elevated serum levels of sFlt-1 and thus inhibiting its actions could provide a therapeutic strategy against this condition. A study by Maynard *et al.,* (2003) suggested that that exogenous therapy of using proangiogenic molecules (VEGF and or PLGF) could restore the endothelium integrity of preeclamptic patients (Maynard *et al.*, 2003).

Nicotine in smoking have also shown to stimulate angiogenesis by lowering the pathogenic effect of sFlt-1 and thus exposure to small amount of smoke in preeclamptic pregnancy may be an effective treatment (Heeschen *et al.*, 2001). However, preeclamptic women who are exposed to smoke are at increased risk of delivering of small for gestational age infant and babies with IUGR.

Chapter 3

MATERIALS AND METHODS

3.1 STUDY DESIGN/SETTING

This hospital-based prospective cohort study was conducted from April to December, 2014 at the Obstetrics and Gynaecology (O & G) department of the Komfo Anokye Teaching Hospital (KATH) in the Ashanti Region of Ghana, which has an average population of 4,780,380 (Ghana Statistical Service, 2012). Komfo Anokye Teaching Hospital is the second largest tertiary hospital in Ghana with a thousand bed capacity and is a major referral centre that provides health services to five regions namely Ashanti, Brong-Ahafo, Northern, Upper East and Upper West of Ghana. The hospital also receives referrals from other regions such as Central, Eastern, Western and some parts of the Volta region of Ghana and this gives fair representation of the Ghanaians population.

3.2 SELECTION OF STUDY PARTICIPANTS

Using a purposive sampling technique, a total of two hundred and thirty five (235) patients comprising 100 preeclamptics (PE), 70 gestational hypertensive (GH) and 65 normal pregnant controls under clinical management at the Obstetrics and Gynaecology Department of the Komfo Anokye Teaching Hospital in the Ashanti Region of Ghana were recruited for this study. One hundred (100) pregnant women with pregnancy–Induced hypertension (50 GH and 50 PE) and 50 aged-matched normotensive pregnant women consented and were finally enrolled. Follow-up was done on these study participants (150) during their course of pregnancy and one hundred and twenty (120) participants (40 controls, 40 GH and 40 PE) showed up and were recruited 48 hours postpartum. The diagnosis of pregnancy induced hypertension during pregnancy was done by qualified

consultant Obstetrician/Gynaecologist using the National High Blood Pressure Education Program Working Group diagnostic criteria (this is known as the American College of Obstetricians and Gynecologists definition) (ACOG, 2000). Structured closed ended questionnaire was given to each participant and based on interview, information on sociodemographic data; maternal lifestyle factors such as smoking and alcohol consumption before and during pregnancy, a complete present and past obstetric outcome of ectopic pregnancies, livebirths, stillbirths, induced abortions and spontaneous abortion were obtained. Recent medical history, antimalarial therapy, antioxidant drug use, contraceptive use, occupational factors, and other relevant information were also obtained. All the information obtained from each subject was assessed through record reviews of hospital database with a 100% rate of accuracy. All biochemical analyses were performed without knowledge of subject's clinical status by means of code numbering.

3.2.1 Inclusion Criteria

Pregnant women both nulliparous and multiparous women aged 18 - 40 years, within the gestational age of ≥ 20 - 40 weeks, singleton pregnant women, those with or without proteinuria but hypertensive were included as study participants. Age-matched apparently healthy pregnant women met the following criteria; pregnancy with normal blood pressure (<140/90 mmHg), absence of proteinuria, without medical and obstetrical complications certified by a consultant Obstetrician/Gynaecologist.

3.2.2 Exclusion Criteria

Pregnant women who are unable to give informed consent (learning disability, mental illness, not Fraser competent) were excluded from the study. Subjects with previous chronic hypertension, sexually transmitted infections, sickle cell anemia, diabetes mellitus, renal disease, gestational diabetes, cardiovascular disorder, malaria and use of antihypertensive medication before the recruitment were excluded from the study.

3.2.3 Sample Size Justification

The estimated minimum sample size for this prospective cohort study was calculated to be 39.9 for each case and control using the formula as described by Charan and Biswas, (2013)

$$n = (\frac{r+1}{r}) \frac{(\bar{p})(1-\bar{p})(Z_{\beta} + Z_{\alpha/2})^2}{(p_1 - p_2)^2}$$

n=sample size. r=the ratio of case to control which was chosen as 1 based on the expected equal number of cases and control. P is the average proportion exposed is equal to the proportion of exposed case + proportion of control exposed/2.

$$p_{case\ \exp} = \frac{ORp_{controls\ \exp}}{p_{controls\ \exp}(OR-1)+1}$$

OR=odd ratio which was calculated as 0.035 based on previous study by Ahenkorah, (2009). Proportion of control exposed =20% and the proportion of case exposed =0.864%. p= 1.04

Z_{β} is the standard normal variate for power. For the purpose of this study 80% was used=$Z_{0.20}$ =0.842 (from the Z statistical table). Zα/2 is the standard normal variate for level of significance at type I error of 5%=$Z_{0.05/2}$=$Z_{0.025}$=1.96 (from the Z statistical table). P1-P2 is the effect size = differences in proportion exposed=. P1 is the proportion in cases and p2 is the proportion in controls

$$n = \frac{(1+1)}{1}\left[\frac{(0.104\ (1-0.104)\ (0.842-1.96)^2}{(0.00864-0.2)^2}\right]$$

n=39.9. Approximately 40 samples in each group were expected.

In order to achieve a stronger statistical power, a larger sample size was used. Fifty (50) participants in each group were considered for this study. Two different case

groups (PE and GH) and one control group were used. In totality this study recruited 150 study participants at baseline (50 controls +50 GH+ 50 PE).

3.2.4 Ethical consideration

Ethical approval for this study was granted by the Committee on Human Research, Publications and Ethics (CHRPE) (CHRPE/AP/365/14), School of Medical Sciences, Kwame Nkrumah University of Science & Technology (KNUST) and the Research and Development Unit of the KATH. Written informed consent in a form of signature or fingerprint was obtained from all the participants prior to enrolment. It was clearly stated that participants were free to withdraw from the study at any time.

3.3 BLOOD PRESSURE

Trained personnel used a mercury sphygmomanometer (Accoson, England) and a stethoscope (3M™ Littmann® Stethoscopes, USA) to measure the blood pressure of participants in accordance with recommendations of the American Heart Association (Kirkendall *et al.*, 1967). The procedure was repeated for each patient between 5-10 minutes. Mean values of duplicate measurements were recorded as the blood pressure to the nearest 2.0 mmHg. Baseline and postpartum BP of each patient was reported.

3.4 ANTHROPOMETRIC MEASUREMENTS

3.4.1 Weight and Height

Patients were made to stand without their sandals, bags or anything of significant weight on the weighing scale (Seca, Hamburg, Deutschland) and against the stadiometer (Seca, Hamburg, Deutschland). The weight was read to the nearest 0.1 kilogram and recorded. The value for the height was recorded to the nearest 0.1 centimeter and then converted to meters. The body mass index (BMI) was calculated using formula (weight/height squared) and expressed in kg/m^2.

Baseline and post-partum weight of each patient was reported. BMI were classified based on WHO definition for adults as underweight (<18.5kg/m2), normal (18.5-24.9kg/m2), overweight (25-29.9kg/m2) and obese (>30kg/m2) (WHO, 1995).

3.4.2 Waist Circumference and Hip Circumference

Gulick II spring-loaded measuring tape (Gay Mills, WI) was used to measure waist circumference midway between the inferior angle of the ribs and the suprailiac crest, whereas hip circumference was measured at the outermost points of the greater trochanters (WHO, 1995). Waist to hip (WHR) and waist to height (WHtR) were recorded to the nearest 2 decimal places. WHR and WHtR were measured during first phase of sample collection.

3.5 SAMPLE COLLECTION AND PROCESSING

3.5.1 Urine sampling and estimation of proteinuria

Ten to twenty (10 -20) milliliters (ml) freshly voided early morning urine in clean, wide mouth and leak proof containers. Samples were collected during pregnancy and 48hrs post-partum. Semi-quantitative proteinuria was immediately assessed using dipstick (URIT 2VPG Medical electronic Co., Ltd. Jiuhua Road, Guilin, Guangxi 541001, PR China). Proteinuria in preeclamptics was defined as the presence of urinary protein in concentrations more than 2+ (>0.3g/L) on urine dipstick.

3.5.1.1 Principle and procedure

The test area of the reagent strip is impregnated with an indicator (tetrabromophenol blue) buffered to pH 3. At this pH it is yellow in the absence of albumin. Protein forms a complex with the dye, stabilizing it in the blue form; it is

green or bluish-green if albumin is present. A fresh strip was used per sample and was dipped into early morning urine collected in clean dry plastic containers. The strip was inserted up to the test area, for not more than two seconds. The edge of the strip was drawn along the brim of the vessel to remove excess urine making sure the test area does not touch the vessel. The strip was turned on its side and tapped on an absorbent paper to remove any remaining urine since excessive urine on the strip may cause interaction of chemicals between adjacent pads leading to incorrect results. The test result on the strip held horizontally and compared with the colour chart on the vial label under good light. Result was read within 60 seconds.

3.5.2 Blood sampling and processing

Six (6) milliliters (ml) venous blood sample was collected from each patient at mid-gestation and post-partum of which 4 ml was dispensed into serum gel separator tube (10 ml MICROPOINT clot activator tube; batch number: GDO4OSGC) and the remaining 2ml into dipotassium ethylene diamine tetra acetic acid (K_2EDTA) tube. Serum was separated from blood after centrifugation (HERMLE Z300K, Labsource, Inc. Romeoville, IL 60446) at 3500 revolution per minute (rpm) for five (5) minutes and stored at - 80°C (Thermo Scientific™ Revco™ UxF −Ultra-Low Temperature Freezers, USA) until assayed. Biomarkers including VEGF-R1, PLGF, and 8-epi-PGF-2 alpha were measured using commercially prepared enzyme-linked immunosorbent assay kit (ELISA) (R&D Systems, Minneapolis, MN, USA) in accordance with the supplier's protocols. Total antioxidant capacity (T-AOC) reagents was obtained from Green stone Swiss Co., Ltd, China and serum levels were estimated spectrophotometrically (Mindray BA-88A; Shenzhen Bio-medical electronics Co., Ltd, China)

3.6 ANALYSIS OF BIOMARKERS

3.6.1 Human vascular endothelium growth factor receptor-1 (VEGFR-1/sFlt-1)

3.6.1.1 Assay principles and procedure

Human VEGFR-1 (sFlt-1) levels in the sample were added to a pre-coated purified human VEGFR1 antibody microliter plate wells. An addition of VEGFR-1 then forms solid-phase antibody. A combination of VEGFR-1 antibody with horseradish peroxidase (HRP) label forms antibody-antigen-enzyme-antibody complex. After washing completely TMB substrate solution was added, which produces a blue color at HRP enzyme-catalyzed? The reaction was terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of Human VEGFR1 in the sample is then determined by comparing the optical density (O.D.) of the samples to the standard curve.

Microtiter wells were secured in the holder, original density standards was diluted as follows:

Table 3. 1 Serial dilution protocol for sFlt-1 assay

Concentrations	Dilutions
Standard 5 (100 pg/ml)	150 µl original density standard + 150 µl standard diluent
standard 4 (50 pg/ml)	150 µl standard 5 + 150 µl standard diluent
standard 3 (25 pg/ml)	150 µl standard 4 + 150 µl standard diluent
standard 2 (12.5 pg/ml)	150 µl standard 3 + 150 µl standard diluent
standard 1 (6.25 pg/ml)	150 µl standard 1 + 150 µl standard diluent

Standard 5= original standard concentration

A Blank well was set separately and 40µl sample diluent was added to test sample wells followed by 10µl of test samples. Without touching the walls of the wells,

samples were then added and gently mixed. After covering plate with closure plate membrane, samples were incubated for 30 minutes at 37^0C. Wells were then washed repeatedly for 5 times and the microtiter well was stroke sharply on an absorbent paper to remove residual water. A 50µl Horseradish peroxidase (HRP)-conjugate reagent was added to each well with exception of blank. Samples were incubated and washed followed by addition of 50 µl each of chromogen A and B solution for 10minute at 37^0C. The reaction was stopped after adding 50µl of the stop solution to each well to change the blue colour to yellow. Absorbances were read at 450nm within 15 minute after adding stop solution using microplate ELISA reader (Mindray MR-96A; Shenzhen Mindray Bio-medical electronics Co., Ltd, China).

3.6.2 Human Placental growth factor (PLGF)

3.6.2.1 Assay principles and procedure

Human PLGF levels in the sample were added to a pre-coated purified human PLGF antibody microliter plate wells. An addition of PLGF then forms solid-phase antibody. A combination of PLGF antibody with HRP labeled forms antibody-antigen-enzyme-antibody complex. After washing completely TMB substrate solution was added, which produce a blue color at HRP enzyme-catalyzed. The reaction is terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450nm. The concentration of Human PLGF in the sample was then determined by comparing the O.D. of the samples to the standard curve.

Microtiter wells were secured in the holder, original density standards was diluted as follows:

Table 3. 2 Serial dilution protocol for PLGF assay

Concentrations	Dilutions
Standard 5 (120 pg/ml)	150 µl original density standard + 150 µl standard diluent
standard 4 (60 pg/ml)	150 µl standard 5 + 150 µl standard diluent
standard 3 (30 pg/ml)	150 µl standard 4 + 150 µl standard diluent
standard 2 (15 pg/ml)	150 µl standard 3 + 150 µl standard diluent
standard 1 (7.5 pg/ml)	150 µl standard 1 + 150 µl standard diluent

Standard 5= original standard

A Blank well was set separately and 40µl sample diluent was added to test sample well followed by 10µl of test samples. Without touching the walls of the wells, samples were then added and gently mixed. After covering plate with closure plate membrane, samples were incubated for 30 minutes at 37^0C. Wells were then washed repeatedly for 5 times and the microtiter well was stroke sharply on an absorbent paper to remove residual water. A 50µl HRP-conjugate reagent was added to each well with exception of blank. Samples were incubated and washed followed by addition of 50 µl each of chromogen A and B solution for 10minute at 37^0C. The reaction was stopped after adding 50µl of stop solution to each well to change the blue colour to yellow. Absorbances were read at 450nm within 15 minute after adding stop solution using microplate ELISA reader (Mindray MR-96A; Shenzhen Mindray Bio-medical electronics Co., Ltd, China).

3.6.3 Human 8-epi-PGF2alpha (F2-Isoprostane)

.6.3.1 Assay principles and procedure

Human 8-epi-PGF2alpha levels in the sample were added to a pre-coated purified human 8-epi-PGF2alpha antibody microliter plate wells. An addition of PLGF then

forms solid-phase antibody. A combination of 8-epi-PGF2alpha antibody with horseradish peroxidase (HRP) labeled forms antibody-antigen-enzyme-antibody complex. After washing completely TMB substrate solution was added, which produce a blue color at HRP enzyme-catalyzed. Reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450nm. The concentration of Human 8-epi-PGF2alpha in the sample was then determined by comparing the optical density of the samples to the standard curve.

Microtiter wells were secured in the holder, original density standards was diluted as follows:

Table 3. 3 Serial dilution protocol for 8-epi-PGF2alpha

Concentrations	Dilutions
Standard 5 (800 pg/ml)	150 µl original density standard + 150 µl standard diluent
standard 4 (400 pg/ml)	150 µl standard 5 + 150 µl standard diluent
standard 3 (200 pg/ml)	150 µl standard 4 + 150 µl standard diluent
standard 2 (100 pg/ml)	150 µl standard 3 + 150 µl standard diluent
standard 1 (50 pg/ml)	150 µl standard 1 + 150 µl standard diluent

Standard 5= original standard

A Blank well was set separately and 40µl sample diluent was added to test sample wells followed by 10µl of test samples. Without touching the walls of the wells samples were then added and gently mixed. After covering plate with closure plate membrane, samples were incubated for 30 minutes at 37^0C. Wells were then washed repeatedly for 5 times and the microtiter well was stroke sharply on an absorbent paper to remove residual water. A 50µl HRP-conjugate reagent was added to each well with exception of blank. Samples were incubated and washed followed by addition of 50 µl each of chromogen A and B solution for 10minute at 37^0C. The reaction was stop after adding 50µl of stop solution to each well to

change the blue colour to yellow. Absorbances were read at 450nm within 15 minute after adding stop solution using microplate ELISA reader (Mindray MR-96A; Shenzhen Mindray Bio-medical electronics Co., Ltd, China).

3.6.4 Total Antioxidant Capacity (T-AOC) Assay

3.6.4.1 Assay principles and procedure

This assay kit is a simple, automated test which estimates the ferric reducing ability of plasma (FRAP). The measurement of the ferric reducing ability of plasma (FRAP) was done by the assay based on the method of Benzie and Strain, (1999). The FRAP assay, is presented as a novel method for assessing "antioxidant power like total antioxidant levels. Under acidic conditions, the ability of reducing Fe^{3+} - tripyridyl triazine (Fe^{3+}-TPTZ) to produce a blue Fe^{2+}-TPTZ reflects the total antioxidant capacity. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. The FRAP assay is inexpensive (cheap), sensitive, reagents are simple to prepare, it requires low blood volume, results are highly reproducible, and the procedure is straightforward and it is not time consuming (speedy). The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects.

$$\text{Fe}^{3+}\text{---TPTZ} \xrightarrow{\text{Acetic acid}} \text{Fe}^{2+}\text{--TPTZ blue}$$

Procedure

Reagents were pre-diluted in a ratio of 10:1:1 by acetate buffer (pH 3.6) with 2, 4, 6-tripyridyl-striazine (TPTZ) solution and $FeCl_3$ solution respectively after 37℃ pre-warmed. Sample and reagents were pipetted into test tubes as follows:

Table 3. 4 Pipetting protocol for estimation of T-AOC

	Control tube	**Measuring tube**
Mixture	**900 µl**	**900 µl**
Sample		**30 µl**
Double distilled water	**120 µl**	**90 µl**

After uniform mixture and incubation at 37℃ temperature for 10 minutes the spectrophotometer was zero with double distilled water. The intensity of the blue colour formed was measured at 593nm using semi-automated analyser (Mindray BA-88A; Shenzhen Bio-medical electronics Co., Ltd, China). The control tube was read once followed by the sample tubes. T-AOC was obtained by the change in absorbance which corresponds to a standard curve at 593nm according to the regression equation (y = 0.6308x + 0.1291) obtained in the standard solution ion concentration. T-AOC was expressed as mmol/l.

3.7 ANTI-HYPERTENSION TREATMENT PROTOCOL

Preeclamptics and gestational hypertensive patients on admission were given prescribed antihypertensive drugs by the specialised nurse. Before treatment procedure blood samples were collected from each participant (PE and GH). Participants with severe preeclampsia received magnesium sulphate (MgSO4) treatments (14g of 50% MgSO4 as start loading dose); thus 10g intramuscular and then 4g intravenous (IV) (prepared as 8mls of MgSO4 +12mls of aqua). Five (5)

grams every 4hr in alternate buttocks was given over a period of 24 hours. Subjects with high diastolic BP (≥110mmHg) were treated with 10mg hydralazine IV start and then 5mg over a period of 30 minutes. Patients with mild PE and GH were given either nifecard xl tablet 30mg with aldomet tablet 500mg combination or only nifecard xl.

3.8 DEFINITION AND CLASSIFICATION OF CLINICAL AND OBSTETRIC TERMS

Participants classified as preeclamptic met one or more of the following diagnostic criteria: systolic blood pressure ≥140 mmHg or a diastolic pressure of ≥90 mmHg taken on two occasions at least six hours apart and proteinuria of ≥ 0.3 g/l or 2+ based on semiquantitative urine analysis (ACOG Committee on Practice Bulletins–Obstetrics, 2002). Patients with GH met the following criteria: systolic pressure of ≥140 mmHg and a diastolic pressure of ≥90 mmHg without proteinuria after 20 weeks of gestation with resolution to baseline by 12 weeks postpartum. Normal pregnant women met the following criteria: pregnancy with normal blood pressure (<140/90 mmHg), absence of proteinuria, without medical and obstetrical complications (Forest *et al.*, 2005). Severe preeclampsia was defined as a systolic blood pressure (SBP) ≥160 mmHg or a diastolic blood pressure (DBP) ≥110 mmHg on 2 occasions recorded 6-h apart in association with proteinuria (≥ +3) (Ohkuchi *et al.*, 2007). Preeclamptic women with early onset preeclampsia was defined by appearance of both hypertension and proteinuria before 34 weeks of gestation while late onset preeclampsia were those diagnosed after 34 weeks (Ohkuchi *et al.*, 2007). We defined intrauterine growth restriction (IUGR) infant as one having a birth weight below the 5th percentile accompanied by abnormal umbilical artery Doppler examination, defined as absence or reverse of end-diastolic velocity (Drewlo *et al.*, 2010). Preterm delivery was defined delivery before 37 completed

weeks of gestation (Lawn *et al.*, 2010). Stillbirth was defined based on International Classification of Diseases, 10th revision (ICD-10) as "death prior to the complete expulsion or extraction from its mother of a product of conception, irrespective of the duration of pregnancy (World Health Organization, 2004). IUFD was defined as the fetal death (where there is no sign of life) at equal or more than 20 weeks of gestation and /or birth weight of equal or more than 500 gram (Jahan *et al.*, 2005). An Abruptio placenta was defined as premature separation of placenta from uterine wall after 20 weeks of gestation and prior to birth (Oyelese and Ananth, 2006). Placenta previa was defined as implantation of the placenta over or near the internal orifices of the cervix after 20 weeks gestation prior to transvaginal or abdominal ultrasonography (Oyelese and Smulian, 2006). Premature prerupture of membrane (PPROM) was defined as defined as rupture of the membrane of the amniotic sac and chorion occurring before 37 weeks gestation (Caughey *et al.*, 2008).

3.9 STATISTICAL ANALYSIS

Data were entered into Excel for window 2007. Shapiro-Wilk normality test was used to test the data for a normal distribution. After exploring the entire data, angiogenic factors and oxidative stress biomarkers did not assume a normal distribution curve and thus non-parametric statistical methods were used. The Mann Whitney U test was used to compare the two groups while Kruskal Wallis one-way analysis of variance (ANOVA) followed by Dunnet test was used to compare more than two groups. Two by two categorical variables were compared using Fischer's exact test while chi-squared test was used for the comparison of three by two categorical variables. For data that assumed Gaussian distribution comparism between two groups was performed using un-paired t-test while more than two groups were performed using one-way ANOVA. Data were reported as mean ± standard deviation (SD) for continuous data, as median (interquartile

range) for non-parametric continuous data and as a frequency (percentage) for categorical data. The Wilcoxon rank-sum test was also applied to compare levels of soluble markers within each group in order to evaluate any changes between the various gestational ages. Statistical analysis was performed using Graphpad Prism® version 5.0 (Graph Pad Software Inc., Los Angeles) for windows.

Spearman correlation was employed to test the association between angiogenic factors and oxidative biomarkers while partial correlation was used to assess the correlational effect after adjusting for maternal age, BMI, gestational age and parity. To assess the clinical utility of individual factor assay (PLGF, sFlt-1, and 8-epi-PGF2α) and the ratio of sFlt-1/PLGF and PIGF/sFlt-1 in the prediction of preeclampsia, we used receiver operating characteristics (ROC) curve analysis to assess the optimal cutoff value of each factor. The optimal cutoffs of the analytes sFlt-1, PLGF, 8-epi-PGF2α, the ratio of PIGF/sFlt-1 and sFlt-1/PLGF ratio were set at the best accuracy (area under the curve) value based on the ROC curve analysis. The sensitivity, specificity, positive and negative predictive values, and accuracy for the optimal cutoff value were recorded. The odds ratio (OR) with a 95% confidence interval (CI) was calculated to consider the predictive competence of the selected cutoff values. Statistical analysis was performed with the Statistical Package for Social Sciences 20.0 (IBM SPSS Inc., Chicago, IL, USA). Statistical significance was accepted at $p<0.05$ for all comparisons

Chapter 4

RESULTS

4.1 SOCIODEMOGRAPHIC CHARACTERISTICS

Table 4.1 shows the sociodemographic features of the study population. Mean age of general study participant was 29.78 ± 0.40 years. There was no statistically significant difference in mean age across the study groups ($p>0.05$). Eighty three percent (83.3%) of the pregnant women were married. The percentage of married participants with PE and GH was significantly lower compared to the normal pregnant women ($p<0.05$). Forty nine (49.3%) percent of the pregnant women had completed Junior high school (JHS) whereas 24.7%, 18.6%, and 6.0% had completed Senior High School, Tertiary, and Primary education respectively. Seventy percent (70.0%) of the JHS leavers were more likely to be associated with PE ($p<0.05$). Pregnant women from the Akans ethnic group reported the highest percentage (75.3%), followed by Mole-Dagbani (18.6%), Ga-Adangbe (4.0%) and Ewe (2.0%). Ninety eight (98) of the study participants representing 65.3% were self-employed (65.3%) while 22.0% and 12.0% of them were government and unemployed respectively. Higher proportion of self-employed study participants were associated with GH and PE ($p<0.05$). Highest proportion (80.6%) of the participants had low economic income (<500.00 GHS). Low economic income earning pregnant women were significantly associated with PE and GH ($p<0.05$) **(Table 4.1).**

Table 4. 1 Sociodemographic characteristics of study Participants

Variables	Total (n=150)	NP (n=50)	GH(n=50)	PE(n=50)
Age (years)	29.78 ± 0.40	30.79 ± 0.69	30.49 ± 0.76	28.85 ± 0.61
Marital Status				
Single	25 (16.7%)	3(6.0%)	12(24.0%)*	10(20.0%)*
Married	125 (83.3%)	47 (94.0%)	38(76.0%)	40 (80.0%)
Highest level of education				
No education	5(3.3%)	0(0.0%)	2(4.0%)*	3(6.0%)*
basic	9(6.0%)	3 (6.0%)	5(10.0%)	1(2.0%)
JHS	74(49.3%)	15 (30.0%)	24(48.0%)	35(70.0%)
SHS	37(24.7%)	12 (24.0%)	19(38.0%)	6(12.0%)
Tertiary	25(16.7%)	20 (40.0%)	0(0.0%)	5(10.0%)
Ethnicity				
Akan	113(75.3%)	43(86.0%)	38(76.0%)	32(64.0%)
Ga-Adangbe	6(4.0%)	0(0.0%)	2(4.0%)	4(8.0%)
Ewe	3(2.0%)	1(2.0%)	1(2.0%)	1(2.0%)
Mole-Dagbani	28(18.7%)	6(12.0%)	9(18.0%)	13(26.0%)
Occupation				
Unemployed	19(12.7%)	1(2.0%)	7(14.0%)*	11(22.0%)*
Self-employed	98(65.3%)	25(50.0%)	38(76.0%)	35(70.0%)
Gov't employed	33(22.0%)	24(48.0%)	5(10.0%)	4(8.0%)
Economic income (GHS)				
<500	121(80.7%)	28(56.0%)	47(94.0%)*	46(92.0%)*
500-1000	23(15.3%)	16(32.0%)	3(6.0%)	4(8.0%)
>1000	6(4.0%)	6(12.0%)	0 (0.0%)	(0.0%)

Values are presented as frequency (proportion) or Mean ± SD. Chi-square or Fischer exact test was used to compare between each groups (GH and PE) to NP group. *p<0.05 is considered statistically significance difference. NP: Normal pregnant control; GH: Gestational hypertension; PE: Preeclampsia; GHS: Ghana cedis; JHS: Junior high school; SHS: Senior high school

4.2 OBSTETRIC CHARACTERISTICS

Obstetric characteristics of the studied participants are shown in **Table 4.2.** Highest percentage (42.7%) of the participants was nulliparous while 35.3% and 22.0% were multiparous and primiparous respectively. Most of the participants were multigravida and higher percentage of them were GH patients compared to PE (p=0.0112). The proportion of abortion, family history of hypertension and previous caesarean section were higher in the PE compared to the controls ($p<0.05$) **(Table 4.2.).**

Table 4. 2 Obstetric characteristics of studied participants

Variables	Total	NP (n=50)	GH (n=50)	PE(n=50)
GA at onset of sampling	24.1 ± 5.47	24.1 ± 4.17	23.81 ± 4.37	24.39 ± 2.55
GA at delivery	36.12 ± 0.71	35.85 ± 0.37	36.91 ± 0.40	35.62 ± 0.28
Parity				
nulliparous	64 (42.7%)	20(40.0%)	19(38.0%)	25(50.0%)
primiparous	33 (22.0%)	12(24.0%)	11(22.0%)	10(20.0%)
multiparous	53 (35.3%)	18(36.0%)	20(40.0%)	15(30.0%)
Gravidity				
primigravida	44 (29.3%)	12(24.0%)	14(28.0%)	18(36.0%)
Secundigravida	47 (31.3%)	17(34.0%)	10(20.0%)	20(40.0%)
multigravida	59 (39.3%)	21(42.0%)	26(52.0%)	12(24.0%)
Family history of HTN				
Yes	22 (14.7%)	1(2.0%)	4(8.0%)	17(34.0%)*
History of Abortion				
Yes	75 (50.0%)	21(42.0%)	22(44.0%)	32 (64.0%)*
Previous Caesarean section				
Yes	39 (26.0%)	6(12.0%)	9(18.0%)	24(48.0%)*
Contraceptive usage				
Yes	31 (20.7%)	15 (30.0%)	8 (16.0%)	8 (16.0%)
Alcohol Intake				
before pregnancy	10 (6.7%)	1(2.0%)	3(6.0%)	6(12.0%)
during pregnancy	2 (1.3%)	0(0.0%)	1(2.0%)	1(2.0%)
First ANT visit (weeks)	4.07 ± 0.57	4.07 ± 0.53	3.43 ± 0.55	4.90 ± 0.51
Urinary protein (g/l)	0.74 ± 0.07	0.00 ± 0.00	0.150 ± 0.001*	2.05 ± 0.11*

Values are presented as frequency (proportion) and Mean ± SD. Chi-square for trend was used to compare between each groups (GH and PE) to NP group. *p<0.05 is considered statistically significance difference. NP: Normal pregnant control; GH: Gestational hypertension; PE: Preeclampsia. HTN: Hypertension; ANT: Antenatal; GA: Gestational age

4.3 ANTHROPOMETRIC MEASUREMENTS

Table 4.3 shows the anthropometric measurements of study participants. Preeclamptics and gestational hypertensive subjects had a significantly increased pregestation body weight, pregestation BMI, body weight at sampling, BMI at sampling, % body weight gain, WC and WHtR compared to normal pregnant controls ($p<0.05$). A significantly higher mean HC were associated with GH pregnant women compared to control ($p=0.0062$). However, there was no statistically significant difference in the mean WHR among the three studied groups ($p>0.05$) **(Table 4.3).**

Table 4. 3 Anthropometric measurements of studied participants

Anthropometrics	NP (n=50)	‡p-value	GH (n=50)	♀p-value	PE (n=50)	φp-value
Body Height (m)	1.61 ± 0.01	0.0073	1.57 ± 0.01	0.4242	1.57 ± 0.01	0.0001
pregestation body weight (Kg)	66.69 ± 4.49	0.0024	74.11 ± 7.83	0.1996	75.22 ± 9.34	0.0362
Pregestation BMI (Kg/m2)	25.91 ± 6.58	< 0.0001	29.97 ± 4.70	0.2621	29.03 ± 2.49	0.0001
Body Weight at sampling (Kg)	74.56 ± 4.49	0.0009	83.26 ± 3.03	0.7033	84.16 ± 4.36	< 0.0001
BMI at sampling (Kg/m2)	28.96 ± 8.58	< 0.0001	33.72 ± 5.84	0.5075	34.33 ± 3.49	< 0.0001
% body Weight gain at sampling	3.05 ± 0.14	0.0375	3.75 ± 0.29	0.0002	5.29 ± 0.25	< 0.0001
WC (cm)	96.31 ± 2.23	< 0.0001	106.30 ± 1.69	0.0003	103.80 ± 2.27	< 0.0001
HC (cm)	104.80 ± 2.63	0.0062	113.30 ± 1.85	0.5597	110.20 ± 2.27	0.1597
WHR	0.89 ± 0.03	0.0545	0.94 ± 0.01	1.0000	0.94 ± 0.01	0.1089
WHtR	0.59 ± 0.02	0.0005	0.67 ± 0.01	0.2037	0.65 ± 0.01	0.0223

Values are presented as Mean ± SD. One way ANOVA followed by Turkey Post Hoc multiple comparisons was used to compare between groups. ‡p-value (NP vs GH); ♀p-value (GH vs PE); φp-value (NP vs PE). p<0.05 shows statistically significant difference. WHR: waist to hip ratio; WHtR: Waist to height ratio; WC: waist circumference; HC: hip circumference

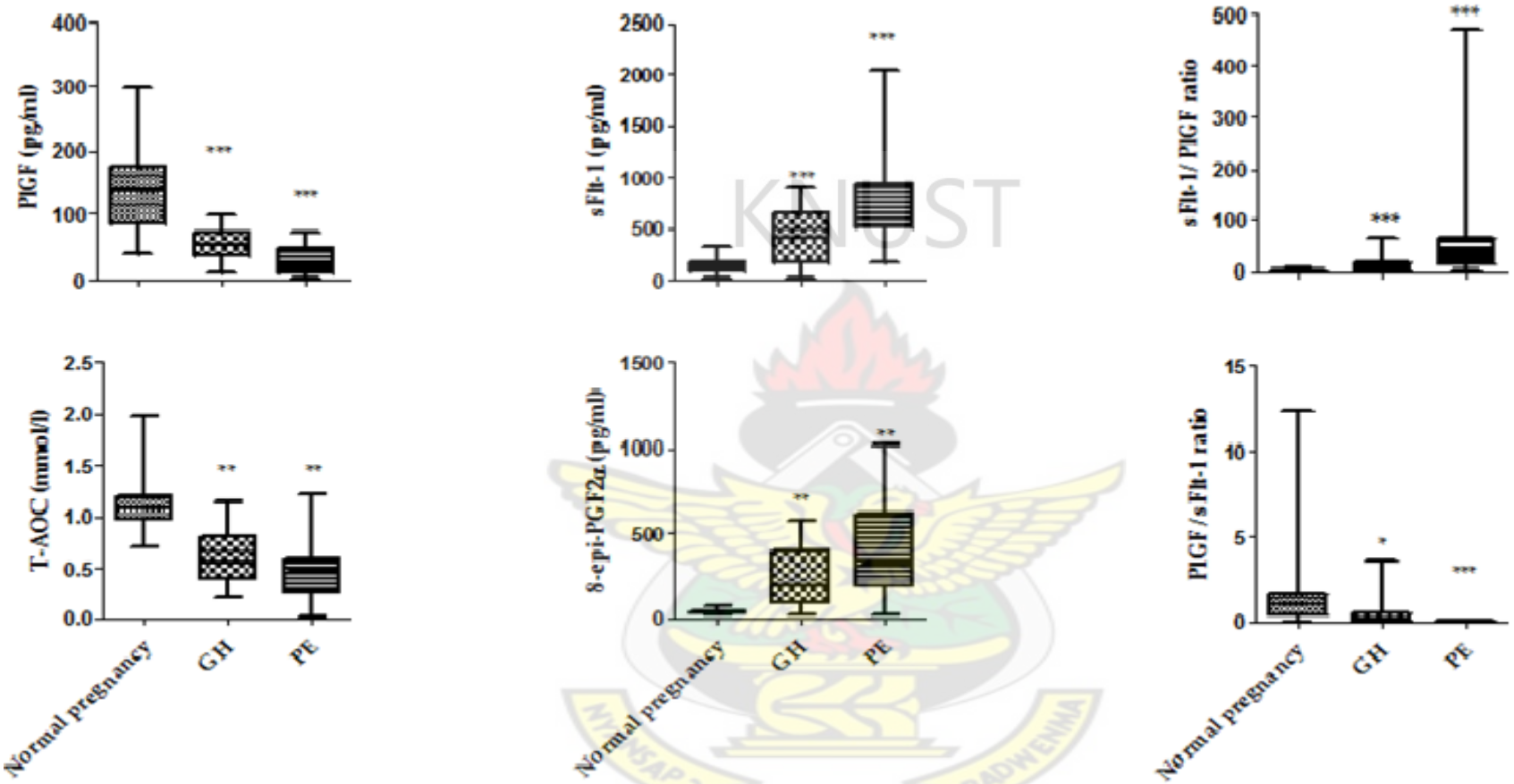

Figure 4. 1 Levels of angiogenic and oxidative stress biomarkers stratified of studied participants at baseline sampling

*Boxplot showing median (interquartile range) *p<0.05 (statistically significant); **p<0.001 (statistically highly significant); ***p<0.0001 (statistically very highly significant) One-Way Kruskal Wallis Test coupled with Dunnet post hoc multiple comparisons to compare each group (GH, PE) to NP (Normal Pregnancy). GH: Gestational hypertension; PE: Preeclampsia*

4.4 ANGIOGENIC AND OXIDATIVE STRESS BIOMARKERS AT SAMPLING

Levels of angiogenic and oxidative stress biomarkers of studied participant at sampling are shown in **Figure 4.1.** Median levels of PLGF, PLGF/sFlt-1 ratio and T-AOC were severely reduced amongst PE but moderately decreased in GH compared to normal pregnant controls ($p<0.0001$). Meanwhile a statistically significant and markedly elevated median levels of sFlt-1, sFlt-1/PLGF ratio and 8-epi-PGF2α were observed among PE with a mild increase in GH compared to control ($p<0.0001$) (**Figure 4.1)**.

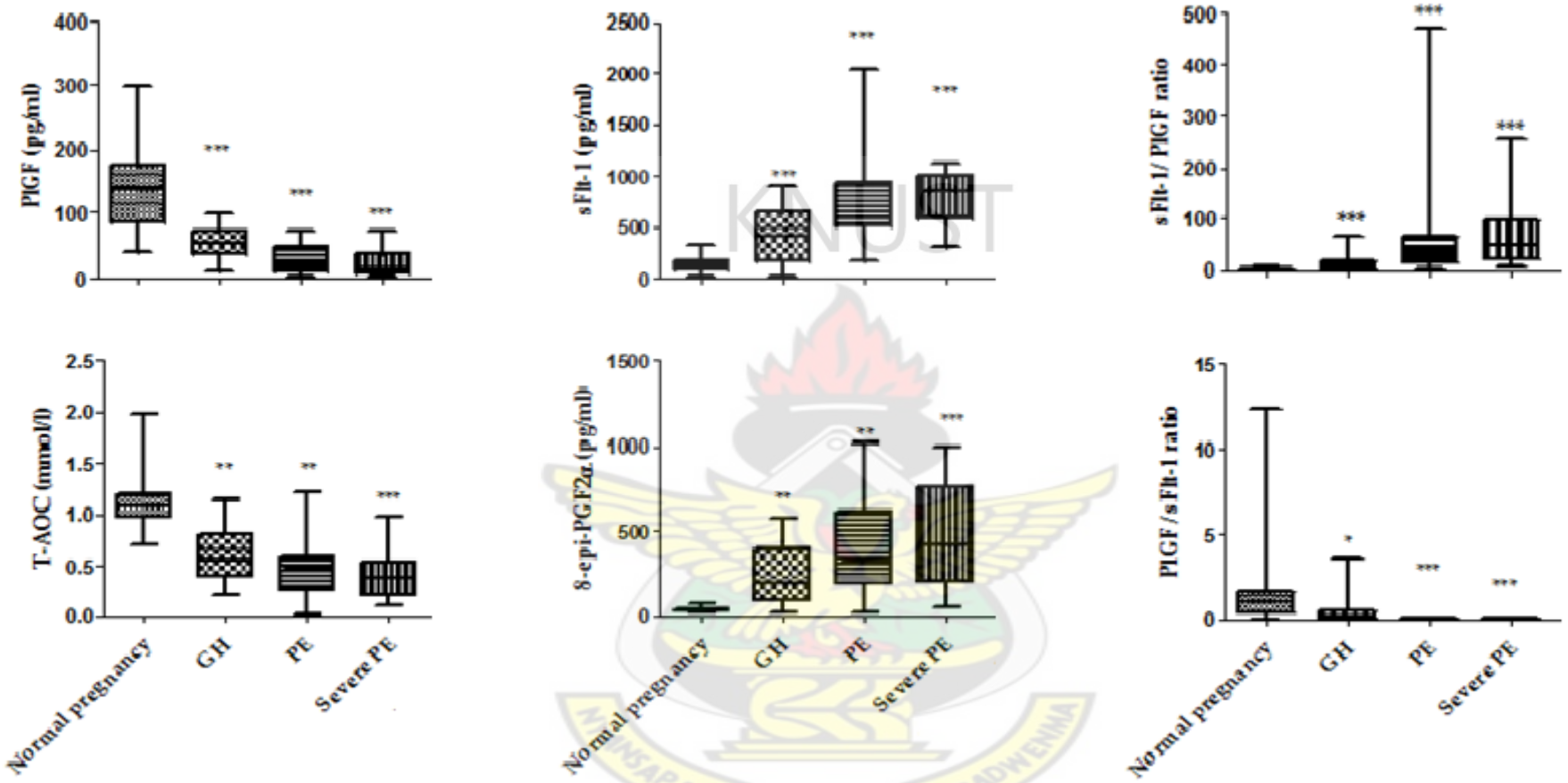

Figure 4. 2 Levels of angiogenic and oxidative stress biomarkers stratified by severity of hypertensive disorders

*Boxplot showing median (interquartile range) *p<0.05 (statistically significant); **p<0.001 (statistically highly significant); ***p<0.0001 (statistically very highly significant) One-Way Kruskal Wallis Test coupled with Dunnet post hoc multiple comparisons to compare each group (GH, PE, Severe PE) to Normal Pregnancy. GH: Gestational hypertension; PE: Preeclampsia.*

4.4.1 ANGIOGENIC AND OXIDATIVE STRESS LEVELS IN RELATON TO SEVERITY OF THE HYPERTENSIVE DISORDER OF PREGNANCY

Median levels of angiogenic factors and oxidative stress markers in relation to severity of hypertensive pregnancy are shown in Figure 4.2. Median levels of levels of PLGF, PLGF/sFlt-1 and T-AOC decreased in the order of Severe PE < PE< GH < NP while sFlt-1, 8-epi-PGF2α and sFlt-1/PLGF ratio levels significantly increased in subjects in the order of Severe PE > PE> GH > NP ($p<0.05$) **(Figure 4.2).**

4.5 COMPARISON OF BLOOD PRESSURE MEASUREMENT, ANGIOGENIC AND OXIDATIVE BIOMARKERS BEFORE AND AFTER DELIVERY

Figure 4.3 depicts baseline and postpartum blood pressure, angiogenic factors and oxidative stress biomarkers levels among studied participants. Preeclamptics and gestational hypertensive women had higher blood pressure (SBP and DBP) compared to the normal pregnant (NP) controls ($p<0.05$). Forty eight (48) hours postpartum BP was significantly lower in PE and GH compared to baseline BP measurement ($p<0.05$) while BP before and 48 hr. after delivery was not statistically significant different in NP controls ($p>0.05$). Levels of PIGF, PLGF/sFlt-1 and T-AOC significantly increased after delivery compared to baseline levels in all the three groups ($p<0.05$). Conversely, levels of sFlt-1, sFlt-1/PLGF ratio and 8-epi-PGF2α significantly decreased after 48 hour postpartum levels compared to baseline levels in all the three groups under study ($p<0.05$) **(Figure 4.3).**

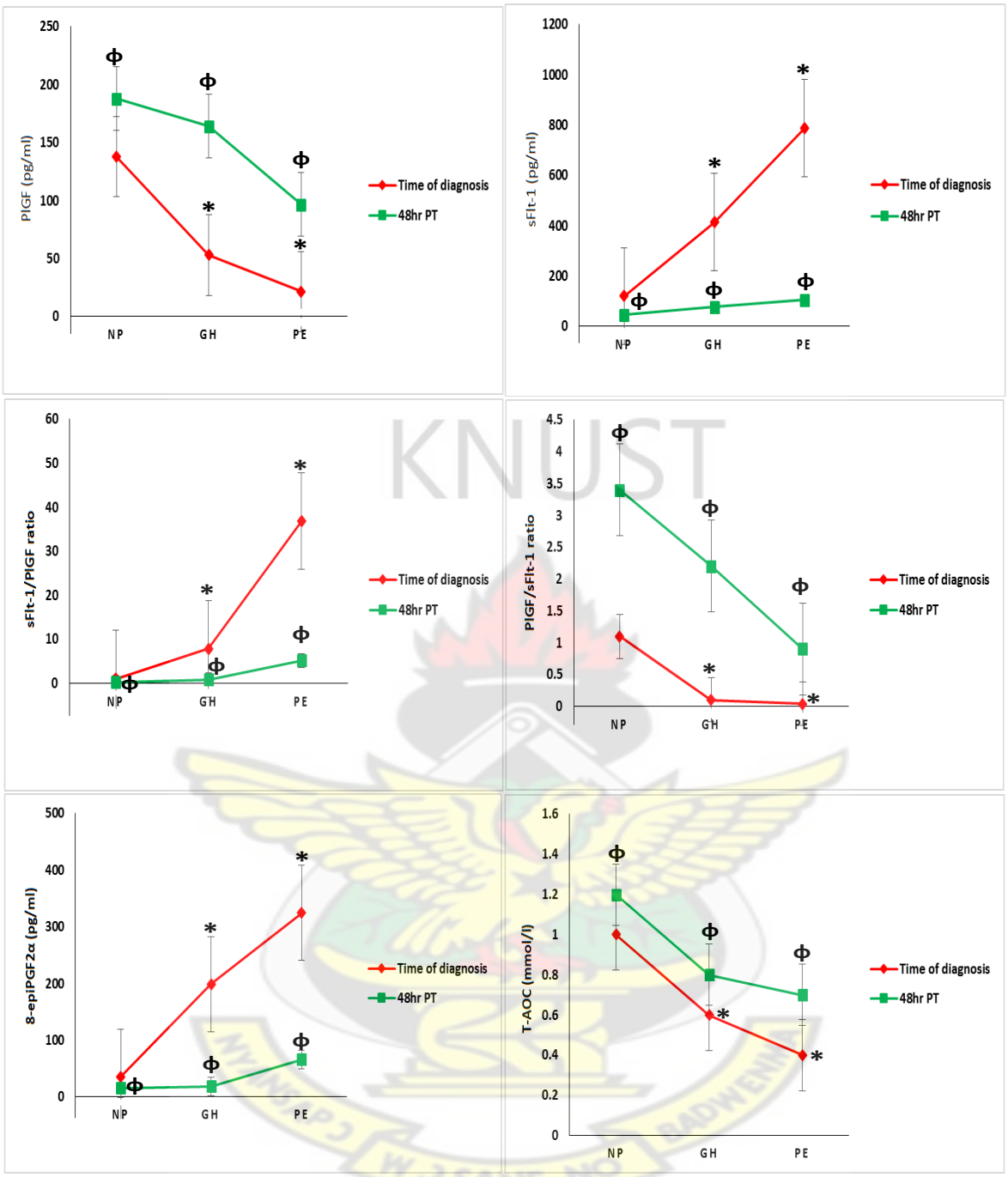

Figure 4. 3 Levels of angiogenic factors and oxidative stress biomarkers at time of diagnosis and 48hr postpartum

*Graphs are presented a scatter line plots of median (interquartile range). *p<0.05 (GH, PE vs NP), ϕ significant compared to baseline/time of diagnosis. PT: Postpartum; NP: Normal pregnancy; GH: Gestational hypertension; PE: Preeclampsia*

4.6 ANGIOGENIC AND OXIDATIVE STRESS MARKERS STRATIFIED BY GESTATIONAL AGE OF PREGNANCY

Figure 4.4 and 4.5 depicts levels of angiogenic and oxidative stress biomarkers in various gestational age of pregnancy. Within each group (NP, GH, and PE) median levels of PLGF and PLGF/sFlt-1 increased up to 22-26 week and began to decrease from 27 – 31week to 32-36 weeks of gestation but transiently increased again from 37- 40 week of gestation until postpartum. Conversely, median levels of sFlt-1, 8-epi-PGF2α and sFlt-1/PLGF ratio were significantly decreased up to 22-26 week and increased orderly from 27-31 week to 32-36 week gestation but transiently decreased at late gestation (37-40 week) until 48 hours postpartum. After post-hoc multiple comparison within each gestational age group and across each studied groups there were statistically significant differences in median levels of angiogenic factors, oxidative stress levels in GH, and PE compared to NP ($p<0.05$) **(Figure 4.4 and 4.5).**

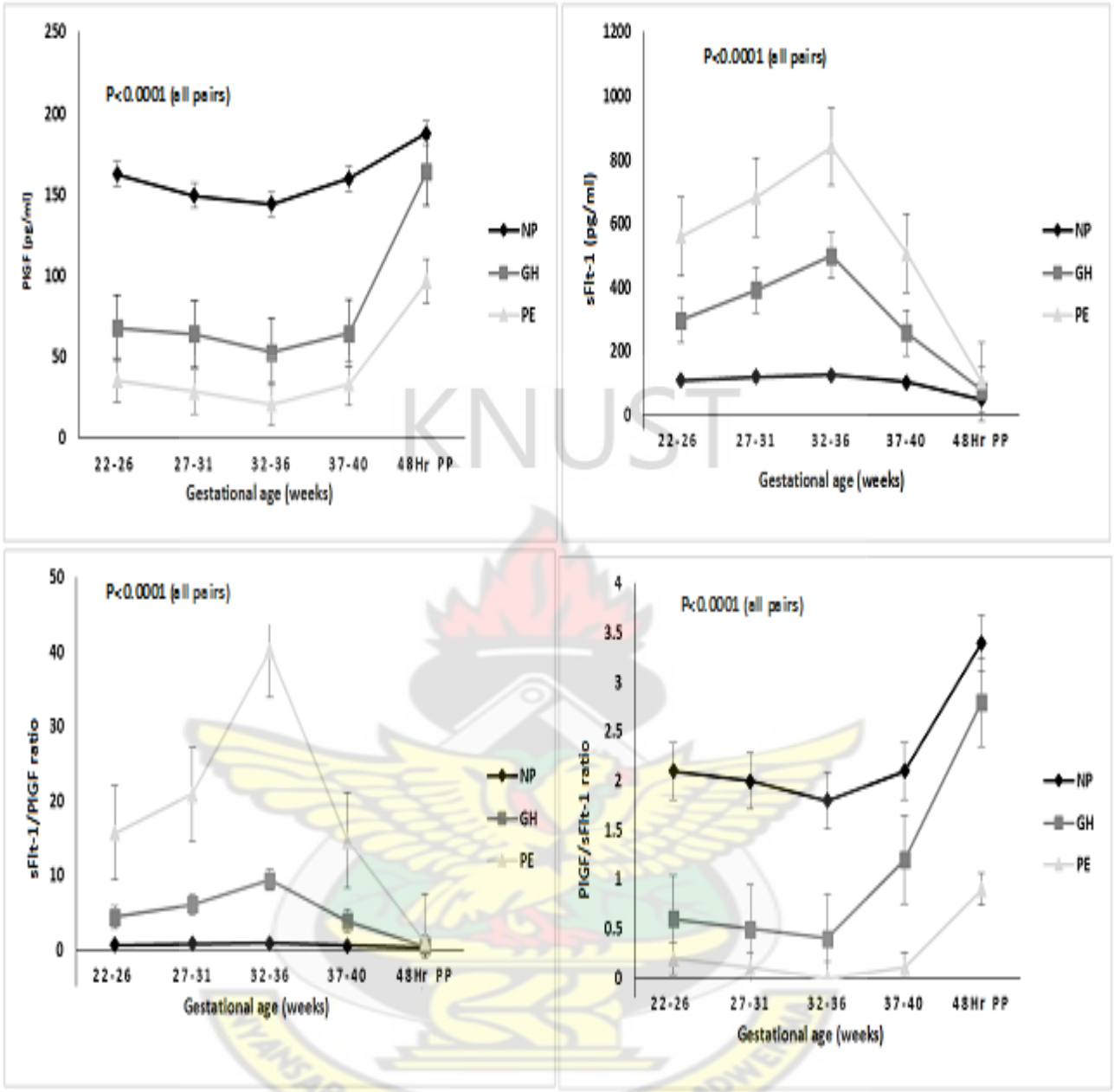

Figure 4. 4 Prospective gestation changes in pro-angiogenic (PlGF and PlGF/sFlt-1 ratio) and anti-angiogenic (sFlt-1 and sFlt-1/PlGF ratio) factors among the study groups

Graphs are presented a scatter line plots of median (interquartile range). Throughout pregnancy women who developed PE showed significantly (p<0.0001) lower levels of PlGF and PlGF/sFlt-1 ratio and higher levels of sFlt-1 and sFlt-1/PlGF ratio compared to GH and NP. Levels of PlGF and PlGF/sFlt-1 ratio increased at 48hrs PT while sFlt-1 and sFlt-1/PlGF ratio decreased at 48hr PP (NP>GH>PE). PP: Postpartum; NP: Normal pregnancy; GH: Gestational hypertension; PE: Preeclampsia. PIGF: Placental growth factor (PlGF), sFlt-1: soluble fms-like tyrosine kinase 1.

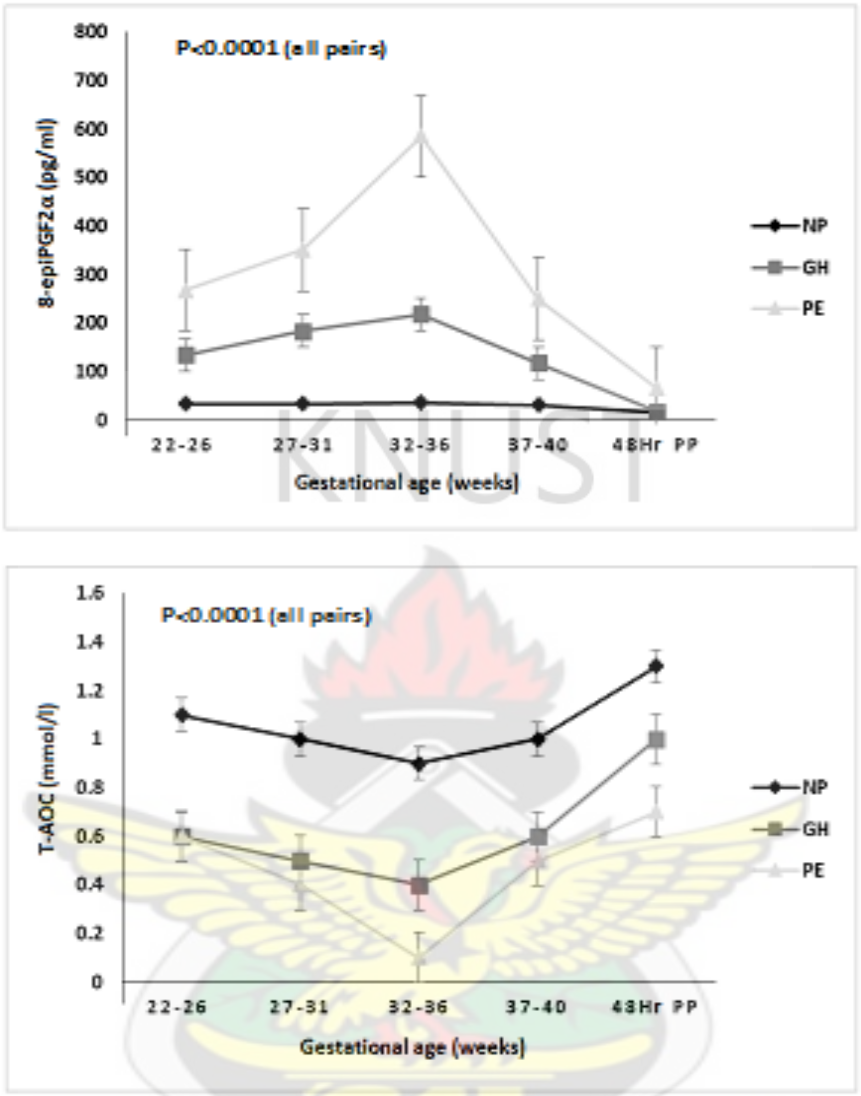

Figure 4. 5 Prospective gestational changes in levels of pro-oxidants (8-epi-PGF2α) and anti-oxidants (T-AOC) markers among the study groups

Graphs are presented a scatter line plots of median (interquartile range). Throughout pregnancy women who developed PE showed significantly (p<0.0001) lower levels of T-AOC and higher levels 8-epi-PGF2α compared to GH and NP. Levels of T-AOC increased at 48hrs PP while 8-epi-PGF2α decreased at 48hr PT (NP>GH>PE) (p<0.0001). PP: Postpartum; NP: Normal pregnancy; GH: Gestational hypertension; PE: Preeclampsia. 8-epi-PGF2α: 8-epi-prostaglandin F2alpha; T-AOC: total antioxidant capacity

4.7 ANGIOGENIC AND OXIDATIVE STRESS MARKERS STRATIFIED BY MATERNAL AGE

Table 4.4 shows levels of angiogenic and oxidative stress markers in relation to maternal age. Within each study group (NP, GH and PE) levels of PLGF, and PLGF/sFlt-1 decreased while sFlt-1, 8-epi-PGF2α and sFlt-1/PLGF ratio levels significantly increased correspondingly with increasing maternal age from 18-24years through 25-29yrs, 30-34yrs to 35-40 years. Despite reduced levels of T-AOC with advancing maternal age was observed there was no statistically significant difference ($p>0.05$) in levels among some study group (NP and GH) but significantly different in PE ($p<0.05$) **(Table 4.4).**

Table 4. 4 Levels of angiogenic and oxidative stress biomarkers in relation to maternal age of pregnancy

Study Groups	Maternal Age Groups (years)				Significant pairs among groups
	(18-24) A	(25-29) B	(30-34) C	(35-40) D	
NP					
PLGF	162.4 (129.0 - 167.0)	152.80 (99.96 - 177.9)	138.7 (79.30 - 177.3)	115.1 (88.88 - 194.7)	<0.0001 (all pairs)
sFlt-1	17.00 (15.40 - 79.00)	101.00 (59.43 - 142.5)	132.4 (88.30 - 174.0)	139.3 (77.10 - 195.4)	0.0001 (all pairs not C vs D)
8-epi-PGF2α	19.80 (16.50 - 27.00)	36.35 (29.68 - 38.90)	38.40 (28.70 - 60.40)	38.5 (29.58 - 69.45)	0.0201 (all pairs not C vs D)
T-AOC	1.19 (0.88 - 1.30)	1.09 (1.06 - 1.19)	1.066 (0.88 - 1.124)	1.04 (0.95 - 1.52)	0.2387
sFlt-1/PLGF	0.09 (0.08 - 0.25)	0.69 (0.42 - 1.94)	0.961 (0.34 - 1.77)	1.22 (29.58 - 0.57)	0.0001 (all pairs)
PLGF/sFlt-1	6.20 (1.49 - 10.97)	1.47 (0.56 - 2.39)	1.040 (0.57 - 2.93)	0.84 (0.35 - 1.46)	<0.0001 (all pairs C vs D)
GH					
PLGF	70.80 (39.70 - 85.20) *	53.95 (36.80 - 75.68) *	51.55 (40.60 - 94.63) *	37.80 (16.35 - 61.05) †	<0.0001 (all pairs)
sFlt-1	192.40 (105.6 - 519.00) *	400.7 (102.6 - 598.8) **	495.1 (205.5 - 775.2) *	718.4 (359.8 - 800.7) *	<0.0001 (all pairs)
8-epi-PGF2α	148.70 (43.05 - 310.3) **	164.2 (71.25 - 351.0) *	301.7 (106.6 - 462.7) *	379.7 (160.8 - 471.9) **	<0.0001 (all pairs)
T-AOC	0.69 (0.43 -0.87)*	0.63 (0.39 - 0.83) *	0.55 (0.38 - 0.82) **	0.50 (0.37 - 0.69) *	0.5931
sFlt-1/PLGF	4.79 (1.29 - 11.47) *	7.52 (1.69 - 17.40) *	8.88 (3.09 - 16.83) *	14.43 (5.023 - 48.85) †	0.0083 (all pairs not B vs C)
PLGF/sFlt-1	0.21 (0.09 - 0.78) *	0.142 (0.06 - 0.34) *	0.133 (0.059 - 0.589) *	0.07 (0.02 - 0.21) **	<0.0001 (all pairs not B vs C)
PE					
PLGF	36.40 (14.41 - 49.40) †	24.60 (8.095 - 45.85) †	16.70 (11.82 - 43.60) †	15.80 (10.10 - 51.33) †	0.0001 (all pairs not C vs D)
sFlt-1	671.3 (499.5 - 854.5) **	768.6 (578.6 - 960.1) †	783.4 (493.7 - 1006) †	806.8 (335.1 - 911.6) †	<0.0001 (all pairs)
8-epi-PGF2α	312.1 (71.95 - 477.6) **	315.4 (159.3 - 356.7) †	401.0 (201.5 - 944.2) †	543.5 (174.0 - 722.8) †	<0.0001 (all pairs)
T-AOC	0.49 (0.32 - 0.79) *	0.49 (0.39 - 0.55) **	0.39 (0.18 - 0.38) **	0.29 (0.19 - 0.61) †	0.0055 (all pairs A vs B)
sFlt-1/PLGF	17.54 (12.68 - 46.27) †	32.97 (14.88 - 124.3) †	43.67 (14.25 - 66.37) †	48.21 (8.671 - 93.85) †	<0.0001 (all pairs)
PLGF/sFlt-1	0.06 (0.02 - 0.08) †	0.03 (0.01 – 0.080) †	0.02 (0.014 – 0.065) †	0.02 (0.009 - 0.141) †	<0.0001 (all pairs not C vs D)

*Values are presented as Median (interquartile range). Kruskal Wallis Test to compare GH and PE to NP within age group: *p<0.05; ** p<0.001 † p<0.0001. One-Way Kruskal Wallis Test coupled with Dunnet post hoc multiple comparisons to compare across each group. NP: Normal pregnancy; GH: Gestational hypertension; PE: Preeclampsia.*

4.8 INTRAPARTUM AND POSTPARTURM OBSTETRIC OUTCOMES

Table 4.5 shows antepartum and postpartum obstetric complications and outcomes among study participants. A greater proportion of the study groups had cephalic fetal presentation. Higher proportion of preeclamptics (24.0%) had breech fetal presentation compared to NP (0.0%) and GH (8.0%). Increased percentage of placental praevia (14.0% vs 0.0%), placental abruptio (22.0% vs 0.0%), IUFD (40.0% vs 0.0%), IUGR (44.0% vs 1.0%), APH (22.0% vs 0.0%) and PPROM (16.0% vs 0.0%) in PE compared to NP control. Higher proportion (86.0%) of PE and 28.0% of GH were more likely to be delivered off by emergency caesarean section compared to NP controls. Presentation such as fresh stillbirth (24.0% vs 2.0%), PPH (26.0% vs 6.0%), preterm delivery (90.0% vs 6.0%) and maternal mortality (10.0% vs 0.0%) were mostly associated with PE pregnancies compared to NP controls. However, prolonged obstructed labour was mostly common in GH pregnancies compared NP pregnancies (58% vs 10.0%) **(Table 4.5)**

Table 4. 5 Intrapartum and Postpartum characteristic and adverse complications

Variables	NP(n=50)	GH(n=50)	PE(n=50)
Fetal presentation			
Cephalic/Vetex	50(100.0%)	46(92.0%)	38(76.0%)
breech	0(0.0%)	4(8.0%)	12(24.0%)
Placental praevia			
Yes	0(0.0%)	4(8.0%)	7(14.0%)
Placental abruption			
Yes	0(0.0%)	1(2.0%)	11(22.0%)
IUFD			
Yes	0(0.0%)	5(10.0%)	20(40.0%)
IUGR			
Yes	1(2.0%)	7(14.0%)	22(44.0%)
APH			
Yes	0(0.0%)	2(8.0%)	11(22.0%)
PPROM			
Yes	0(0.0%)	0(0.0%)	8(16.0%)
Mode of delivery			
Vaginal			
Spontaneous	45(90.0%)	15(30.0%)	0(0.0%)
Induced	5(10.0%)	20(40.0%)	1(2.0%)
Caesarean section			
Emergency	0 (0.0%)	14(28.0%)	43(86.0%)
Elective	0(0.0%)	1(2.0%)	7(14.0%)
Stillbirth			
Fresh	1(2.0%)	5(10.0%)	12(24.0%)
Macerated	0(0.0%)	1(2.0%)	2(4.0%)
Live birth			
Yes	49(98.0%)	44(88.0%)	36(72.0%)
Prolong labour			
Yes	5(10.0%)	29(58.0%)	13(26.0%)
PPH			
Yes	3(6.0%)	5(10.0%)	13(26.0%)
Status of delivery			
Term	47(94.0%)	12(24.0%)	5(10.0%)
preterm	3(6.0%)	38(76.0%)	45(90.0%)
Maternal mortality			
Yes	0(0.0%)	0(0.0%)	5(10.0%)

Values are presented as frequency (proportion); IUFD: Intrauterine fetal death; IUGR: Intrauterine growth retardation; APH: Antepartum haemorrhage; PPROM: Preterm premature ruptures of membrane. PPH: Postpartum haemorrhage

4.9 FETAL CHARACTERISTICS AND OUTCOMES

Table 4.6 shows fetal characteristics and outcomes. There was a statistically significantly low birthweight and Apgar score in PIH (GH and PE) compared to NP control ($p<0.05$). Fetal respiratory distress and birth asphyxia were significantly associated with PE (38.0% vs 2.0%) and GH (16.0% vs 4.0%) pregnancies respectively compared to NP control ($p<0.05$). Fetal anomalies such a webbed feet and deformation of nose and ear were observed in PE pregnancies compared to NP control (6.0% vs 0.0%). A significantly higher proportion (64.0% vs 0.0%) of PE babies had APGAR score less than seven (7) after 5 minutes compared to controls ($p<0.05$) **(Table 4.6).**

Table 4. 6 Fetal Characteristics features and adverse outcomes among various studied groups

Variables	NP(n=50)	GH(n=50)	PE(n=50)	p-value
Birth weight (kg)	2.87 ± 0.06	2.75 ± 0.11	2.06 ± 0.09	< 0.0001
Fetal gender				
Male	34(68.0%)	33(66.0%)	24(48.0%)	0.0676
female	16(32.0%)	17(34.0%)	26(42.0%)	
Male/ female ratio	2.13:1	1.94:1	0.92:1	
Fetal distress				
Yes	1(2.0%)	3(6.0%)	19(38.0%)	< 0.0001
Birth asphyxia				
Yes	2(4.0%)	4(16.0%)	4(8.0%)	0.1175
APGAR score				
1st min	6.54 ± 0.13	5.59 ± 0.29	2.71 ± 0.19	< 0.0001
5th min	8.56 ± 0.08	7.32 ± 0.35	5.99 ± 0.34	< 0.0001
APGAR score ≤ 7 after 5 min				
Yes	0(0.0%)	9(18.0%)	32(64.0%)	< 0.0001

Values are presented as frequency (proportion);. p<0.05 was considered statistically significant difference. APGAR: Appearance, pulse, grimace, activity, respiration.

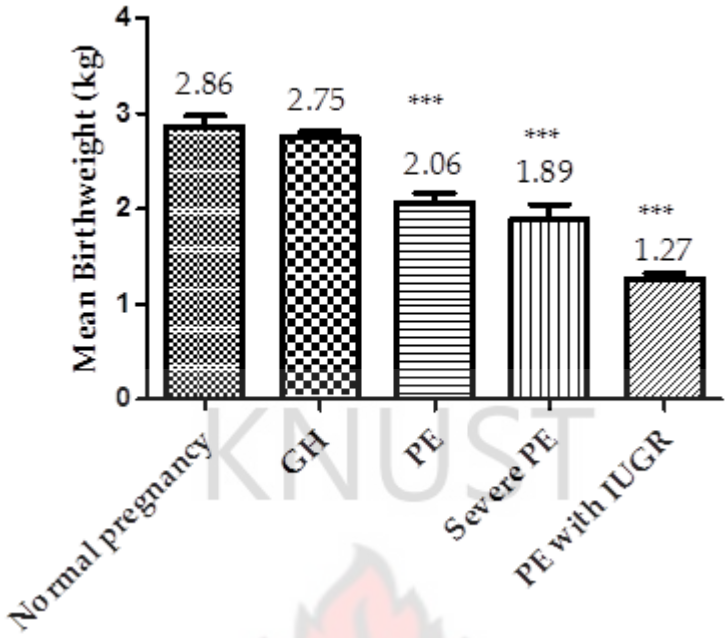

Figure 4. 6 Birthweight of babies stratified by the severity of the condition

**** p<0.05 (statistically significant); ** p<0.001(statistically highly significant) *** p<0.0001 (statistically Very highly significant). One-Way Kruskal Wallis Test coupled with Dunnet post hoc multiple comparisons to compare each group (GH, PE, Severe PE and PE + IUGR) to NP. IUGR: Intrauterine growth retardation; GH: Gestational hypertension; PE: Preeclampsia.***

Babies from mothers with PE associated IUGR had a significantly lower birthweight (LBW) than those with severe PE, PE and GH compared to NP controls ($p<0.0001$) **(Figure 4.6).**

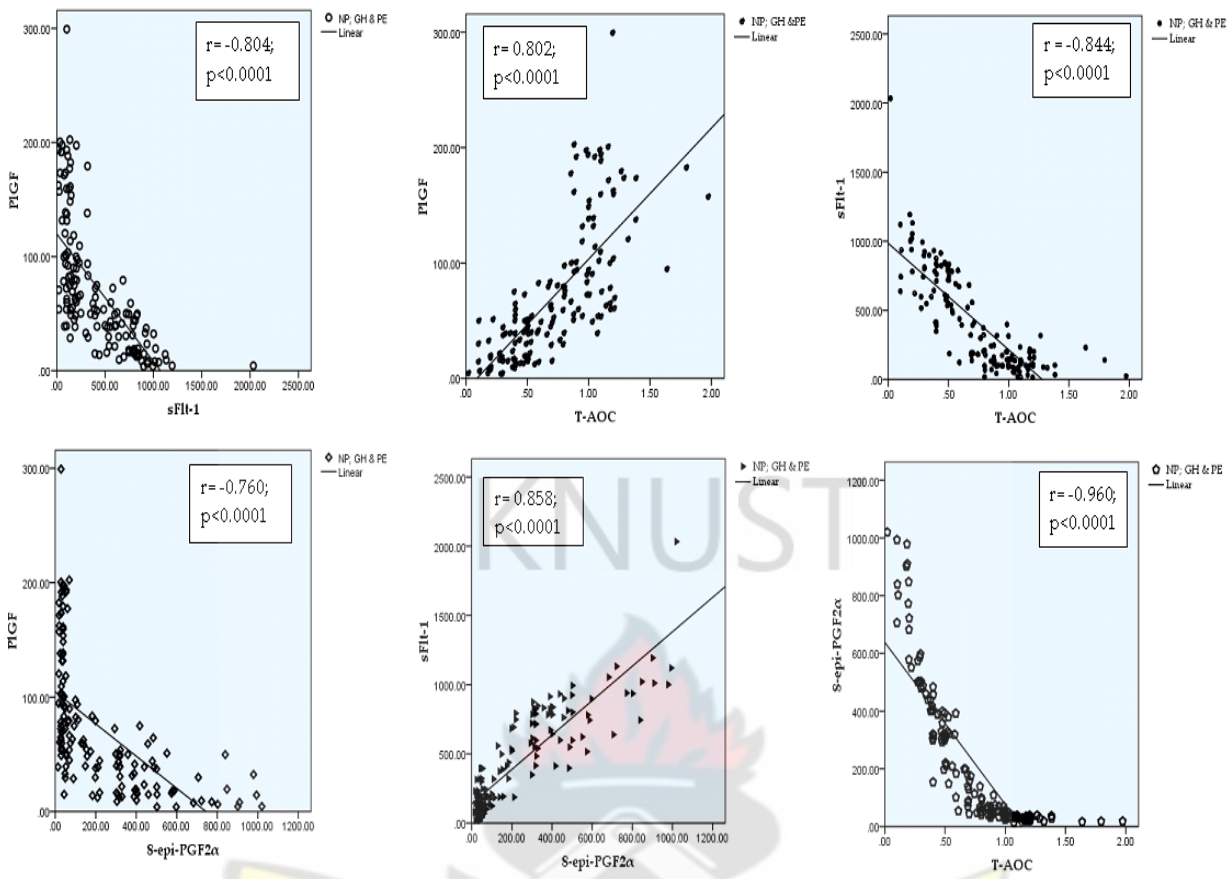

Figure 4. 7 Scatter plots of a correlation between Angiogenic factors and oxidative biomarkers

r= correlation coefficient

4.10 CORRELATION BETWEEN ANGIOGENIC AND OXIDATIVE STRESS BIOMARKERS

Correlation between angiogenic factors and oxidative stress markers are shown in Figure 4.7. A statistically significantly positive correlation was observed between PIGF and T-AOC (r=0.802; p<0.0001) and sFlt-1 and 8-epi-PGF2α (r=0.858; p<0.0001) in all the three study groups. Meanwhile, a negative correlation was observed between PLGF and sFlt-1 (r= -0.804; p<0.0001), sFlt-1 and T-AOC (r= -0.844; p<0.0001), PLGF and 8-epi-PGF2α (r= -0.760; p<0.0001), T-AOC and 8-epi-PGF2α (r= -0.960; p<0.0001) in all the three groups understudied **(Figure 4.7).**

4.10.1 PARTIAL CORRELATION BETWEEN ANGIOGENIC AND OXIDATIVE STRESS BIOMARKERS AFTER ADJUSTING FOR MATERNAL AGE, GESTATIONAL AGE, PREGESTATIONAL BODY MASS INDEX (BMI) AND PARITY

Table 4.7 shows partial correlation between angiogenic and oxidative stress biomarkers. After, adjusting for maternal age, gestational age (GA) and pregestational BMI there was a positive significant correlation between PIGF and T-AOC (r=0.700; p<0.0001) and sFlt-1 and 8-epi-PGF2α (r=0.873; p<0.0001) while a negative correlation was observed between PLGF and sFlt-1 (r= -0.638; p<0.0001), sFlt-1 and T-AOC (r= -0.807; p<0.0001), PLGF and 8-epi-PGF2α (r= -0.581; p<0.0001), T-AOC and 8-epi-PGF2α (r= -0.845; p<0.0001) **(Table 4.7).**

Table 4. 7 Partial correlation between angiogenic and oxidative stress biomarkers

	PIGF	sFlt-1	8-epi-PGF2α	T-AOC
PLGF	1	r=-0.638 p<0.0001	r=-0.581 p<0.0001	r=0.700 p<0.0001
sFlt-1		1	r=0.873 p<0.0001	r=-0.807; p<0.0001
8-epi-PGF2α			1	r=-0.845; p<0.0001
T-AOC				1

r=correlation coefficient; Correlation was adjusted for maternal age, gestational age, pregestational body mass index and parity. 1 indicates a perfect correlation

4.11 BIVARIATE AND PARTIAL CORRELATION OF ANGIOGENIC AND OXIDATIVE STRESS MARKES WITH BP, GESTATIONAL AGE (GA), PARITY BMI AND MATERNAL AGE

Correlation of angiogenic and oxidative markers with BP (SBP and DBP), GA, parity BMI and maternal age are shown in **Table 4.8.** Analysis on Spearman rho moment correlation indicated that there was a statistically significant ($p<0.05$) negative correlation of BP, GA, parity, BMI and maternal age with PLGF, T-AOC and PLGF/sFlt-1 ratio, while a statistically significant positive correlation was observed with sFlt-1, 8-epi-PGF2α, and sFlt-1/PLGF ratio ($p<0.05$).

The effect of each component of BP, GA, Parity, and BMI on angiogenic factors and oxidative stress markers was assessed after adjusting for maternal age and pregestational BMI using partial correlation. The strength of the relationship of BP with all the angiogenic and oxidative stress biomarkers, GA with sFlt-1 and 8-epi-PGF2α and that of BMI with PLGF, sFlt-1, T-AOC and PLGF/sFlt-1 ratio were also statistically significant ($p<0.05$) after adjusting for maternal age **(Table 4.8).**

Table 4. 8 Bivariate and Partial correlation of angiogenic and oxidative biomarkers with Blood pressure, Gestational age, parity and body mass index in GH and PE subjects

	PLGF	sFlt-1	8-epi-PGF2α	T-AOC	sFlt-1/PIGF	PIGF/sFlt-1
SBP	r= -0.688; p<0.0001	r= 0.644; p<0.0001	r= 0.627; p<0.0001	r= -0.660; p<0.0001	r= 0.702; p<0.0001	r= -0.451; p<0.0001
	r= -0.690; p<0.0001	**r= 0.628; p<0.0001**	**r= 0.534; p<0.0001**	**r= -0.674; p<0.0001**	**r= 0.652; p<0.0001**	**r= -0.748; p<0.0001**
DBP	r= -0.694; p<0.0001	r= 0.647; p<0.0001	r= 0.677; p<0.0001	r= -0.684; p<0.0001	r= 0.709; p<0.0001	r= -0.459; p<0.0001
	r= -0.708; p<0.0001	**r= 0.635; p<0.0001**	**r= 0.575; p<0.0001**	**r= -0.699; p<0.0001**	**r= 0.667; p<0.0001**	**r= -0.853; p<0.0001**
GA	r= -0.132; p= 0.107	r= 0.208; p=0.011	r= 0.090; p= 0.275	r= -0.049; p=0.554	r= 0.174; p=0.0330	r= -0.116; p=0.0330
	r= -0.140; p= 0.088	**r= 0.211; p=0.010**	**r= 0.177; p= 0.031**	r= -0.066; p=0.424	r= 0.417; p=0.073	r= -0.120; p=0.1440
Parity	r= 0.021; p= 0.794	r= -0.021; p= 0.794	r= -0.081; p=0.3250	r= 0.080; p=0.330	r= -0.005; p=0.949	r= 0.037; p=0.651
	r= 0.015; p= 0.855	r= -0.012; p= 0.881	r= -0.020; p= 0.813	r= 0.035; p=0.670	r= -0.019; p=0.819	r= 0.047; p=0.567
BMI	r= -0.327; p<0.0001	r= 0.299; p<0.0001	r= 0.271; p=0.001	r= -0.303; p<0.0001	r= 0.332; p<0.0001	r= -0.221; p=0.0070
	r= -0.313; p<0.0001	**r= 0.245; p= 0.003**	r= 0.147; p= 0.073	**r= -0.315; p<0.0001**	**r= 0.253; p=0.0460**	**r= -0.269; p=0.0040**
AGE	r= -0.205; p= 0.0371	r= 0.319; p= 0.0038	r= 0.279; p= 0.0150	r= -0.226; p= 0.0193	r= 0.397; p= 0.0026	r= -0.146; p= 0.0497

r: correlation coefficient. r<0.5 (weak correlation); r>0.5 (strong correlation). SBP: Systolic blood pressure; DBP: Diastolic blood pressure; GA: Gestational age; BMI: Body mass index. Values without box shows Pearson moment correlation and values within box are partial correlation after adjusting for maternal age and pregestational BMI. p<0.05 (statistically significant) p<0.001(statistically highly significant) p<0.0001 (statistically very highly significant).

4.12 CORRELATION OF ANGIOGENIC AND OXIDATIVE BIOMARKERS WITH INTRAPARTUM AND POSTPARTUM ADVERSE PREGNANCY OUTCOME

Table 4.9 shows Spearman rho correlation of angiogenic and oxidative stress markers with Intrapartum and adverse maternal outcomes in Preeclamptic pregnancy. There was a significant ($p<0.05$) negative correlation of PIGF and T-AOC with IUGR, placental abruptio, IUFD, stillbirth, and PPH. Conversely, the correlation of sFlt-1 and 8-epi-PGF2α with IUGR, placental abruptio, IUFD, stillbirth, and PPH was positive and statistically significant ($p<0.05$) **(Table 4.9)**

Table 4. 9 Spearman rho correlation of Angiogenic and Oxidative biomarkers with adverse pregnancy outcome in PE.

	Biomarkers			
Adverse Outcomes	PLGF	sFlt-1	8-epi-PGF2α	T-AOC
IUGR	r= -0.416; **p= 0.0030**	r= 0.549; **p<0.0001**	r= 0.610; **p<0.0001**	r= -0.635; **p<0.0001**
Placental abruptio	r= -0.663; **p<0.0001**	r= 0.680; **p<0.0001**	r= 0.579; **p<0.0001**	r= -0.740; **p<0.0001**
IUFD	r= -0.694; **p<0.0001**	r= 0.730; **p<0.0001**	r= 0.629; **p<0.0001**	r= -0.819; **p<0.0001**
Stillbirth	r= -0.366; **p=0.0183**	r= 0.732; **p<0.0001**	r=0.691; **p<0.0001**	r= -0.815; **p<0.0001**
PPH	r= -0.297; **p=0.0420**	r= 0.479; **p=0.0043**	r=0.410; **p=0.0039**	r= -0.509; **p=0.0044**

r: correlation coefficient; p<0.05(statistically significant) p<0.001(statistically highly significant) p<0.0001 (statistically very highly significant). r<0.5 (weak correlation); r>0.5 (strong correlation). IUGR: Intrauterine growth retardation; PPH: Postpartum haemorrhage

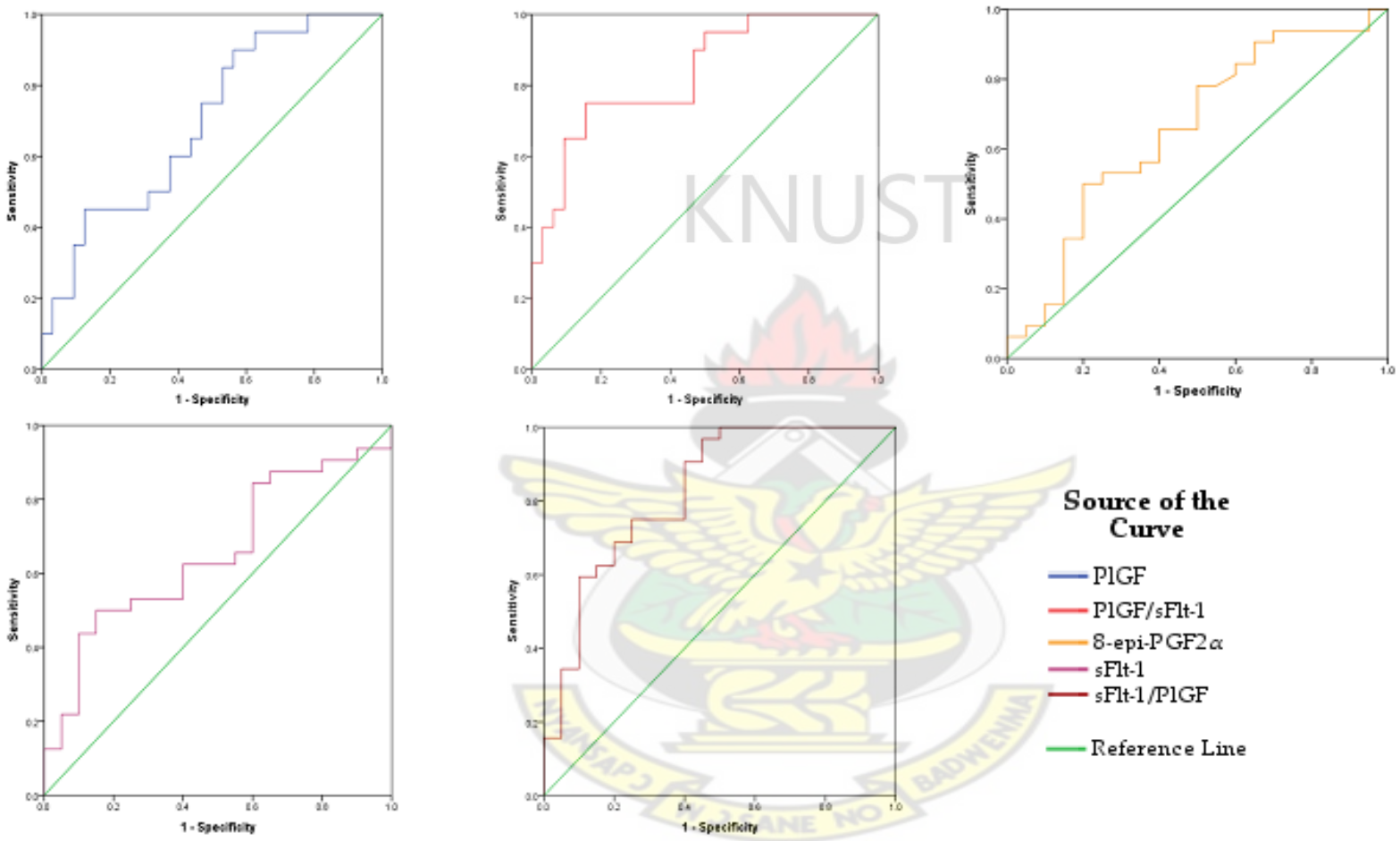

Figure 4. 8 Receivers operating Characteristics (ROC) curve on Angiogenic and Oxidative markers showing area under the curve (AUC).

4.13 DIAGNOSTIC ACCURACY OF ANGIOGENIC AND OXIDATIVE STRESS MARKERS

Diagnostic accuracy of angiogenic and oxidative stress biomarkers for early onset of preeclampsia are shown in **Figure 4.8** and **Table 4.10.**

ROC curve **(Figure 4.8)** applied to the third trimester biomarkers indicated best diagnostic profile for PIGF/sFlt-1 ratio [AUC=0.88; 95% CI (0.72–0.95)] followed by sFlt-1/PLGF [AUC=0.84; 95% CI (0.71- 0.93)], PLGF [AUC=0.69; 95% CI (0.55 – 0.84)], sFlt-1 [AUC=0.66 95% CI (0.50 - 0.80)], and 8-epi-PGF2α [AUC=0.65; 95% CI (0.49 - 0.81)] as depicted by area under the curve (AUC)

The optimal thresholds were 0.014 for PLGF/sFlt-1 ratio, 39.9 for sFlt-1/PLGF ratio, 14.55 pg/ml for PLGF, 618.65 pg/ml for sFlt-1 and 300.80 pg/ml for 8-epi-PGF2α. However PLGF/sFlt-1 ratio (96.9% sensitivity, 87.9% specificity, 98.3% PPV and NPV of 88.9%; p<0.0001) sFlt-1/PLGF ratio (95.7% sensitivity, 80.5% specificity, 94.4% PPV and NPV of 89.1%; p<0.0001) and PIGF (85.1% sensitivity, 78.1% specificity, 87.96% PPV and NPV of 80.4%; p=0.0180) were found to be the best predictor of early onset third trimester preeclampsia than using the individual markers **(Table 4.10).**

Table 4. 10 Diagnostic accuracy of angiogenic factors and oxidative stress biomarkers in predicting early onset of preeclampsia

Biomarkers	Cut-off point	Area under curve (AUC) (95% CI)	Sensitivity	Specificity	PPV	NPV	p-value
PlGF	14.55	0.69 (0.55– 0.84)	85.1%	78.1%	87.9%	80.4%	**0.0180**
sFlt-1	618.65	0.66 (0.50-0.80)	81.3%	68.0%	81.9%	75.3%	0.0650
8-epi-PGF2α	300.80	0.65 (0.49-0.81)	78.5%	65.0%	80.5%	78.5%	0.0610
sFlt-1/PlGF	39.90	0.84 (0.70-0.95)	95.7%	80.5%	94.4%	88.9%	**<0.0001**
PlGF/sFlt-1	0.014	0.88 (0.72–0.93)	96.9%	87.5%	98.3%	89.1%	**<0.0001**

PPV: positive predictive value; NPV: negative predictive value; CI: confidence interval; AUC: Area under the curve

Chapter 5

DISCUSSION

This study evaluated antepartum and postpartum levels of angiogenic factors and oxidation stress markers among Ghanaian women presenting with GH and PE. Angiogenic factors were assessed by measuring sFlt-1 and PLGF whereas 8-epi-PGF2α and T-AOC were assessed as oxidative stress biomarkers. The results indicated that anti-angiogenic and lipid peroxidation markers were significantly elevated while pro-angiogenic and antioxidant capacity was significantly reduced in GH and PE compared to healthy normal pregnant controls at baseline. Again, postpartum levels of anti-angiogenic and lipid peroxidation markers significantly reduced while pro-angiogenic and antioxidant capacity increased in all studied participants. As anti-angiogenic and lipid peroxidative markers peaked at 32-36 weeks gestation and fall transiently from 37-40 weeks up to 48 hours post-delivery, pro-angiogenic and antioxidant capacity reduced and rises steadily in the same pattern. Moreover, correlation of angiogenic factors, oxidative biomarkers and adverse maternal outcomes were observed. The ratio of PLGF/sFlt-1 proved to be the most accurate marker for the diagnosis of the early onset of PE.

5.1 TIME OF DIAGNOSIS AND POSTPARTUM LEVELS OF ANGIOGENIC FACTORS AND OXIDATIVE STRESS BIOMARKERS

Accumulating evidence suggests that PE, a multisystem disorder may be associated with imbalance in the circulatory angiogenic factors (Bdolah *et al.*, 2005; Teran *et al.*, 2007). Some studies (Yelumalai *et al.*, 2010; Maynard and Karumanchi, 2011; Petla *et al.*, 2013) have shown that circulating levels of sFlt-1 may increase in preeclamptic women compared to normal pregnant women (Levine *et al.*, 2006). A study by Wang *et al.*, (2004) reported that the pathogenic effect associated with

increased sFlt-1 may culminate in endothelial dysfunction by interfering with pro-angiogenic activity initiated by VEGF and PLGF. This in no doubt confirms the findings of the present study which showed significantly lower levels of PLGF and a correspondingly increased concentration of sFlt-1 in GH and PE compared to normal pregnancy (Figure 4.1). However, elevated levels of sFlt-1 were significantly associated with PE compared to GH. The increased levels of sFlt-1 observed in PE and GH could be explained by the increased evidence of hypoxia of the placenta due to shallow trophoblasts invasion and reduced placental perfusion (Maynard and Karumanchi, 2011) though this study observed an increased concentration of sFlt-1 among newly diagnosed GH and PE. Palm *et al.,* (2012) have also observed that levels of sFlt-1 may be elevated before the clinical onset of PE and GH. The mechanism underlying the similarity in results though at different stage of sampling is not well understood but the pathophysiological changes associated with pregnancy and its demands may be implicated.

For this study, reduced concentration of sFlt-1 was observed after delivery which indicates that the placenta may play a key role in the pathogenesis of GH and thus PE. This is consistent with findings of (Noori *et al.*, 2010). This study also observed that despites the reduction in sFlt-1 levels after delivery in GH and PE, the levels of sFlt-1 was significantly higher in hypertensive groups compared to controls. This supports the findings that endothelial dysfunction can last for a period after the episode of preeclampsia (McDonald *et al.*, 2008). However, our results are inconsistent with workdone by Yelumalai *et al.*, (2010) who indicated that postpartum levels of sFlt-1 in PE, GH and NP were not statistically significant different compared to each other. This discrepancy could be explained by the fact that Yelumalai and colleagues assessed levels of sFlt-1 6 weeks after delivery whereas in this study sFlt-1 was evaluated 48 hours post-delivery.

The pathogenic role associated with increased sFlt-1 culminates into endothelial dysfunction by interfering with pro-angiogenic activity initiated by VEGF and

PLGF (Wang *et al.*, 2004). This further confirms the findings of the present study which showed a significantly lower level of PLGF in GH and PE compared to normal pregnant women (Figure 4.1). Increased levels of sFlt-1 may be responsible for the decreased PIGF in hypertensive pregnancy. We observed that as PLGF levels reduced significantly at baseline and rise steadily after delivery, levels of sFlt-1 increased and reduced correspondingly. Whereas the lowering of P1GF can be related to a decreased syncytiotrophoblast synthesis in predelivery, lower levels of sFlt-1 postpartum may be compensatory as the syncytiotrophoblast may be producing more PLGF and less sFlt-1 enabling binding to ligand receptors on the endothelium. Forty eight (48) hour postpartum levels of PLGF which was significantly different and did not return to normal levels among all studied groups could be due to similar trend observed in concentration of sFlt-1. Serum sFlt1 concentrations have been shown to be elevated at an average of 18 months postpartum in women presenting with preeclampsia compared to those with normotensive pregnancies only (Wolf *et al.*, 2004) thus providing a supportive evidence as observed in our study that these women may tend toward a persistent antiangiogenic state even after delivery.

Again this study observed an increased lipid peroxidation activity as depicted by an increased serum level of 8-epi-PGF2α. Women with preeclampsia had an elevated levels of 8-epi-PGF2α followed by gestational hypertensives and then normal control at baseline. This result is in agreement with studies by Palm *et al.*, (2009) and Gubaljevia and Aceauaievia, (2013). Most studies (Mohanty *et al.*, 2006; Suhail and Faizul-Suhail, 2009) measured malondialdehyde (MDA), a secondary by-product of lipid peroxidation which is unstable and affected by several factors. The current study used 8-epi-PGF2α which is considered a potent and stable lipid peroxidation marker responsible for abnormalities such as hypertension, endothelial cell dysfunction, renal vasoconstriction, placental vasoconstriction, and cerebral vasospasm of eclampsia (Walsh *et al.*, 2000; Gubaljevia and Aceauaievia,

2013). The increased serum levels of 8-epi-PGF2α in this study could be explained by these abnormalities. It is possible that the elevated amount of 8-epi-PGF2α secreted in serum reflect the severity of maternal oxidative stress which was predominantly observed among PE participants.

This study has established that postpartum levels of 8-epi-PGF2α does not return to normal and thus was significantly different between all studied groups. Phagocytosis of placental debris by endothelial cells after delivery may have activated the endothelium and cause release of reactive oxygen species and thus the increased in postpartum OS as indicated by Chen *et al.*, (2012a).

Consistent with previous reports (Chappell *et al.*, 2000; Palm *et al.*, 2009), this study observed a reduced concentration of T-AOC in hypertensive pregnancy (GH and PE) compared to controls (Figure 4.1). The observed decrease in T-AOC is given further impetus by the lipid peroxidation observed in the participants with PE and GH compared to controls. The levels of T-AOC decreased significantly in PE than in GH indicating that preeclamptics participants may suffer a huge compromised antioxidant system and may be associated with increased widespread endothelial dysfunction compared to GH participants. Concentrations of T-AOC in normal postpartum period were increased compared to pre-delivery levels in normal pregnancy. This association implies that normal pregnancy is associated with oxidative stress which worsens in GH and PE (Aebovia *et al.*, 2013). Although delivery of placenta remains the outstanding therapy for PE, antioxidant supplementation during pregnancy has also been shown to be beneficial however, its usefulness is still unclear (Aebovia *et al.*, 2013). Like the results of this study, the increased levels of sFlt-1/PIGF in PE and GH compared to controls have been reported by some studies (Noori *et al.*, 2010; Yelumalai *et al.*, 2010).

However, no data in previous studies reported decreased levels in the ratio of PIGF/sFlt-1 in GH and PE as observed in this study. The observation made in this study clearly shows that PIGF/sFlt-1 ratio may give a better prediction of GH and

thus PE than using the individual markers alone. Investigating into the usefulness of PIGF/sFlt-1 as an early onset diagnostic tool would thus be imperative.

The results of this study which observed an elevated anti-angiogenic factors and pro-oxidant levels in GH and thus PE warrant the need of using pharmacologic remedies such as exogenous proangiogenic molecules and antioxidant supplements and or inhibiting the action of anti-angiogenic molecules as part of treatment and management approach to help alleviate the adverse complications suffered by these patients.

5.2 ANGIOGENIC FACTORS AND OXIDATIVE STRESS MARKERS IN RELATION TO GESTATIONAL AGE OF PREGNANCY

The exact gestation where angiogenic factors and oxidative stress biomarkers peak have received little or no attention. To understand the changes over time of angiogenic factors and oxidative stress biomarkers in pregnancy, this study also evaluated levels in relation to the gestational age of pregnancy.

In this study levels of sFlt-1 and 8-epi-PGF2α levels began to increase from 22-26 weeks, 27-31 weeks, peaked at 32-36 week and transiently decreased from 37-40 week until 48 hours postpartum while PLGF and T-AOC decreased and increased correspondingly in the same pattern. Some researchers (Palm *et al.*, 2009; Palm, 2012) observed that angiogenic profile and oxidative stress markers fluctuate throughout gestation in normal pregnancy. Results of this study clearly showed that increased and shift of the imbalance in favour of sFlt-1 and 8-epi-PGF2α were significantly associated with third trimester pregnancies specifically at 32-36 week of gestation than the second trimester in all studied group and the levels tend to decrease slowly after birth up to 48 hours (Figure 4.4 and 4.5). This finding is consistent and confirms reports from Hertig et al., (2004) who observed in previous prospective study that levels of sFlt-1 peak in third trimester (Hertig *et al.*, 2004). Hung *et al.*, (2010) have also reported increased oxidative stress in the third trimester. Clinical onset of PE usually arising in the third trimester of pregnancy long after initiation of the underlying process could explain the elevated levels of

sFlt-1 and 8-epi-PGF2α and a reduced levels of PLGF and T-AOC (Hertig *et al.*, 2004). Nevertheless, a previous study by Palm, (2012) which indicated that sFlt-1 peak from the onset of PE till term is not consistent with the current result of this study which observed sFlt-1 to peak at 32-36 week, followed by a transient decrease from 37-40week gestation until postpartum. This disparity could be explained by the fact that levels of angiogenic factors and oxidative stress markers fluctuate throughout pregnancy making it difficult to identify the actual gestational age associated with increases or decreases (Palm, 2012). However, levels of sFlt-1 which peaked at 32-36 week with a corresponding reduced PLGF is consistent with the findings of Yelumalai *et al.,* (2010). Previous studies have however not extensively explore the changes in oxidative stress markers in relation to gestational age of preeclamptic and gestational hypertensive pregnancy as shown in this study.

One of the perceived limitations of this study was that blood samples were not taken from PE and GH pregnant women after antihypertensive treatment with intravenous magnesium sulphate (MgSO4) and hydralazine respectively before delivery to assess the effect of these drugs on angiogenic and oxidative stress profile. MgSO4 treatment is known to reduce eclamptic seizures (Altman *et al.*, 2002) but does not act through angiogenic or oxidative stress pathways to decrease or correct the imbalance effect or the risk of adverse pregnancy outcome (Vadnais *et al.*, 2012). Another study by Fei *et al.*, (2011) observed that MgSO4 therapy may inhibit the progression of preeclampsia by modulating endothelial dysfunction mediators. Eshkoli *et al.*, (2013) have indicated that perfusion of MgSO4 increases sFlt-1 secretion on the fetal side of the placenta in preeclamptic women although not much published data have confirmed this finding. The latter study confirms the finding of this study which observed that postpartum levels of angiogenic factors in PE and GH pregnant women did not revert to normal and were associated adverse pregnancy outcomes despite antihypertensive treatment and delivery. This necessitates this study to put forward the hypothesis that neither

antihypertensive treatment nor delivery is capable enough to prevent all complications associated with hypertensive disorders of pregnancy.

5.3 ANGIOGENIC FACTORS AND OXIDATIVE STRESS MARKERS IN RELATION TO MATERNAL AGE

This study demonstrated a significant association of maternal age with angiogenic and oxidative stress biomarkers. To establish this association, the study assessed the angiogenic profile and oxidative stress markers in maternal age categories from a young age (18-24years) to advanced maternal age (35-40years) in all studied participants.

The results showed that levels of PLGF, PLGF/sFlt-1 and T-AOC were reduced while sFlt-1, 8-epi-PGF2α and sFlt-1/PLGF ratio were significantly increased correspondingly with increasing maternal age from 18-24years through 25-29yrs, 30-34yrs to 35-40 years. A search through literature could not however, find any supportive publication confirming our findings thus showing that this study is probably the first to report this trend. However, the possible explanation to these finding is typical of angiogenic and oxidative imbalance originating from an incomplete invasion and placental underperfusion. Apart from the demands of pregnancy and its associated changes, aging is associated with increased production of reactive oxygen species with a higher probability of inducing oxidative stress and angiogenic imbalance.

Per the observations of this study a higher proportion of adverse pregnancy outcomes were associated with advanced maternal age (AMA). This proves to support the idea that AMA is an independent risk factor for pregnancy complications (Ahenkorah, 2009). Laopaiboon *et al.*, (2014) have indicated that myometrial function deteriorates with age and this could be an alternate

explanation to the advanced maternal age-related angiogenic and oxidative stress imbalance

5.4 INTRAPARTUM AND POSTPARTUM ADVERSE MATERNAL AND FETAL PREGNANCY OUTCOMES IN PE AND GH

The causes of poor maternal and fetal outcomes have been attributed to preeclampsia, gestational hypertension, advanced maternal age, and gestational diabetes by some previous studies (Allen *et al.*, 2004b; Bdolah *et al.*, 2005). In the present study the most prevalent occurring adverse maternal outcome was preterm delivery (90%) out of which 86% were emergency caesarean delivery attributed to PE. The exact cause of preterm delivery is not understood. Several studies attribute this to advanced maternal age (Laopaiboon *et al.*, 2014), preeclampsia (Sibai, 2006), defective angiogenic profile (Andraweera *et al.*, 2012), oxidative stress (Menon and Bonney, 2014) which are consistent with findings of this present study. The findings by Kim *et al.,* (2003) which indicated that a significantly higher mean percentage spiral arteries with failure of physiological transformation in the myometrial and decidual segments observed among preterm delivery in PE participants may explain a similar mechanism associated with increased proportion of preterm deliveries in this study (Kim *et al.*, 2003).

Intrauterine growth restriction, a common complication associated with severe preeclampsia and HELLP syndrome was also seen among PE women than GH compared to normal pregnant women. Forty four (44) percent of preeclamptics compared to 14% of gestational hypertensive women presented with IUGR which indicates that this complication is common in severe than mild hypertensive disorders of pregnancy. This findings is consistent with several others published data (Smith *et al.*, 2002; Villar *et al.*, 2006b). The possible explanation of increased

IUGR may be placental insufficiency due to poor placental perfusion or transplacental flux of nutrients (Villar *et al.*, 2006b). The alternative reason may be an imbalance in angiogenic (Ghosh *et al.*, 2012) and oxidative stress profile (Biri *et al.*, 2007) which also confirms the findings of this present study that PE complicated by IUGR were significantly associated with angiogenic and oxidative imbalance as depicted by elevated sFlt-1 and 8-epi-PGF2α concentrations respectively.

There is no doubt that due to incomplete cytotrophoblast invasion and placental underperfusion the fetus is likely to lose oxygen and a resulting intrauterine death. This study showed that 40% of babies who died in the womb were associated with the preeclamptic condition. Although previous studies have not extensively address the association between IUFD and hypertensive disorders (PE and GH) (Many *et al.*, 2002) explained that IUFD may be may be due to inherited thrombophilia in PE women.

Previous work by Osei-Nketiah, (2001) indicated that 40% of maternal mortality in Ghana were associated with antepartum and postpartum haemorrhage. The present study observed 26% incidence of PPH and 22.0% of APH among women with preeclampsia than those with gestational hypertension and normal pregnancy. The likely cause of PPH observed in this study could be caesarean delivery, retained placenta, induction of labour, placental praevia, and suspected placental abruptio among other though the probable cause of APH has been related to placental abruptio (Bateman *et al.*, 2010). Rosenthal and Paterson-Brown, (1998) also explain that uterine atony is the most common cause of postpartum haemorrhage. The present study have also identified a significant correlation between PPH an imbalance in the angiogenic and oxidative stress profile (Table 4.9) indicating that these markers play a significant role in the etiology of PPH.

Impaired placental function has already been discussed as a possible cause for the increased incidence of IUGR, IUFD and may have also contributed to the increased

rate of fresh stillbirth (24.0%) observed among PE patients in this study. Some study speculated that long gestational exposure of the foetus to hypoxic environment coupled with a number of mechanism including the exposure of gametes to increased oxidative stress, increase apoptosis and necrosis of intervillous space which could also be relevant in the increased risk of fresh stillbirth (Peter-Stein *et al.*, 2008). Other adverse outcomes such as breech fetal presentation (24.0%), PPROM (16.0%), placental previa (14.0%) and 10% of maternal mortality were significantly associated to preeclampsia than gestational hypertension compared to normal pregnancy

Babies born to women presenting with PE were mostly associated respiratory distress, low birth weight (LBW), birth asphyxia and APGAR score below 7 after 5 minutes than GH and normal pregnancies (Table 4.6). All these adverse outcomes could be attributed to placental insufficiency.

5.5 CORRELATION BETWEEN ANGIOGENIC FACTORS AND OXIDATIVE STRESS BIOMARKERS

In this study the significantly elevated levels of both sFlt-1 and 8-epi-PGF2α and a reduction in both T-AOC and PLGF levels in women presenting PE compared to GH and normal pregnancy called for an investigation in the association between angiogenic factors and oxidative stress biomarkers.

Previous studies have not published data on the association of angiogenic factors with oxidative stress biomarkers. For the first time a more sensitive oxidative stress biomarkers were used to assess an association with angiogenic factors. This study for the first time has established a positive correlation between sFlt-1 and 8-epi-PGF2α levels while both were inversely associated with T-AOC and PLGF (Figure 4.7). This may explain the synergistic role antiangiogenic and lipid peroxidation markers play in the pathogenesis of hypertensive pregnancy. This established relationship also buttress the earlier findings in the present study which indicated

that significantly elevated levels of both sFlt-1 and 8-epiPGF2α and a subsequent reduction in concentrations of PLGF and T-AOC were associated with the severity of the hypertensive condition (De Vivo *et al.*, 2008).

A negative correlation between sFlt-1 and PIGF has been established previously (Kim *et al.*, 2007; Ghosh *et al.*, 2012). However, the significant association of 8-epiPGF2α with T-AOC, sFlt-1, and PLGF and that of T-AOC with PLGF and sFlt-1 observed in this study has not yet been described in previous studies.

An additional strength of the association is the use of partial correlation to control for the confounding effects of maternal age, prepregnancy BMI, gestational age and parity. The significant correlation observed between angiogenic factors and oxidative biomarkers after adjusting for these confounding factors indicates that their role in the pathogenesis of hypertensive disorders of pregnancy cannot be discounted.

5.6 CORRELATION BETWEEN ANGIOGENIC FACTORS, OXIDATIVE STRESS MARKERS AND ADVERSE PREGNANCY OUTCOME

In this study the increased levels of sFlt-1 and 8-epiPGF2α and a reduced levels of T-AOC and PLGF among patients presenting with adverse pregnancy outcome, necessitated the quest to assess the correlation between angiogenic factors, oxidative stress and adverse pregnancy outcomes. Angiogenic factors and oxidative stress biomarkers correlated significantly with adverse pregnancy outcomes such as IUGR, IUFD, placental abruptio, stillbirth and PPH.

Previous studies (Lam *et al.*, 2005; Agarwal and Karumanchi, 2011) indicated that pro and anti-angiogenic proteins may be associated with pregnancy complications such as pre-eclampsia and IUGR. Ghosh *et al.,* (2012) have reported a significant negative correlation between PLGF and IUGR which is consistent with the finding

of this study which found a significant negative correlation between PIGF and IUGR (Table 4.9).

In addition to previous findings this study is the first to observe a significant negative correlation between PLGF and IUFD, placental abruptio, stillbirth, and PPH and a positive correlation between sFlt-1 and IUGR, IUFD, placental abruptio, stillbirth, and PPH. This indicates that the degree of sFlt-1 is elevated while PLGF is reduced proportionately to the hypertensive pregnancy complicated with adverse outcomes. The markedly increased sFlt-1 and correspondingly decreased PLGF levels proportional to the severity of PE condition indicates that imbalance in angiogenic factors plays an important role in the aetiology of these adverse outcomes. With the exception of IUGR the associations of angiogenic profile with IUFD, placental abruptio, stillbirth and postpartum haemorrhage have not been reported in previous studies. Hypoxia induced by incomplete trophoblasts invasion and placental underperfusion may be the probable explanation for the imbalances in angiogenic factors and oxidative stress biomarkers and its association with adverse pregnancy outcomes.

This present study has established the relationship between oxidative stress indicated by increased 8-epiPGF2α and decreased levels of T-AOC with adverse pregnancy outcome (Table 4.9). As 8-epiPGF2α correlated positively with adverse pregnancy outcome (IUGR, IUFD, placental abruptio, stillbirth, and PPH) in preeclamptic participants, the correlation of T-AOC with IUGR, IUFD, placental abruptio, stillbirth, and PPH levels were significant and inversely correlated. Hypoxic intervillous space may have triggered increased inflammatory response and a subsequent release of reactive oxygen species (ROS) resulting in these complication. PE co-existing adverse outcome may be using up the antioxidants in circulation for its metabolic processes and this could explain the reduced concentration of T-AOC.

From this study antiangiogenic and pro-oxidant positively correlated adverse pregnancy outcome (Stillbirth, placental abruption, IUGR, IUFD, PPH). Early identification of adverse complications could provide innovative approaches to the early management.

5.7 CORRELATION BETWEEN BLOOD PRESSURE (SBP, DBP) GESTATIONAL AGE, PARITY, BMI WITH ANGIOGENIC FACTORS AND OXIDATIVE STRESS BIOMARKERS

Some studies have explored the association between angiogenic factors with maternal arterial pressure, parity, BMI, maternal age, SBP and DBP in the first and second trimester of normotensive pregnancies (Faupel-Badger *et al.*, 2011). A very few of these studies assessed these relationship in the second and third trimesters pregnancy of normotensive, gestational hypertension and preeclamptic women. The results of the study has demonstrated a significant independent correlation between levels of angiogenic factors and oxidative stress biomarkers with selected maternal features such as blood pressure (SBP and DBP), BMI and maternal age, but not parity and gestational age. Blood pressure were significant and negatively associated with PLGF, T-AOC, and PLGF/sFlt-1 ratio but positively correlated with 8-epiPGF2α, sFlt1 and sFlt1/PLGF ratio (Table 4.8).

A significant positive correlation of blood pressure with sFlt1 and sFlt1/PLGF ratio and a negative correlation with PLGF have been observed in uncomplicated pregnancy but not preeclampsia in a previous study by Troisi *et al.*, (2008) which is inconsistent with the findings of the present study. The most likely explanation of the latter disparity may be due to the fact that Troisi and colleagues used a small sample size and sampled only patients scheduled for caesarean section at the point of delivery while this study took a baseline sampling of PE and GH subjects before antihypertensive treatment.

However, the positive correlation of blood pressure with sFlt1 and sFlt1/PLGF ratio and a negative correlation with PLGF have been observed in previous studies (Molvarec *et al.*, 2010b; Noori *et al.*, 2010; Varughese *et al.*, 2010). The strength of the association apart from adjusting for maternal age is the use of baseline subjects who were not on antihypertensive. The shift of balance in favor of sFlt-1 in PE and GH and the corresponding evidence of a significant positive correlation with SBP and DBP supports the argument that angiogenic imbalance plays a pivotal role in the pathogenesis of PE and GH.

Furthermore, the significant negative correlation between SBP and DBP with T-AOC assessed by FRAP levels supports the importance of serum antioxidant status in blood pressure modulation. This finding although agrees with workdone by Rattan *et al.,* (2013) who observed a similar association in general hypertensive patients with preserved renal function although this study used pregnancy induced hypertensive patients Rattan *et al.*, (2013). The mechanism underlying the significant correlation of blood pressure with angiogenic profile and oxidative stress biomarkers is not understood however, the following reasons may be implicated. Firstly, the loss of endothelial control of vascular development due to imbalance in angiogenic factors and oxidative markers (Petla *et al.*, 2013) and secondly, disturbed renin-angiotensin signalling by activation of angiotensin receptors 1 (AT-1) on various organs (kidney, placenta etc) increasing sFlt-1, reactive oxygen species, and NADPH oxidase and finally resulting into endothelial cell dysfunction and vascular damage (Mustafa *et al.*, 2012). A further study may be needed to explore the latter reason.

With the exception of a significant positive correlation observed between sFlt-1 and gestational age, PLGF, 8-epiPGF2α and T-AOC were not significantly associated with gestational age and parity. In another population where angiogenic factors were measured at nulliparity the association of parity with anti-angiogenic profile was not statistically significant (Staff et al., 2009). Kasdaglis *et al.*, (2010) also

observed no significant correlation between parity and PLGF. All these studies support the findings of this study.

In this current study however, gestational BMI was positively correlated with 8-epiPGF2α, sFlt1 levels and sFlt1/PLGF ratio, and inversely associated with T-AOC, PLGF and PIGF/sFlt-1 levels at these same time points. These results of a direct association of BMI with sFlt1 and sFlt1/PLGF ratio and an inverse association with PLGF and PIGF/sFlt-1 are novel and are inconsistent from earlier studies. In a Norwegian cohort study, BMI at delivery was not associated with angiogenic factors measured in PE or normotensive pregnancies (Staff *et al.*, 2009). A more recent workdone by Mijal and colleagues found a negative association of second trimester sFlt-1 concentrations in maternal serum with BMI in 668 normotensive pregnancies (Mijal et al., 2011). Zera *et al.*, (2014) also observed a negative correlation of BMI with sFlt-1 and PIGF in pregnant women with evidence of placental dysfunction which is not consistent with findings in this present study Despite the numerous conflicting findings observed by these previous studies, there are still some supportive literature confirming a significant positive association between BMI and sFlt-1 and a corresponding negative correlation (Moore Simas *et al.*, 2011). In addition, the factors such as higher maternal age and maternal BMI assessed in this study could be associated with greater blood pressure measures, and this in turn could explain the noted associations with angiogenic profile and oxidative stress markers.

Moreover, literature searches were conducted to determine if these clinical characteristics and their correlation with serum levels of PLGF/sFlt-1, T-AOC and 8-epiPGF2α in PE and GH have ever been reported but no such publication were found. Thus this study is the first to observe a significant and positive association of SBP and DBP with 8-epiPGF2α and this provide scientific prove of the role the role of lipid peroxidation in the pathogenesis of PE and GH pregnancies. After adjusting for maternal age and pregestational BMI individually, the strength of the

association of angiogenic factors and oxidative biomarkers with blood pressure and gestational BMI remained the same with largely unchanged statistical significance.

5.8 ANGIOGENIC FACTORS AND OXIDATIVE BIOMARKERS AS PREDICTORS OF PREECLAMPSIA

Various studies have indicated that the diagnosis of PE using BP measurement, urine protein analysis and other biochemical profiles have not proven to be optimal due to the fact that most of these markers have low sensitivity and specificity with respect to prediction of the course of the disease or maternal and perinatal pregnancy outcomes (Ganzevoort *et al.*, 2006; von Dadelszen *et al.*, 2011). This study therefore evaluated the diagnostic ability of angiogenic factors and oxidative stress biomarkers in predicting the occurrence of PE. Results of this study showed that levels of sFlt-1/PLGF, sFlt-1 and 8-epiPGF2α increases with decreasing PLGF, PLGF/sFlt-1 and T-AOC levels after 20 week of gestation in all studied participants.

The pathogenic effect of the antiangiogenic factor and the lipid peroxidation marker significantly peaked at the third trimester of pregnancy and thus prompting the quest to assess the diagnostic accuracy of angiogenic and oxidative biomarkers in early (<34 weeks) and late onset (>34 weeks) third trimester preeclamptic. The results of this study indicated that sFlt-1/PLGF, sFlt-1 and 8-epiPGF2α concentrations were significantly higher with lower levels of PLGF, PLGF/sFlt-1 and T-AOC in early onset preeclampsia compared to late onset suggesting their role as a predictor of preeclampsia in the third trimester. Again, their utility in identifying women at high risk of developing pre-eclampsia in the third trimester of pregnancy could be particularly important, since intensified monitoring and referral to a specialized perinatal care centre during this period could substantially reduce maternal and fetal morbidity.

This study also observed that in pre-eclampsia, the median levels of sFlt-1 and 8-epiPGF2α marker in the third trimester of gestation were found to be thrice the median values of the control group while PLGF and T-AOC were three times less the values for the control. The best area under the curve obtained on analysis using the ROC curve showed that the ratio of PLGF/sFlt-1 followed by the ratio of sFlt-1/PLGF and PLGF could be used as predictive markers (Figure 4.8). This study found the sFlt-1/PLGF ratio as the second best predictor of early onset third trimester preeclampsia although several others researchers (Carty *et al.*, 2008; De Vivo *et al.*, 2008; Rizos *et al.*, 2013) proposed this marker as the best in predicting preeclampsia, which disagrees with the findings of this study. This disparity may be due to the difference in gestational age in assessing the diagnostic accuracy of these biomarkers. This present study assessed the predictive ability of sFlt-1/PLGF in the third trimester while most of the previous studies used first trimester or early gestations

Nevertheless, this present study is the first to report PLGF/sFlt-1 ratio as the most accurate marker for predicting early onset third trimester preeclampsia with a 96.9% sensitivity, 87.5% specificity, 98.3% PPV and 89.1% NPV. (Hertig and Liere, 2010) had previously reported PLGF/sFlt-1 ratio as a predictor of first trimester preeclampsia before the clinical onset. Although Hertig and colleagues made reference to the first trimester their findings are consistent with that of the current study. PLGF/sFlt-1 ratio being found to be more strongly associated with preeclampsia reflects the modified balance between sFlt-1 and PLGF observed in the preeclamptic group in this study. PLGF levels were found to be significantly low in the preeclamptic group in the early stage of the third trimester and this supported its ability to predict preeclampsia. This finding is consistent with the findings of a study conducted by Ohkuchi *et al.*, (2007). This outcome may explain the role PLGF plays in the pathogenic process in the development of preeclampsia. Although sFlt-1 was found to be higher in the preeclamptic group in early stage of the third trimester its use as a predictor of preeclampsia in clinical practice appears

to be less than the other three markers described above, since its diagnostic accuracy, sensitivity and specificity were lower than those for the PLGF/sFlt-1 ratio, sFlt-1/PLGF ratio and PLGF. The onset threshold levels for sFlt-1 are relatively higher in earlier gestations and begins to deviate for reference range in preeclamptic patients suggesting their low levels in early onset preeclampsia (Unal *et al.*, 2007). A study by De Vivo *et al.,* (2008) also observed similar finding. Based on these findings it may be suggested that, sFlt-1 should be considered as a late marker of pre-eclampsia than an early onset marker.

This study observed that all the biomarkers evaluated may play a role in the onset of preeclampsia and might therefore be used as biomarkers for predicting PE. However, the PLGF/s-Flt1 ratio proved to be a most accurate marker for predicting preeclampsia. Further studies are required to evaluate its role in the pathogenesis of preeclampsia

Chapter 6

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The findings of the present study clearly show that imbalance in the concentrations of angiogenic factors and oxidative stress markers are associated with PE than in GH compared to normal pregnancy.

Imbalance in angiogenic factors was depicted by an increase productions of antiangiogenic factors and a correspondingly decrease in proangiogenic factors while an increase pro-oxidant and a decrease antioxidant capacity indicated oxidative stress.

Third trimester pregnancies particularly, at 32-36 week gestation, advanced maternal age (35-40 years), PE with co-existing adverse maternal complications were mostly associated with a significant imbalance in angiogenic factors and oxidative stress biomarkers. Higher proportions of adverse maternal and fetal outcomes were more associated with PE than GH pregnancies compared to normal pregnancy.

A significant correlation between angiogenic and oxidative profile was identified. Angiogenic and oxidative markers correlated with blood pressure and gestational BMI.

Again, an imbalance in the angiogenic factors (increased sFlt-1 and decreased PLGF) and oxidative markers (increased 8-epiPGF2α and decreased T-AOC) was proportionate to the adverse pregnancy outcomes.

The ratio of PLGF/sFlt-1 proved to be the most accurate marker for early onset diagnosis of PE.

6.2 RECOMMENDATION

Recommendations from this work to the Obstetric and Gynaecological unit

1. A combination therapy of pro-angiogenic molecules and anti-oxidant are required in early perinatal care
2. Incorporating testing of angiogenic factors in early pregnancy could help identify patients at risk and thus prevent future hypertension in pregnancy

Recommendations for Furthers studies

A positive correlation of gestational BMI with anti-angiogenic and pro-oxidant markers depict the role BMI play in the etiology of GH and PE. In order to understand the depth of this association further studies are needed to evaluate placental taurine transporter activity in obese pregnant women.

Another important finding was the direct association of blood pressure with anti-angiogenic factor and pro-oxidant marker. Further investigation is needed to explore the role of the renin-angiotensin pathway in the pathogenesis of hypertensive pregnancy.

The marked imbalance in angiogenic factor and oxidative stress biomarkers in PE compared to GH and normal pregnancy is also an evidence of endothelial dysfunction. Further studies are therefore needed to explore the levels of endothelial dysfunctions mediators in hypertensive pregnancy.

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APPENDIX