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ROLE OF ANGIOGENIC FACTORS AND CIRCULATING MONOCYTES IN THE PATHOGENESIS OF PREECLAMPSIA AND INTRAUTERINE FETAL GROWTH RESTRICTION

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I declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Ethics approval by The Human Research Ethics Committee, Sydney West Local Health District, Sydney West Area Health Service and The University of Sydney.

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Abstract

The pathogenesis of preeclampsia and intrauterine fetal growth restriction, common pregnancy complications, has eluded researchers over decades. The current paradigm suggests defective placentation in early gestation and inadequate vascular remodelling of maternal spiral arteries leading to ischemia as the central cause of these pregnancy complications. Placental ischemia is thought to release soluble factors such as sFlt-1 and sEndoglin that enter the maternal circulation, resulting in endothelial dysfunction and the clinical presentation of preeclampsia. While the pathogenesis of preeclampsia has been extensively studied, the causative mechanisms underlying IUGR are less well understood.

A collection of studies into the pathogenesis of preeclampsia (PE) and intrauterine fetal growth restriction (IUGR) are presented, focusing on factors involved in the angiogenesis and maintenance of the microcirculation of the placental villi as well as maternal factors such as circulating monocytes and lipid status. A key feature of the research is the use of abnormal umbilical artery Doppler resistance in the definition of IUGR.

The research presented has examined the placental expression and circulating levels of pro and anti-angiogenic factors. The focus has been on the VEGF family and its receptors (Flt-1, KDR) as well as Endoglin, a transforming growth factor β receptor. A comparison of the angiogenic factor milieu has been made between normal pregnancy and pregnancies complicated by PE, IUGR and PE+IUGR.

We found moderately strong placental expression of VEGF and Flt-1 in all clinical groups with no major differences between the groups. Placental expression of PlGF and KDR were significantly reduced in IUGR as compared to normal and preeclampsia only pregnancies. The results of this study raise the possibility that changes in VEGF and Flt-1 expression may

be a consequence rather than the cause of placental pathology, and that reduced expression of PlGF and KDR may be a main cause for the development of intrauterine fetal growth restriction.

This is the first description of digital image analysis techniques in the assessment of angiogenic factor expression in the placenta. The study has shown that automated digital image analysis is an alternative and more reproducible method to manual reading of placental immunohistochemical staining.

The findings on the circulating angiogenic factors clearly establish that the deranged angiogenic profile of elevated sFlt-1 and sEndoglin and low PlGF is a feature of not only pregnancies complicated by preeclampsia, but also intrauterine fetal growth restriction. Pro and anti-angiogenic factors and their ratios were assessed as biomarkers in identifying normal vs pathological pregnancies complicated with preeclampsia and/or intrauterine fetal growth restriction. The ratios sEng / PlGF and sFlt*sEng/PlGF were identified as possible biomarkers of placental disease that should be assessed in future longitudinal studies.

To investigate possible causes of the disparity in the clinical presentation of PE and IUGR with concordant circulating pro and anti-angiogenic factor levels, the research was extended to study the contribution of maternal peripheral and fetal monocytes to the angiogenic profile. The findings of this study suggest that maternal and fetal monocyte-derived Flt-1 and maternal monocyte Endoglin expression are unlikely to significantly contribute to the pathogenesis of PE or IUGR.

Maternal and fetal monocyte phenotype and polarization were examined in the placental diseases of PE and IUGR. This data suggested a trend towards a lower circulating maternal percentage of classical monocytes and a higher percentage of intermediate and non-classical

monocytes in pregnancies complicated by preeclampsia and intrauterine fetal growth restriction. For the first time, a new distribution of monocyte subsets with intermediate monocytes as the dominant subtype has been documented in the fetal circulation.

CD86, CD163 and the CD86/CD163 ratios were examined as a surrogate of inflammatory (M1) and healing (M2) phenotypes in pregnancies complicated by PE and IUGR. Whilst a gestational related change in M1/M2 phenotype in third trimester normal pregnancy was not detected, pregnancies complicated by IUGR showed a clear shift towards M2 (healing) monocyte phenotype.

Recent work has suggested that the maternal metabolic syndrome or lipid status may predispose to preeclampsia and may be a possible explanation for the disparity in clinical presentation of PE and IUGR. The present work explored the differences in maternal and fetal lipid profiles between PE, IUGR and PE+IUGR. The maternal and fetal triglyceride levels were significantly higher in preeclampsia compared to normal pregnancy and thus suggested an increased maternal risk of preeclampsia during the pregnancy. This study documented the first description of elevated Apo lipoprotein B levels in cord blood at delivery in PE and IUGR, suggesting a useful link in identifying newborns at risk of cardiovascular disease in later life.

This research contributes to the literature on pathogenesis of preeclampsia and intrauterine growth restriction, demonstrating similarities and differences between the two conditions which has lead us closer towards an understanding of their pathogenesis. The findings are in line with recent opinions, suggesting a move away from the traditional diagnosis of preeclampsia using hypertension and proteinuria and embracing the use of biomarkers in the diagnosis.

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Publications

Journal Articles

Alahakoon TI, Zhang W, Trudinger BJ, Lee VW. Discordant clinical presentations of preeclampsia and intrauterine fetal growth restriction with similar pro- and anti-angiogenic profiles. J Matern Fetal Neonatal Med. 2014; 27(18): 1854-1859.

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- Alahakoon TI, Medbury H, Williams H, Fewings N, Xin M Wang, Lee VW Distribution of Cord Blood Monocyte Subtypes in Preeclampsia and Intrauterine Fetal Growth Restriction. Society of Reproductive Investigation, 62nd Annual Scientific Meeting, San Francisco CA, USA March 2015

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Abbreviations

AEDF	Absent end diastolic flow
ADAM-12	A metalloprotease
ADMA	Asymmetric dimethylarginine
ANG	Angiopoietin
ApoAI	Apolipoprotein AI
ApoB	Apolipoprotein B
ATP	adenosine triphosphate
ASSHP	Australasian Society for the Study of Hypertension in Pregnancy
AUC	Area under the curve
BD	BD Biosciences, USA
BMI	Body Mass Index
CRP	C reactive protein
EDC	Expected date of confinement
EVT	Extra-villous trophoblast
Flt	Fms-like tyrosine kinase
sFlt	Soluble Fms-like tyrosine kinase
GA	Gestational age
HDL	High density lipoprotein
HIC	hypoxic/ischemic change
IHC	Immunohistochemistry
HUVEC	Human umbilical vein endothelial cells
Ip	Positive Intensity
Isp	Strong positive intensity

ISSHP	International Society for Study of Hypertension in Pregnancy
IUGR	Intrauterine growth retardation
Iwp	Weak positive intensity.
KDR	Kinase insert domain receptor
LDL	Low density lipoprotein
LMP	Last menstrual period
MFI	Mean Fluorescence Intensity (geometric)
Normal	Normal pregnancy
PAPP-A	Pregnancy associated plasma protein -A
PBS	phosphate-buffered saline
PE	Preeclampsia
PE+IUGR	Preeclampsia and intrauterine fetal growth restriction
PlGF	Placenta growth factor
RBC	Red blood cells
R&D	R&D Systems, USA
ROC	Receiver operating characteristic
SD	Standard deviation
SGA	Small for gestational age
SOMANZ	Society of Obstetric Medicine of Australia and New Zealand
STBM	Syncytiotrophoblast Membrane Microparticles
TC	Total cholesterol
TC/HDL ratio	Total cholesterol/High density lipoprotein
TG	Triglyceride
VEGF	Vascular endothelial growth factor-A
WBC	White blood cells

LITERATURE REVIEW

Chapter 1 - Angiogenesis and monocytes in preeclampsia and intrauterine fetal growth restriction

1.1 Introduction

"The (umbilical) vessels join the uterus like the roots of plants and through them the embryo receives its nourishment."

Aristotle, *On the Generation of Animals*, ca. 340 B.C.

1.2 Human pregnancy and placentation

1.2.1 Placental development in human pregnancy

In mammalian reproduction, the placenta is the organ through which nutrients, wastes and respiratory gases are exchanged between the maternal and fetal circulations, providing all the metabolic demands for fetal growth and development. The rate of this trans-placental exchange depends mainly on rates of uterine (maternal placental) and umbilical (fetal placental) blood flow (1).

Early pregnancy is a critical period of gestation because of the major developmental events that take place, including embryonic organogenesis as well as formation of the placenta, a process known as placentation (2, 3).

1.2.2 Haemochorionic placentation

Human placenta is described as a haemochorial, a type of placenta having the maternal blood in direct contact with the chorionic trophoblast.

1.2.3 Placental histology- Villous architecture of the human placenta

The implantation of the embryo is an invasive process in human reproduction. The cells of the implanting blastocyst consist of the outer wall (trophoblast), forerunner of the fetal membranes, including the placenta. The inner cell mass that forms the embryoblast develops into the embryo, umbilical cord and the amnion.

The early embryo erodes the maternal tissues leading to implantation. The proliferating cytotrophoblast starts trophoblast invasion, formation of the villi of the placenta and adaptation of maternal blood vessels for pregnancy and anchorage of the developing placenta. The structure of the placental villi is shown in Figure 1.1.

All placental villi have the same basic structure.

Syncytiotrophoblast is an epithelial layer that covers the external surface of the villi, separating the interior of the villi from the maternal blood that surrounds the villi. The syncytiotrophoblast represents a continuous multinucleated surface layer without separate cell borders.

Cytotrophoblast lies beneath the syncytiotrophoblast. This is composed of individual or aggregated cells also called Langerhan cells. Cytotrophoblast comprises the stem cells of the syncytiotrophoblast that supports the growth and regeneration of the latter.

The trophoblast basement membrane separates syncytiotrophoblast and cytotrophoblast from the stromal core of the villi. The stroma is composed of varying number and type of connective tissue cells, connective tissue fibers and ground substance. The fetal vessels are seen within the villous stroma in various sizes and calibers, ranging from arteries and veins in the stem villi to capillaries and sinusoids in the peripheral branches.

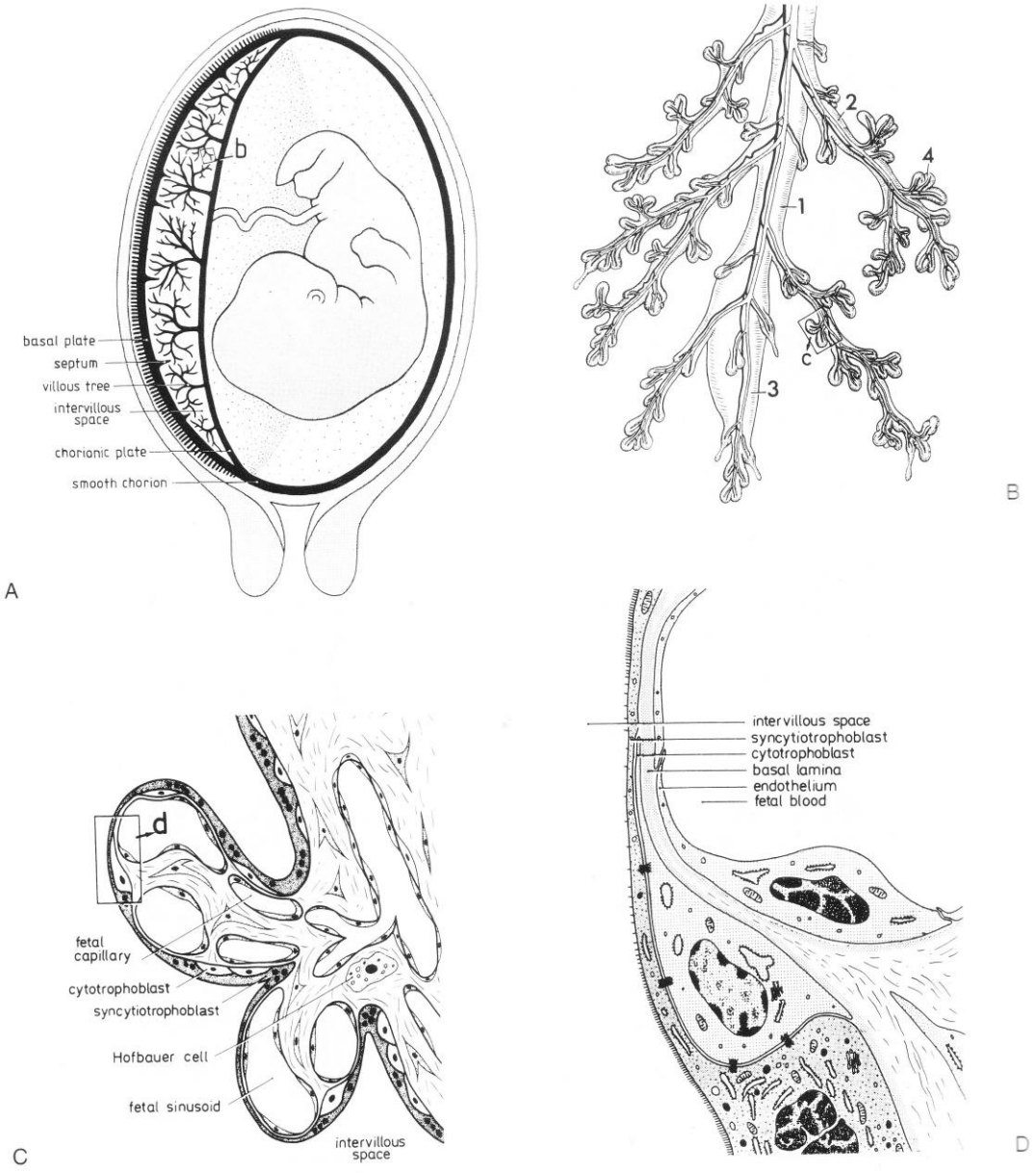

Figure 1.1 Structure of the placental villi. Adopted from Benirschke (2).

Hofbauer cells are oval eosinophilic histiocytes of mesenchymal origin, found in the placenta, in mesoderm of the chorionic villi, particularly numerous in early pregnancies. They are believed to be a type of macrophage and are most likely involved in preventing the transmission of pathogens from the mother to the fetus (4).

1.3 Placental angiogenesis and blood flow through gestation

1.3.1 Angiogenesis and vasculogenesis

The vascular system develops through two separate processes: vasculogenesis and angiogenesis (5). Vasculogenesis forms blood vessels in place by the aggregation of angioblasts into a cord and a primitive vascular network. Vasculogenesis is thought to have three major steps: induction of haemangioblast angioblasts, assembly of primordial vessels and transition from vasculogenesis to angiogenesis (6).

Angiogenesis is the formation of new vessels by sprouting of capillaries from existing vessels. Two forms of angiogenesis have been described: sprouting and non-sprouting angiogenesis (7). Angiogenesis is essential for normal mammalian development and is controlled by the local balance of pro- and anti-angiogenic factors.

These two processes, vasculogenesis and angiogenesis, are involved in placental development. Vasculogenesis is responsible for establishment of the primitive vascular network, whereas during angiogenesis the existing vasculature is remodeled (8).

The vasculature is the first organ to arise during development, since its function is required for growth. Blood vessels run through every organ of the body, ensuring metabolic homeostasis by the supply of nutrients and the removal of waste products. During development, blood vessel formation is fine-tuned by several families of angiogenic factors; some stimulate and others inhibit vessel growth in order to obtain a vascular bed adapted to the specific needs of each organ. Malformation or dysfunction of blood vessels can lead to compromised organ function, resulting in congenital and acquired diseases (9).

1.3.2 Placental angiogenesis

Adequate placental angiogenesis is critical for the establishment of the placental circulation and thus for normal fetal growth and development. Extensive angiogenesis in maternal and fetal placental tissues is accompanied by a marked increase in uterine and umbilical blood flow (10). The rate of placental blood flow depends on the extent of placental vascularization, which is determined by placental angiogenesis.

Placental growth increases with gestational age as evident by the increasing placental weight with gestation. Vascular growth, or angiogenesis, is indeed a major component of the increase in placental growth throughout gestation (10). Extensive angiogenesis in maternal decidua and fetal villi with expansion of the tertiary villi and small arterial channels results in an increase in vascular density and a decrease of the vascular resistance of the placental circulation (11). While vascular density of maternal placental tissues continues to increase slowly throughout gestation, vascular density of the fetal placental cotyledons remains relatively constant through mid-gestation and increases dramatically during the last third of gestation in association with dramatic fetal growth (1).

1.4 Placental vascular disease

1.4.1 Placental supply to the fetus

The exponential increase in fetal growth during the last half of pregnancy depends primarily on the growth of the placental vascular beds and the resultant increases in uterine and umbilical blood flow (10). Uterine and umbilical blood flow that represents the circulation to the maternal and fetal portions of the placenta, respectively, increases exponentially

throughout gestation, essentially keeping pace with fetal growth (10). Internal and external factors such as maternal and fetal genotype, the number of fetuses, maternal under-nutrition, maternal age, parity, environmental factors can have effects on placental size and function, affecting the rates of fetal oxygen and nutrient uptakes (10).

1.4.2 Placental insufficiency

Placental insufficiency secondary to placental vascular disease is a major cause of fetal death in-utero, fetal growth restriction, preterm delivery as well as neonatal and long term morbidity. In the mother, placental changes can cause preeclampsia, the maternal syndrome associated with placental vascular disease. Increased uterine vascular resistance and reduced uterine blood flow can be used as predictors of high-risk pregnancies and are associated with fetal growth restriction (12, 13). Thus, factors that influence placental vascular development and function will have a dramatic impact on fetal growth and development, and thereby on neonatal survival and growth.

1.4.3 Umbilical artery Doppler in identifying placental vascular disease.

In a clinical setting, maldevelopment of the angiogenesis of the placenta can be demonstrated by using Doppler ultrasound techniques (14, 15). Studies into Doppler evaluation of the umbilical vasculature have shown that significant placental insufficiency clinically manifesting as the fetal syndrome of intrauterine growth restriction and/or the maternal syndrome of preeclampsia (16) can be clinically diagnosed by an increased resistance pattern of the umbilical circulation as evident by increased systolic diastolic ratio or resistance index (Figure 2.1 and Figure 2.2) on flow velocity waveforms (12, 15). The level of this increased resistance has been identified at the small arteries and arterioles. These clinical and Doppler findings have been correlated with pathological changes in the placenta. Examination of

placentas of normal pregnancies and pregnancies with high resistance Doppler indices has shown a significant reduction in the number of small arteries and arterioles, correlating the clinical and ultrasound findings with histological changes (14). This reduction in small vessels could be due to failure of formation or obliteration of existing vessels. Evidence of increasing systolic/diastolic ratios in compromised fetuses with advancing gestation (15) supports the hypothesis of obliterative vasculopathy as a cause of placental insufficiency (11). This vessel obliteration has been associated with luminal thrombosis, intimal endovasculitis and fibro-proliferative changes in the arterial vessel media. The underlying cause of this placental vascular obliteration has not been elucidated but factors thought to be involved include rejection of feto-placental tissue or aberration in the angiogenesis process (17, 18).

1.5 Definition and Pathogenesis of hypertensive disorders in pregnancy

1.5.1 Definition of hypertension in pregnancy

Hypertension in pregnancy may develop as a result of the pregnancy or following pre-existing hypertension (either essential or secondary). Hypertension arising for the first time after 20 weeks may be an isolated finding, such as gestational hypertension or part of a multisystem disorder, i.e. preeclampsia.

Following the consensus statement by The Australasian Society for the Study of Hypertension in Pregnancy (ASSHP) (19) and statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP) (20), hypertension in pregnancy is diagnosed when systolic blood pressure is $\geq$ 140 mmHg and/or diastolic blood pressure is $\geq$ 90 mmHg.

The hypertension in pregnancy guidelines have been revised twice since the ISSHP 2001 guidelines, by SOMANZ in 2008 and ISSHP/ SOMANZ 2014 (21).

1.5.2 Classification of hypertension in pregnancy

Gestational hypertension

Gestational hypertension is hypertension arising in pregnancy after 20 weeks gestation without any other feature of the multisystem disorder preeclampsia and which resolves within 3 months postpartum.

Preeclampsia

Preeclampsia is usually first detected by the measurement of high blood pressure but features other than hypertension are required to make a diagnosis. It is now recognized that preeclampsia is a disorder which affects other organ systems including the feto-placental unit. Proteinuria is the most commonly recognized feature of preeclampsia after hypertension but should not be considered mandatory to make the clinical diagnosis.

The definition of preeclampsia has changed over the last 15 years with the realisation that preeclampsia is a multisystem disorder and the different definitions are listed below.

2001 ISSHP and 2008 SOMANZ guidelines for diagnosis of preeclampsia (20, 22)

A clinical diagnosis of preeclampsia can be made when the following criteria are fulfilled:

- Hypertension arising after 20 weeks gestation and the onset after 20 weeks of gestation of one or more of:

- Proteinuria ≥ 300mg/24 hours or spot urine protein/creatinine ratio ≥30mg/mmol
- Renal insufficiency-serum/plasma creatinine ≥0.09mmol/L or oliguria
- Liver disease-raised serum transaminases and/or severe epigastric/right upper quadrant pain
- Neurological problems-convulsions (eclampsia); hyperreflexia with clonus; severe headache with hyperreflexia; persistent visual disturbances (scotoma)
- Haematological disturbances- thrombocytopenia; disseminated intravascular coagulation; haemolysis
- Fetal growth restriction

2014 ISSHP/SOMANZ guidelines for diagnosis of preeclampsia (21)

- A diagnosis of preeclampsia can be made when hypertension arises after 20 weeks gestation and is accompanied by one or more of the following signs of organ involvement:
- Renal involvement
    - Significant proteinuria –a spot urine protein/creatinine ratio ≥ 30mg/mmol
    - Serum or plasma creatinine > 90 μmol/L
- Oliguria: <80mL/4 hr Haematological involvement
    - Thrombocytopenia <100,000 /μL

    - Haemolysis: schistocytes or red cell fragments on blood film, raised bilirubin, raised lactate dehydrogenase >600mIU/L, decreased haptoglobin
    - Disseminated intravascular coagulation
- Liver involvement
    - Raised serum transaminases
    - Severe epigastric and/or right upper quadrant pain.
- Neurological involvement
    - Convulsions (eclampsia)
    - Hypereflexia with sustained clonus
    - Persistent, new headache
    - Persistent visual disturbances (photopsia, scotomata, cortical blindness, posterior reversible encephalopathy syndrome, retinal vasospasm)
    - Stroke
- Pulmonary oedema
- Fetal growth restriction (FGR)

Chronic hypertension

Presence or history of hypertension prior to conception or in the first half of pregnancy. It is considered “essential” if there is no underlying cause or “secondary” if associated with a definitive aetiology.

Preeclampsia superimposed on chronic hypertension.

Development of new signs and/or symptoms associated with preeclampsia after gestational week 20, as above, in a woman with chronic hypertension.

White coat hypertension

White coat hypertension is defined as hypertension in a clinical setting with normal blood pressure away from this setting when assessed by 24 hour ambulatory blood pressure monitoring or home blood pressure monitoring using an appropriately validated device.

Research definition of PE

The ASSHP also suggested a research definition of preeclampsia for investigators, restricted to new onset hypertension after 20 weeks with properly documented proteinuria, stating it will be less sensitive but more specific, ensuring recruitment of true preeclamptic patients into scientific research studies (19).

1.6 Preeclampsia – Pathophysiology and clinical implications

1.6.1 Introduction and clinical Implications

Preeclampsia affects 2–7% of pregnant women (23). This condition results in substantial perinatal morbidity and mortality across the world, especially in developing countries. While hypertension and proteinuria are the commonest features, preeclampsia is a multi-organ disorder, progressing to HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome and eclampsia (seizures) in some women. Preeclampsia only occurs in the presence of the placenta (24). Therefore, currently the only successful treatment remains the delivery of

the placenta and hence the fetus, which may lead to significant morbidity and even death of the baby in the case of early-onset preeclampsia.

1.6.2 Pathophysiology of preeclampsia

Speculation as to the aetiology of preeclampsia has been ongoing for many years. While progress has been made in describing the placental and maternal circulatory changes that are present in preeclampsia, the pathophysiologic mechanism of the cause of preeclampsia is still not clear. Two broad classes of preeclampsia have been described, maternal and placental, although an individual case can be a mix of the two aspects (25). The placental preeclampsia is postulated to result from a failure of villous trophoblast differentiation, which can on the placental side ultimately lead to an abnormal release of trophoblast material into the maternal circulation (26). Maternal preeclampsia is thought to result from endothelial dysfunction secondary to circulating factors released from the placenta (see section 1.6.2.3). Preeclampsia has also been further subdivided into early (< 34 weeks) preeclampsia with signs of fetal growth restriction and late onset preeclampsia (>34 weeks) with normally grown fetuses (27).

1.6.2.1 Genetics

A familial increase in the susceptibility to develop preeclampsia has been described, but extensive studies to date have not identified any particular gene or loci that can be used as a screening test, suggesting the involvement of multiple genetic factors (28, 29).

1.6.2.2 Abnormal placentation

Impaired extra villous cytotrophoblast invasion leading to inadequate vascular remodeling of the spiral arteries has been postulated to be central to the pathophysiology of preeclampsia (30). These suboptimal physiological changes result in reduced uteroplacental perfusion

leading to placental hypoxia and the release of placental circulatory factors that produce the maternal syndrome of preeclampsia (25, 31).

1.6.2.3 Endothelial dysfunction and activation

Endothelial activation in the maternal circulation has been described in preeclampsia and is thought to lead to the clinical manifestations in the mother (32, 33). The hypothesis was advanced that alterations of endothelial function could explain much of the pathophysiology of preeclampsia. Extensive data have been generated to support the hypothesis (34). Markers of endothelial activation can be demonstrated in women with preeclampsia, many preceding clinically evident disease and disappearing with resolution of the disease. It was postulated that factors produced by the poorly perfused placenta, entered the systemic circulation and altered endothelial cell activity. This was proposed to change vascular sensitivity to circulating vasopressors, activate coagulation, and reduce vascular integrity resulting in the pathophysiological changes of preeclampsia (33).

The hypothesis has been expanded to include maternal susceptibility to preeclampsia by the generation of endothelial injury and injurants. This concept is stimulated by the observation that reduced placental perfusion per se is not sufficient to generate the maternal syndrome. Women with growth restricted fetuses and placentas from preterm deliveries also demonstrate failure of the physiological remodeling of decidual vessels resulting in reduced placental perfusion in preeclampsia.

This has led to the concept that preeclampsia is secondary to an interaction of reduced placental perfusion and maternal factors such as obesity, insulin resistance, lipid status, ethnicity, hypertension, and elevated plasma homocysteine concentration. These factors are also risk factors for atherosclerosis in later life (33).

1.6.2.4 Immunology of preeclampsia

It is suggested that the placental aspects of preeclampsia may have an immunological basis, with maternal immune responses to trophoblast determining normal and abnormal placentation (25). It is hypothesised that the maternal immune system 'learns' to accommodate the fetus, and that pre-eclampsia results from a relative failure to develop maternal tolerance to paternal allo-antigens (35).

1.6.2.5 Placental histopathology

There is no single placental lesion pathognomonic of preeclampsia. The histopathological changes seen in preeclampsia can be categorized into several different groups of pathologies (36).

- Uteroplacental vascular pathologic features and secondary villous damage - These changes are seen in 26% of preeclamptic placentas and include villous fibrosis, hypovascularity, increased syncytiotrophoblast knotting, villous infarcts and abruption placentae (37). Placental infarcts seen in preeclamptic placentas are thought to be due to occlusion of maternal spiral arteries (37).

- Lesions involving coagulation - The changes within the placenta include thrombi within chorionic and fetal stem vessels and microcirculatory occlusion demonstrated by avascular terminal villi (36).

- Lesions involving chronic inflammation-Changes include chronic uteroplacental vasculitis, decidual plasma cell infiltrates, decidual eosinophil infiltrates, dense basal lymphocytic infiltrates and villitis of anchoring villi (36).

- Lesions involving coagulation and chronic inflammation have been documented independent of uteroplacental vascular pathologic features and may represent a part of a repair response to placental ischaemic damage (36).

1.6.2.6 Involvement of other organs

Glomerular endotheliosis involving glomerular hypertrophy, narrowing or occlusion of capillary lumens by swelling of endothelial cells and mesangial cells is the most characteristic renal lesion seen in preeclampsia and is a specific variant of thrombotic microvasculopathy (38). Glomerular endotheliosis is not a lesion specific to preeclampsia. The more severe lesions are seen in preeclampsia while milder pathology is described in normal pregnancy and gestational hypertension. Pathological changes seen in other organs such as petechial haemorrhages in the brain, liver, adrenal and heart are thought to be most likely due to hypoperfusion (38).

1.7 Intrauterine fetal growth restriction (IUGR)

1.7.1 Incidence, morbidity and mortality

Intrauterine growth restriction is a common complication that occurs in 4-7% of births in developed countries (12). It carries an increased risk of perinatal morbidity and mortality (12, 39, 40). Growth restricted fetuses have a 4-8 fold increase in perinatal mortality compared with fetuses in the normal range of growth (13). Fifty percent of those that survive experience short and long term morbidity including intrapartum fetal distress, hypoglycaemia, hypocalcaemia, meconium aspiration pneumonia (41) and abnormal neurological impairment (42). At least 20% of stillborn fetuses have evidence of fetal growth restriction, a disproportionately high rate that indicates a higher risk of fetal demise (43). The majority of SGA babies are born to normotensive mothers (81.7%), but some are born to women with

preeclampsia (10.7%) or gestational hypertension (7.6%) (44). Small for gestational age neonates at birth can also be associated with impaired neural development and cognition (45, 46). Epidemiological studies have highlighted a correlation between low birth weight and adult vascular disease such as coronary artery disease, stroke, type 2 diabetes and hypertension (47, 48).

1.7.2 Intrauterine growth restriction (IUGR): Clinical definitions

Fetal growth depends on modulation of the genetically predetermined growth potential by fetal, placental, maternal and external factors. Fetuses who are not reaching their genetic growth potential are considered growth restricted. There is still debate over a precise definition. Birth weight $<10^{th}$ centile is described as small for gestational age (SGA) while varying thresholds ($<3^{rd}$, $<5^{th}$ or $<10^{th}$ centile) have been used as intrauterine growth restriction (49). The causes of SGA and IUGR are varied including congenital abnormalities, infections, substance abuse and constitutionally small fetuses. SGA fetuses may not all be growth restricted (constitutionally small) and growth restricted fetuses may not all be SGA.

Although the etiology of fetal growth restriction can be varied, IUGR resulting from placental insufficiency is very relevant to clinical management as the outcome for the fetus can be altered by appropriate timely diagnosis and delivery. Ultrasound assessment of fetal anatomy, biometry, liquor volume and Doppler studies are central to the management of a suspected IUGR fetus. Umbilical artery Doppler assessment to determine impedance in the feto-placental circulation has become the clinical standard for identifying IUGR in preterm pregnancies <34 weeks (50-52). In a review by the Cochrane collaboration, the only investigation proven by randomized controlled trials to reduce perinatal mortality was umbilical artery Doppler ultrasound (53). Unfortunately umbilical artery Doppler is not

reliable in late onset fetal growth restriction >34 weeks of gestation and significant morbidity and mortality occurs even in fetuses with normal Doppler waveforms (54).

1.7.3 IUGR Pathogenesis /Abnormal placentation

Disorders of placental implantation are thought to be central to the pathogenesis of IUGR as well as preeclampsia. Large numbers of non-transformed spiral arteries as well as obstructive lesions such atherosis and thrombosis can be seen in the maternal vascular bed in IUGR (55). Suboptimal maternal adaptation to pregnancy and inadequate vascular remodeling of maternal spiral arteries are suggested to increase placental vasoactive substances, leading to vascular reactivity (30). If hypoxia stimulated angiogenesis cannot overcome these challenges, placental auto regulation becomes deficient. Maternal placental floor infarction, villous obliteration and fibrosis increase the resistance to blood flow, producing a fetal-maternal mismatch that decreases placental mass and the effective exchange area. The balance of compensatory and non-compensatory mechanisms determines the fetal outcome. If compensatory mechanisms are unsuccessful, stillbirth can be the result. With successful compensation, the shortage of nutrients could be subclinical, unmasked by the exponential requirement for nutrients in late second and third trimester. Vascular studies may appear normal while asymmetrical growth may be the only manifestation of fetal growth restriction (56).

1.7.4 IUGR Placental histopathology

Histopathological changes associated with the clinical syndromes of preeclampsia and intrauterine fetal growth restriction have been well described, although placental findings in IUGR are often varied, from morphologically unremarkable through to severe uteroplacental vasculopathy, with no single pathological feature associated with high sensitivity or

specificity (2, 36, 57).. The morphologic features such as maternal vascular changes, villous infarction, shrinkage and loss of villi, excessive numbers of syncytiotrophoblastic knots, and accelerated peri-villous fibrin deposition, are generally presumed to result from impaired utero-placental blood flow. Extensive infarction and thrombosis can also be noted in severe disease (58).

Pregnancies complicated by IUGR secondary to placental vasculopathy share many pathogenic abnormalities with preeclampsia (24), including maternal endothelial cell dysfunction (59-61) and leukocyte (neutrophil) activation (62). Uteroplacental fibrinoid necrosis, circulating nucleated erythrocytes, avascular terminal villi, villous fibrosis and villous infarction were significantly represented in placentae of fetal growth restriction (57). Decidual vasculopathy and acute atherosis have been described in the preeclamptic and IUGR maternal spiral arteries as a contributor to pathophysiology (63, 64). This is especially relevant in view of the possible predisposition to preeclampsia in the presence of the maternal metabolic syndrome (55, 65).

1.7.5 Preeclampsia and fetal growth restriction-shared and disparate components

Preeclampsia and IUGR are pregnancy specific disorders characterized by abnormal placentation. There is evidence of endothelial dysfunction in IUGR and preeclampsia. Pre-existing conditions such as hypertension, renal disease, systemic lupus erythematosis and older age that involve endothelial dysfunction are common to IUGR and preeclampsia (66). Family history of hypertension, coronary heart disease and stroke elevates the susceptibility to preeclampsia (67). Endothelial activators such as vascular cellular adhesion molecule-1 (VCAM), intercellular adhesion molecule-1 (ICAM) and endothelin-1 are elevated in the

maternal plasma and serum of women with both conditions (60, 68, 69), although more extensively in preeclampsia. These lines of evidence support the view that endothelial dysfunction predisposes to IUGR and preeclampsia (66). With similar predisposition and placental pathology, why some pregnant women develop maternal disease of preeclampsia while other pregnancies show IUGR only is a question yet to be answered.

It has been postulated that both preeclampsia and IUGR arise from a maternal predisposition to endothelial dysfunction, which contributes to shallow implantation. Release of placental cytokines from an endothelial induced placental abnormality in the presence of the maternal metabolic syndrome of adiposity, insulin resistance, hyperglycaemia, hyperlipidaemia and coagulopathies may lead to preeclampsia, while absence of the maternal metabolic syndrome can lead to isolated IUGR (66). This theory focuses on maternal predisposition only. Contribution of a potential fetal susceptibility and circulation to the risk of IUGR and preeclampsia has not been well addressed in research to date.

1.7.6 Preeclampsia and IUGR: long term maternal and neonatal outcomes

The long term maternal and neonatal cardiovascular risks of preeclampsia have attracted recent research interest. Women who have had preeclampsia appear to be at increased risk of premature death, mortality from ischaemic heart disease, cardiovascular diseases including ischaemic heart disease, hypertension, stroke, venous thromboembolism, renal failure and type 2 diabetes while being relatively protected from cancer (47, 70).

Children born to mothers affected by preeclampsia during the pregnancy appear to be more prone to hypertension, insulin resistance, diabetes, stroke and neurological problems (70).

Similar increases in maternal and fetal risks have been identified with a history of isolated intrauterine fetal growth restriction (71).

1.8 Angiogenic Factors and their function

1.8.1 Angiogenic factors

Vascular Endothelial Growth Factor family

The best-characterized group of angiogenic growth factors is the vascular endothelial growth factor (VEGF) family. VEGF, also referred to as VEGF-A, is the founding member of this family, which also includes VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF), and a recently identified tissue-specific endothelial growth factor (endocrine gland-derived vascular endothelial growth factor, EG-VEGF) (9, 72, 73). The VEGF family also includes their receptors VEGFR-1 (also called Flt-1), VEGFR-2 (also called KDR, in humans and fetal liver kinase, Flk in mice) and VEGFR-3 (Flt-4), as well as the co-receptors, neuropilin-1 (NRP-1) and NRP-2 (74, 75).

1.8.1.1 Vascular endothelial growth factor-A

Vascular endothelial growth factor (VEGF**)** was originally described as "vascular permeability factor" (76) and as "vascular endothelial cell growth factor" (77). It is a heparin binding angiogenic growth factor which is an endothelial cell-specific mitogen *in vitro* and an angiogenic inducer in a variety of *in vivo* models (78, 79). For more than a decade, the role of vascular endothelial growth factor (VEGF) in the regulation of angiogenesis in physiological and pathological processes has been the object of intense investigation (80). Multiple studies have characterized the actions of VEGF and its receptors to be mainly mitogenic for

endothelial cells as well as playing a major role in mediation of vasculogenesis, angiogenesis, control of microvascular permeability, vasodilatation and anti-apoptotic activity (78, 80, 81). VEGF mRNA is produced by tumour cells as well as in non-malignant cells in response to hypoxia (82) and inflammation.VEGF mediates its effects mainly by interacting with two tyrosine kinase receptors, VEGF receptor-1 (Flt-1) and VEGF receptor-2 (KDR) (81).

1.8.1.2 Placental growth factor (PlGF)

Placental growth factor (PlGF) is an angiogenic factor that shares a 42% sequence homology with VEGF based on amino acid and cDNA sequences (83). Found on human chromosome 14, PlGF consists of seven exons. Alternative mRNA splicing of the primary PlGF transcript results in four isoforms, PlGF-2 shown to be the clinically active form (84). Genetic deficiency of PlGF does not significantly affect vascular development in the embryo (85). Although PlGF is mainly expressed in trophoblasts (86), a contribution from human umbilical vein endothelial (HUVEC) cells, placenta, heart and lungs has also been shown (87, 88). Interaction between the VEGF family angiogenic factors and their receptors has been summarized in Figure 1.2.

1.8.2 Angiogenic factors and their function

1.8.2.1 Vascular endothelial growth factor activities

Mitogenesis, angiogenesis, and endothelial survival

VEGF promotion of vascular endothelial cell (EC) growth derived from arteries, veins, and lymphatics has been well documented *in-vitro* (78). The vascular progenitors differentiate to ECs in response to VEGF. While VEGF-A is a key regulator of blood vessel growth, VEGF-C and VEGF-D regulate lymphatic angiogenesis (89). Although endothelial cells are the primary targets of VEGF, several studies have reported mitogenic effects also on certain non-endothelial cell types, such as retinal pigment epithelial cells (90), pancreatic duct cells (85), and Schwann cells (86). VEGF has also been shown to stimulate surfactant production by alveolar type II cells, resulting in a protective effect from respiratory distress syndrome in mice (91).VEGF is a survival factor for endothelial cells, both *in vitro* and *in vivo* (92-94). *In vitro*, VEGF prevents endothelial apoptosis induced by serum starvation. Such activity is mediated by the phosphatidylinositol 3-kinase (PI3 kinase)/Akt pathway (93, 94).

Effects of VEGF on bone marrow cells and hematopoiesis

The earliest evidence of VEGF activity on blood cells was a report describing its ability to promote monocyte chemotaxis (95). Associations between monocyte function and angiogenic factors have been detailed further in Section 1.12.3. Subsequently, further effects of VEGF on hematopoietic cells have been described, including inducing colony formation by mature subsets of granulocyte-macrophage progenitor cells (79), increase production of B cells and the generation of immature myeloid cells (80). VEGF delivery to adult mice inhibits dendritic cell development (81), leading to the hypothesis that VEGF facilitates tumor growth by allowing escape of tumors from the host immune system (57).

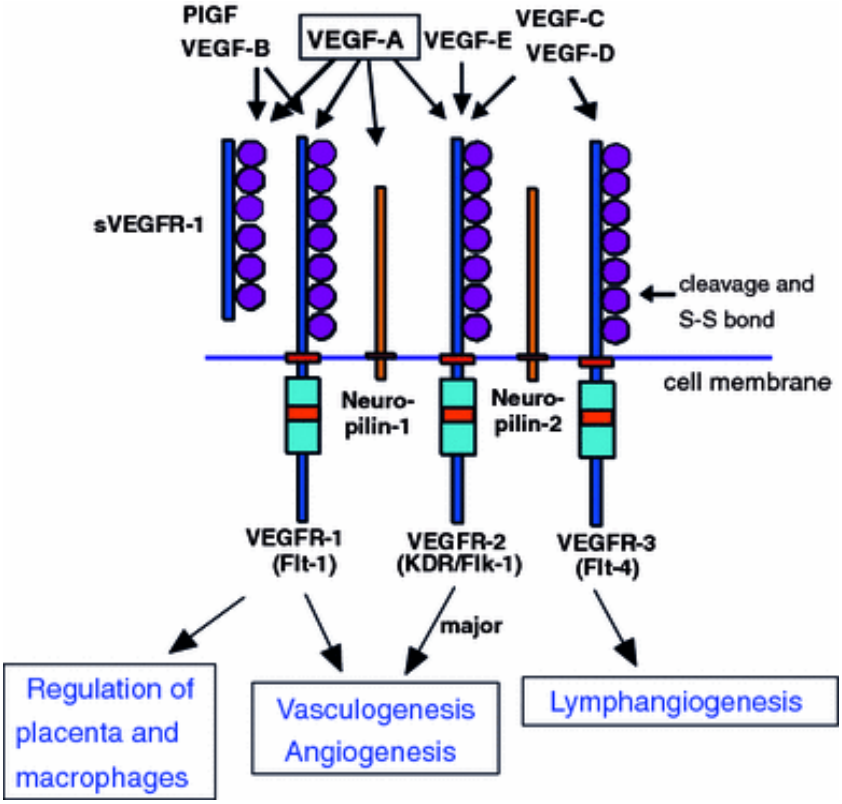

Figure 1.2 VEGF and the VEGF-receptor system.

VEGF-A, a major contributor to angiogenesis, binds and activates VEGFR-1 as well as VEGFR-2, and regulates vasculogenesis, angiogenesis, inflammatory responses, and carcinogenesis. The soluble form of VEGFR-1 appears to be an important modulator for the placental vasculature. (With permission from Shibuya 2006 (96)).

Enhancement of vascular permeability and hemodynamic effects

The ability of VEGF to induce microvascular permeability is a step necessary for angiogenesis, by providing extravasation of fibrin, which represents a scaffold for endothelial cell proliferation and migration (97). This function may play an important role in inflammation and other pathological circumstances (80).

Several studies have pointed to the critical role of nitric oxide (NO) in VEGF-induced vascular permeability, as well as angiogenesis (98-100).

1.8.2.2 Role of VEGF in Physiological Angiogenesis

VEGF has been identified as a major determinant of angiogenesis in normal physiological processes.

VEGF A has been shown to have an essential role in vasculogenesis and angiogenesis in the mouse embryonic and postnatal development. VEGF C regulates lymphatic development (80). Inactivation of a single or both alleles of VEGF –A and C result in embryo lethality by day 12. VEGF is required not only for proliferation but also for survival of endothelial cells (101). VEGF gradient is needed for directional growth and cartilage invasion by metaphyseal blood vessels (102, 103). VEGF-dependent blood vessel recruitment is essential not only for coupling cartilage resorption with bone formation, but also for bone homeostasis (104).

VEGF plays an important role in reproductive homeostasis, being involved in the cyclic proliferation and regression of blood vessels in the endometrium, during ovarian follicle development (105, 106), oocyte fertilization and development and homeostasis of germ cells (9). VEGF has been established as the principal regulator of ovarian angiogenesis and plays a key role in corpus luteal development and function by establishing vasculature for delivery of cholesterol to luteal cells for progesterone biosynthesis (105, 107). Recently, VEGF-induced capillary network has been identified as essential for pancreatic cell angiogenesis and fine tuning blood glucose regulation (108).

VEGF and KDR expression has been found on type II pneumocytes, and VEGF directly stimulated surfactant production of type II pneumocytes *in vitro* (9, 109). These data indicate an important role for VEGF in fetal lung development via its effect on epithelial maturation,

independent of its pro-angiogenic effects. Genetic studies in mice have revealed an important role for VEGF and PlGF in renal development and disease. During glomerular development, podocytes express VEGF, Flt-1 and KDR (110). Conditional deletion of VEGF in glomerular podocytes resulted in nephron malformation and nephrotic syndrome (111).

1.8.2.3 Role of VEGF in Pathological Conditions

Solid tumors and hematological malignancies

In situ hybridization studies have shown that VEGF mRNA is upregulated in many human tumours, including carcinoma of the lung, breast, gastrointestinal tract, kidney, ovary, endometrium and glioblastoma multiforme (80). Clinical trials in cancer patients are ongoing with several VEGF inhibitors, including a humanized anti-VEGF monoclonal antibody and an anti-KDR antibody bevacizumab (Avastin) (80). Thrombosis, increased blood pressure, and proteinuria were among the side effects of treatment in initial studies (112).

Intraocular neovascular syndromes

Neovascularization and vascular leakage are major cause of visual loss in diabetes mellitus, occlusion of central retinal vein, oxygen exposure in prematurity as well as age-related macular degeneration (113, 114). Animal studies have clearly shown the role of VEGF as a mediator of ischaemia induced intraocular neovascularization (115).

Inflammation and brain edema.

VEGF up-regulation has been implicated in various inflammatory disorders such as psoriasis, rheumatoid arthritis, brain edema as well as in keratinocytes in wound healing (80).Vascular endothelial growth factor has been shown to have anti-apoptotic activity, and whether it plays a role in abnormal placental apoptosis is unknown.

1.8.2.4 Placental growth factor activities

Although the exact physiological actions of PlGF are not clear, evidence suggests a pivotal role for PlGF in regulating VEGF-dependent angiogenesis under pathological conditions (85). PlGF has been hypothesized to play a role in placental development and angiogenesis. PlGF is a very weak stimulator of endothelial chemotaxis and proliferation, and when binding competition studies are performed with extracellular domains from either Flk-1/KDR or Flt-1, PlGF appears to be able to bind to Flt-1 but not to Flk-1/KDR (116). PlGF homodimers bind Flt-1 and NRP-1 while PlGF/VEGF-A heterodimers bind KDR and Flt-1/KDR heterodimers *in vitro* (24).

Evidence to date suggests that, whereas the VEGF/KDR axis is important for physiological angiogenesis and pathological angiogenesis, the PlGF/Flt axis appears to be specifically crucial for pathological angiogenesis by modulating the effects of the VEGF/KDR axis (9).

Proposed mechanisms by which PlGF potentiates angiogenesis include stimulating endothelial cells via Flt-1, separating VEGF-A from Flt-1 by competing for the receptor, allowing VEGF-A to activate KDR, recruiting monocytes/macrophages which have a crucial role in vessel growth (88) and inducing the secretion of VEGF-A from monocytes (117).

Monocytes/macrophages have been proposed as critical players in the process of angiogenesis and wound healing. The strong placental expression of PlGF could contribute to the increased demand for angiogenesis in the growing placenta which may be partially mediated by chemo-attraction of peripheral blood monocytes (9).

1.8.3 Angiogenic factors and their control

1.8.3.1 VEGF Isoforms

The human VEGF-A gene is localized in chromosome 6p21.3 (118). It is organized into eight exons, separated by seven introns. Alternative exon splicing of the VEGF-A gene results in the generation of four different isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$ (119, 120). Bioavailable native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa (121). Such properties closely correspond to those of $VEGF_{165}$, which has been identified as the major VEGF isoform (122). The two VEGF monomers are oriented side-by-side and head-to-tail and held together by one interchain disulfide bond to form the dimeric structure which is stabilized by a hydrophobic core region (123, 124).

1.8.3.2 Regulation of VEGF Gene Expression

Hypoxia is a potent stimulus for the expression of *VEGF-A* mRNA and is mediated via hypoxia-inducible-factor-1α (125, 126). In addition, several growth factors including fibroblast growth factor, transforming growth factors (TGF-α and TGF-β), keratinocyte growth factor, insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor, as well as the inflammatory cytokines, interleukin (IL)-1α and IL-6, hormones such as TSH, βhCG are also known to up-regulate VEGF-A expression (24, 81).

1.9 Angiogenic factor receptors: VEGR Receptors

VEGF binding sites were initially localized to the vascular endothelial cell surface *in vitro* (127) and *in vivo* (128, 129). Subsequently, it has been shown that VEGF binding sites also occur on bone marrow derived cells such as monocytes (130).

The biological effects of VEGF are mediated by two high affinity receptor tyrosine kinases (RTKs) VEGFR-1/ fms-like tyrosine kinase (Flt-1) and VEGFR-2/ kinase insert domain receptor (KDR). These differ considerably in signaling properties.

Both VEGFR-1 (Flt-1) and VEGFR-2 (KDR) have seven extracellular immunoglobulin-like domains, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (131, 132).

1.9.1 VEGFR-1 / fms-like tyrosine kinase (Flt-1)

Fms-like tyrosine kinase receptor (Flt-1) is a transmembrane receptor with its gene on chromosome 13q12-q13 (133). Flt-1 has been localized to several human tissues including endothelial cells, placental trophoblast, monocytes and macrophages (24, 80). Flt-1 expression is up-regulated by hypoxia via a HIF-1-dependent mechanism (134). Flt-1 binds VEGF-A, VEGF-B and PlGF with high affinity (116, 135). The latter molecules do not bind KDR (80), the main functional receptor of VEGF-A. Although the binding site for VEGF and PlGF has been identified as primarily the second Ig-like domain on the receptor (136-138), binding of VEGF leads to a weak tyrosine auto-phosphorylation, while activation of Flt by PlGF results in the expression of distinct target genes (139, 140). The binding-affinity of Flt-1 for VEGF-A is higher in magnitude than that of KDR, whereas the kinase activity of Flt-1 is about 10-fold weaker than that of KDR (96).

Recently, it has been shown that PlGF regulates inter- and intra-molecular cross-talk between the VEGF receptors. Activation of Flt-1 by PlGF resulted in trans-phosphorylation of KDR (141). The observed potentiation of the action of VEGF by PlGF could be explained, at least in part, by displacement of VEGF from Flt-1 binding (116) and also partially by trans-phosphorylation of KDR, thus amplifying VEGF-driven angiogenesis through KDR.

In adulthood, Flt-1 tyrosine kinase transduces several downstream signals including cell migration and mild DNA synthesis (Figure 1.3). However, signal transduction pathways from Flt-1 leading to vascular permeability and migration of macrophages as well as vascular endothelial cells remain to be clarified (96).

1.9.1.1 Soluble VEGFR-1/Flt-1 (sFlt-1)

Alternative splicing of the Flt-1 pre-mRNA results in the production of sFlt-1, a soluble form comprising the ligand-binding domain of Flt-1 but lacking the membrane-spanning and intracellular domains (142). Soluble Flt-1 has been shown to be secreted by endothelial cells, monocytes and placental tissues (96). Flt-1 binds not only VEGF-A but also PlGF (116) and VEGF-B (135). sFlt-1 has potent antagonistic action of VEGF-A and PlGF, by inhibiting their binding to cell surface receptors as well as by forming heterodimers with the other VEGF receptor KDR (143).

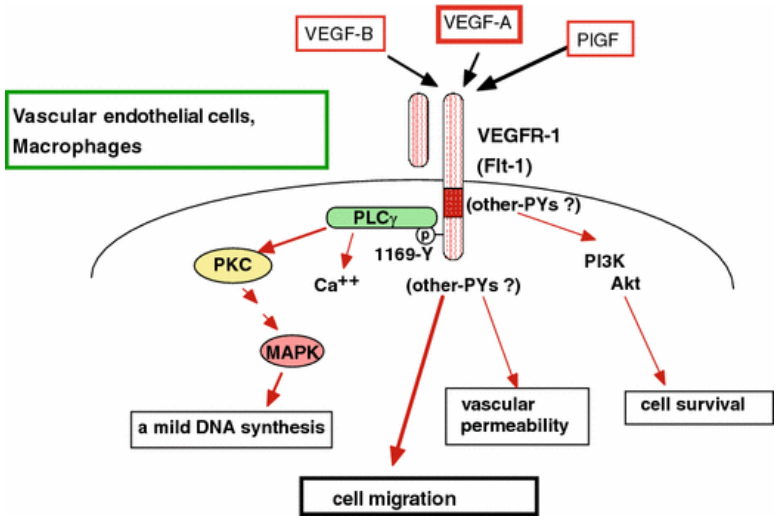

Figure 1.3 Possible signal transduction pathways from VEGFR-1. VEGFR-1(Flt-1) tyrosine kinase transduces several downstream signals including cell migration and mild DNA synthesis in adulthood (Included with permission from Shibuya 2006 (96)).

The evidence to date show that synergism exists between VEGF and PlGF *in vivo*, in a complex maze of interactions involving Flt-1, KDR, sFlt and sKDR. The known mechanisms of interactions are summarized in Figure 1.2.

It has been proposed that Flt-1 may not be primarily a receptor transmitting a mitogenic signal, but rather a "decoy" receptor, able to regulate in a negative fashion VEGF activity on the vascular endothelium, by sequestering VEGF and rendering this less available to VEGFR-2/KDR (116). The membrane-bound form of Flt-1, as well as the soluble form sFlt-1, has the ability to perform such a decoy function (85).

Gene-targeting studies on Flt-1 have demonstrated its essential role during embryogenesis. Flt-1 knockout mice show excessive proliferation of angioblasts with failure to organize vascular channels, leading to fetal death in early gestation (144, 145).

It has been shown that the migration of monocytes/macrophages in response to VEGF or PlGF is mediated by Flt-1 (146, 147) and requires the tyrosine kinase domain of Flt-1 (148). Since Flt-1 mRNA was detected in the early mouse trophoblast before day 13, it is possible that Flt-1 may regulate the migration of trophoblast cells in implantation (142). Key functions of Flt-1 signaling in the vascular endothelium could be the paracrine release of tissue-specific growth/survival factors, possibly in a vascular bed-specific fashion (149), as well as the regulation of angiogenesis.

These characteristics of Flt-1 suggest that it has a dual function in angiogenesis (80), as both a negative regulator by sequestering VEGF via its ligand-binding domain, and a positive regulator by signaling towards migration/proliferation via its tyrosine kinase activity (96). sFlt-1 mRNA has been detected using RT-PCR analysis in adult lung, liver, kidney, and

uterus, suggesting that sFlt-1 may have a role in maintaining endothelial cells in a quiescent state in the adult.

1.9.2 VEGFR-2 / Kinase domain region (KDR human, Flk-1 mouse)

KDR is a transmembrane receptor with its gene localized to chromosomes 4q11-q12 (133, 150). KDR binds VEGF-A, VEGF-C and VEGF-D and is expressed by endothelial cells, neuronal cells and megakaryocytes. The binding affinity of VEGF-A for KDR is lower than that of Flt-1 (dissociation constant (K_d) 75–250 pm *vs.* 25 pm) (151). The receptor plays a key role in developmental angiogenesis and hematopoiesis as evidenced by lack of vasculogenesis and failure to develop blood islands and organized blood vessels in Flk-1 null mice, resulting in death in-utero between day 8.5 and 9.5 (152). The pro-angiogenic biological activities of VEGF and PlGF appear to be mediated exclusively by the KDR/Flk-1 receptor and include the mitogenic, angiogenic, and permeability-enhancing effects of VEGF on the endothelium (153). The expression of KDR appears to be auto-regulated, being up-regulated by VEGF-A, VEGF-C and VEGF-D.

sKDR is a soluble form of the KDR receptor detected in human plasma. *In vitro* studies have determined that the sKDR fragment can be found in the conditioned media of mouse and human endothelial cells, thus suggesting that it may be secreted, similar to sFlt-1, or proteolytically cleaved from the cell (154). While soluble KDR is also considered to have anti-angiogenic properties (155), its mechanism of action has not been defined. Similar to sFlt-1, sKDR may have regulatory consequences with respect to VEGF-mediated angiogenesis as well as potential to serve as a quantitative biomarker of angiogenesis and anti-angiogenic drug activity.

1.9.3 Neuropilins (NRP)

In addition to the receptor kinases, VEGF interacts with a family of co-receptors, the neuropilins. NRP-1 binds VEGF-A, VEGF-B and PlGF while NRP-2 binds VEGF-A, VEGF-C and PlGF (156). In endothelial cells, NRPs are thought to increase VEGF signaling by ensuring optimal presentation of ligands to the receptors and by stabilizing VEGF/VEGFR complexes (24, 157).

The interaction between VEGF-A, PlGF and the two tyrosine kinase receptors FLT and KDR are represented in Figure 1.4. Figure 1.5 summarizes the role of the VEGF receptor tyrosine kinases in different cell types. The angiogenic response to VEGF varies between different organs and is dependent on the genetic background of the animal. Inactivation of the genes for VEGF-A and VEGF receptor-2 / KDR leads to embryonic death due to the lack of endothelial cells. Inactivation of the gene encoding VEGF receptor-1/Flt-1 leads to an increased number of endothelial cells, which obstruct the vessel lumen. VEGF receptor-1/Flt-1 exerts a negative regulatory effect on VEGF receptor-2/KDR, at least during embryogenesis (158).

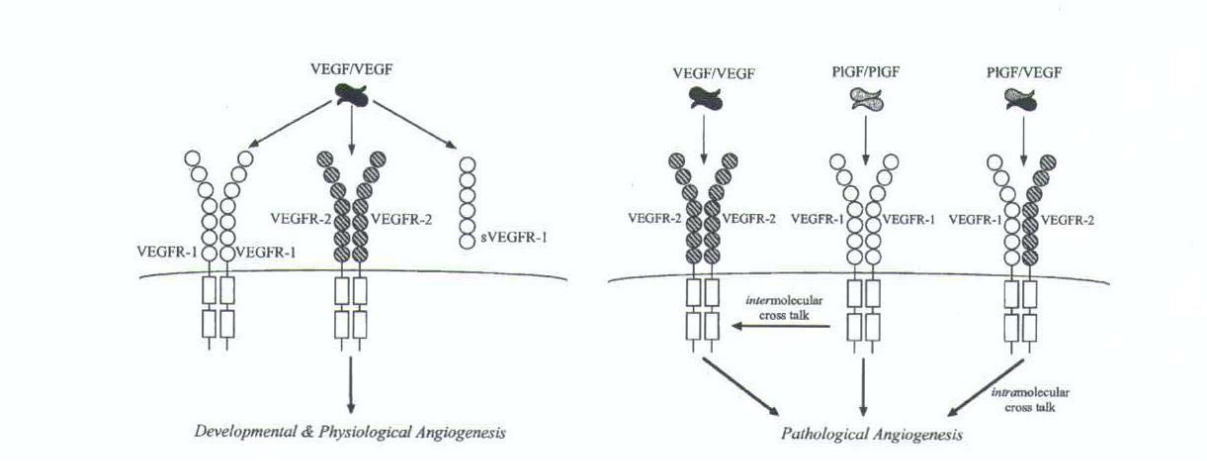

Figure 1.4 Schematic representation of the role of VEGF and PlGF in angiogenesis. The VEGF/VEGFR-2 axis is crucial for developmental and physiological angiogenesis, whereas VEGFR-1 (during development mainly present in its soluble form, sVEGFR-1) regulates VEGF bioavailability (left panel). In contrast, PlGF is a master switch of pathological angiogenesis after birth (printed with permission from Tjwa (9).

1.9.4 Soluble Endoglin (sEng)

In 2006, a novel placenta-derived soluble TGF-beta co-receptor Endoglin (sEng) was identified. Endoglin also known as CD105 is an 180kDa homodimeric transmembrane glycoprotein, a co-receptor for members of the TGF-β superfamily TGF-β_1 and TGF-β_3 (159). This co-receptor may have roles in hematopoiesis, cardiovascular development and angiogenesis. Endoglin-/- knockout mice die in utero at day 10-11 and display weak embryonic vasculature and abnormal cardiac development (160, 161). In humans, mutations of the Endoglin gene are responsible for hereditary telangiectasia type 1 (162).

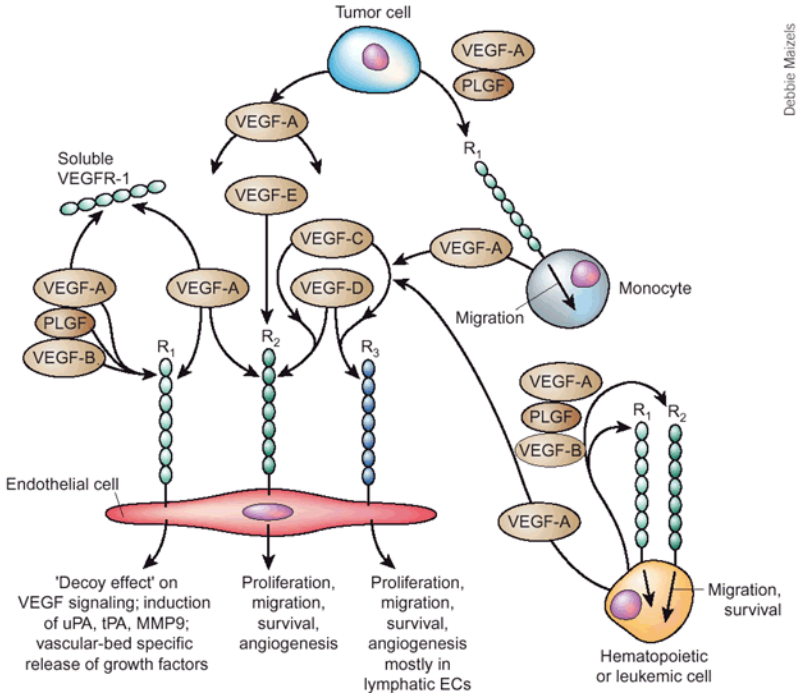

Figure 1.5 Role of the VEGF receptor tyrosine kinases in different cell types.

Reproduced with permission from Ferrara N 2003 (89). VEGFR-1 and VEGFR-2 are expressed in the cell surface of most blood endothelial cells. However, VEGFR-3 is largely restricted to lymphatic EC.

Endoglin has been shown to be highly expressed in vascular endothelial cells (163), chondrocytes (164), erythroid precursors, a subpopulation of hematopoietic stem cells, monocytes (165) and term placental syncytiotrophoblast (166). Although its function has not been clearly elucidated, increased levels of soluble form of Endoglin have been demonstrated in atherosclerosis (167), certain cancers including breast (168), colorectal and myeloid malignancies (169). sEng, the extracellular domain of the co-receptor Endoglin released by proteolytic cleavage of the membrane-bound Endoglin (170), impairs binding of transforming growth factor-β_1 to cell surface receptors, leading to decreased endothelial nitric oxide signaling, hence inhibiting angiogenesis and promoting vascular dysfunction (171).

1.10 Role of angiogenesis and angiogenic factors in placental vascular disease associated with preeclampsia and intrauterine fetal growth restriction

1.10.1 VEGF family angiogenic factors and receptors in normal placenta.

Angiogenesis and vascular transformation are integral processes in the normal development of the placenta. The successful establishment requires the development of a fetal placental circulation and vascular remodeling of the maternal vessels. Several factors have been shown to play a significant role in placental angiogenesis including VEGF, PIGF, FGF, insulin like growth factor (IGF), the angiopoietin (ANG) protein families, as well as their respective receptors (80) .

In the human placenta VEGF has been identified in villous trophoblast in the first trimester and in the syncytiotrophoblast and extra-villous trophoblast at term (1). VEGF is implicated in trophoblast proliferation, differentiation and invasion of the uterine vessels as well as ongoing angiogenesis of the umbilical circulation (172). PlGF belongs to the VEGF family and plays an important role in enhancing VEGF-driven angiogenesis. In contrast, loss of PlGF impairs angiogenesis, wound healing and inflammatory responses (139, 173).

KDR is the major signal transducer of VEGF in endothelial cells, and mediates most known VEGF's bioactivities including cell proliferation, migration, and permeability (89). In contrast, Flt-1 has been shown to have an inhibitory role in VEGF dependent endothelial function (153). VEGF, PlGF and their receptors Flt-1 and KDR are expressed in the placental trophoblast throughout gestation (153). Early studies into the expression and localization of angiogenic factors in the placenta have described varying results that may have been dependent on the techniques used.

In first trimester placenta, VEGF immunoreactive protein has been detected in the cytotrophoblast shell suggesting a role in the regulation of cytotrophoblast growth and differentiation as they also expressed VEGF receptor (Flt-1) protein (174). VEGF and Flt-1 immunoreactive proteins were expressed in Hofbauer cells within the villous mesenchyme, macrophages and in maternal decidual cells while weak VEGF immunoreactive protein was seen in syncytiotrophoblast surrounding the placental villi in first and second trimester placentae. Extra villous trophoblast showed immunostaining for Flt-1 but no staining for VEGF (174). Smooth muscle cells surrounding the vein and arteries of the umbilical cord showed weak VEGF immunoreactivity while no immunoreactivity was localized in endothelial cells in this study.

Immunostaining of PlGF protein has been localized to the vascular endothelium and syncytiotrophoblast membrane and in the media of large blood vessels of the placental villi, while staining within the mesenchyme was weak and diffuse (175). PlGF mRNA was predominantly expressed by the villous trophoblast, whilst there was no apparent expression of PlGF mRNA within the villous mesenchyme. These results suggest that PlGF may be an important paracrine factor for vascular endothelial cells in placental angiogenesis and an autocrine mediator of trophoblast function (175).

A study into localization and expression of VEGF and PlGF in placentas of normal pregnancy revealed that immunostaining localized the VEGF and PIGF proteins mainly in the vascular endothelium, while expression of mRNA was found in chorionic mesenchyme and villous trophoblast. These data imply that VEGF and PIGF are produced by different cells but that both target the endothelial cells of normal human term placenta (176). Another study on VEGF expression found mRNA to be produced by cells within the villous mesenchyme, decidual macrophages and decidual glands but not by trophoblast. The mRNA encoding PlGF

was produced in large amounts by villous cytotrophoblast, syncytiotrophoblast and extra villous trophoblast (177). The discrepancies are likely due to staining techniques used in the early studies into VEGF staining as more recent analyses have confirmed the expression of angiogenic factors using several techniques.

Placenta is thought to be the likely source of plasma sFlt-1 during pregnancy (178). sFlt-1 has been identified in conditioned medium of cultured mouse placenta. These results indicate that sFlt-1 is produced in the placenta (142). In a mouse model, the expression of Flt-1 and sFlt-1 in placental syncytiotrophoblast during pregnancy was stage dependent with mRNA encoding full-length membrane-bound Flt-1 detected in early gestation and high levels of sFlt-1 mRNA being expressed with increased gestation (142).

It is clear that sFlt-1 is a potent antagonist of VEGF *in vivo* and that the physiological alternative splicing of Flt-1 pre-mRNA to generate Flt-1 and/or sFlt-1 is used for the regulation of placental angiogenesis. sFlt-1 is also produced by human umbilical vein endothelial cells *in vitro* (143) suggesting that an alternative splicing mechanism for Flt-1 pre-mRNA also exists in endothelial cells. The balance of the locally expressed VEGF and sFlt-1 appear to be important in the regulation of placental endothelial cell function.

Previous studies localized Flt-1 protein and messenger RNA (mRNA) to extra villous trophoblast, syncytiotrophoblast, and villous cytotrophoblast in normal pregnancy (174, 179). It has been demonstrated that both Flt-1 isoforms can be detected in the placenta (180), and that sFlt-1 protein could be found in supernatant of explants of the placental villi. These observations suggest that sFlt-1 is produced by the human placenta and could be released into the maternal circulation. In 1990, a study by Shibuya et al. (131) showed that the short mRNA of the Flt-1 gene is highly expressed in normal placental tissue. Helshe et al. (181) showed that this mRNA is expressed on trophoblast in the placenta. *In situ* hybridization

using probes specific for the two forms of fms like tyrosine kinase and Northern blot analysis have shown that sFlt-1 expression increased but membrane bound Flt-1 decreased in placenta in late gestation (142).

The expression of the angiogenic growth factors, VEGF and PIGF has been demonstrated using RNase protection assays in isolated human term cytotrophoblast and in-vitro differentiated syncytiotrophoblast (182). VEGF expression increased approximately eightfold in trophoblast cultured under hypoxic conditions while PIGF expression decreased (182). These results suggest that distinct regulatory mechanisms govern expression of VEGF and PIGF in trophoblast. Characterization of the VEGF/PIGF receptors, KDR and Flt-1, revealed the presence of Flt-1 mRNA in isolated cytotrophoblast and *in vitro* differentiated syncytiotrophoblast. KDR was not detected in the isolated trophoblast. The authors suggested that trophoblast-derived VEGF/PIGF could act in a paracrine fashion to promote uterine angiogenesis and vascular permeability within the placental bed. In addition, presence of Flt-1 on normal trophoblast suggests that VEGF/PIGF functions in an autocrine manner to perform an as yet undefined role in trophoblast invasion, differentiation, and/or metabolic activity during placentation (182). The localization of angiogenic factors and their receptors in placental tissue has been summarized in Table 3.1.

Angiogenic factors	Positive tissue
VEGF	Cytotrophoblast, Syncytiotrophoblast, Vascular endothelium macrophages
PlGF	Cytotrophoblast, Syncytiotrophoblast, Extra villous Trophoblast Vascular endothelium, Villous stroma
Flt-1	Cytotrophoblast, Syncytiotrophoblast, Extra villous Trophoblast macrophages
KDR	Vascular endothelium

Table 1.1 Angiogenic factors and their receptor localisation in placental tissue (175, 176, 179).

1.10.2 Angiogenesis in placental vascular disease

Although it is the common belief that late spontaneous abortion, preeclampsia, intrauterine growth restriction and abruption share a common aetiology, these conditions give rise to different villous morphologies. In the model proposed by Kingdom and Kaufmann (30) it is suggested that high altitude and preeclampsia lead to reduced intra-placental oxygen concentration, which in turn lead to compensatory, predominantly branching angiogenesis of terminal villi and reduced vascular impedance. This might explain the low sensitivity of umbilical artery Doppler in the diagnosis of IUGR in late pregnancy (183). In pre-term IUGR with absent umbilical artery end-diastolic flow, there is a failure of terminal villi formation leading to impairment of feto-placental blood flow and transplacental gas exchange (16, 184).

Kingdom and Kaufmann suggest that in this situation, the feto-placental circulation is compromised to a greater extent than the uteroplacental circulation and that rising intra-placental oxygen content results in suppression of angiogenic drive to form terminal villi. This leads to abnormal non-branching angiogenesis, leading to increase in vascular impedance (30).

As growth is a function of cellular destruction as well as replication, cell apoptosis has been studied in the placenta. It has been observed that placental apoptosis increased as pregnancy progressed and that pregnancies complicated by intraIUGR and preeclampsia exhibited a higher degree of apoptosis compared to normal pregnancies (185, 186). Angiogenic factors such as VEGF have been shown to have anti-apoptotic activity, and whether it plays a role in abnormal placental apoptosis is unknown.

1.10.3 Placental tissue expression of VEGF family angiogenic factors in preeclampsia and IUGR.

Published literature has suggested a role for VEGF, its receptors and antagonists in the pathogenesis of preeclampsia and IUGR. Several research groups have demonstrated an increase in the maternal circulating splice variant of Flt-1 receptor (sFlt-1), a decrease in placental growth factor and soluble kinase domain receptor as well as a reduction in free VEGF with the development of preeclampsia (178, 187-189). Increased production of anti-angiogenic factors sFlt-1 and soluble Endoglin (sEng) by the placenta is postulated to contribute to the pathophysiology in preeclampsia (PE) by leading to endothelial dysfunction (190). Recent data on anti-angiogenic factors have demonstrated similar results in both PE

and IUGR, with no clear explanation as to why the maternal syndrome is not evident in isolated IUGR (189).

Studies on VEGF immunoreactivity of placental trophoblast in preeclampsia have shown inconclusive results (191-194). While some studies have demonstrated reduced VEGF immunoreactivity (195-197) compared with uncomplicated pregnancies, others have reported an increase in VEGF staining (191, 198). This study included a mix of patients with early and late onset of preeclampsia as well as intrauterine fetal growth restriction complicating preeclampsia (199). Placental VEGF expression has been demonstrated in placentas from both normotensive and preeclamptic women at term (191). The immunostaining in this study was localized to the syncytiotrophoblast and decidual trophoblast. The expression of VEGF was found to be further increased in amount and intensity of immunostaining in preeclampsia as compared to normal pregnancy. IUGR was specifically excluded in the patient selection of this study.

Discrepant findings have been published on placental VEGF mRNA levels in preeclampsia as compared to normal pregnancy. Variable results have been described including increased VEGF levels (193), reduced VEGF levels (200), and no difference in VEGF expression at delivery in decidua basalis or placenta in pregnancies complicated by preeclampsia (201).

Placental production and expression of sEng, Flt-1, and PlGF in normal and preeclamptic pregnancies were further assessed by immunohistochemistry and trophoblast cell culture under normal and hypoxic conditions (190). Immunohistochemical staining of trophoblast from preeclamptic placentas showed significantly higher sEng, sFlt-1, and PlGF compared with those from normal pregnancies. Under lowered oxygen conditions, trophoblast from PE, but not normal pregnancy released more sFlt-1 and less PlGF. Enhanced Flt-1 and soluble Flt-1 expression has been demonstrated in the placental tissues in preeclampsia using

Northern Blot analysis (155). A comparative morphological study of the placentas with preeclampsia showed increased expression of Endoglin and Flt-1 in all placental structures. The intensity of Endoglin and Flt-1 expression was maximum in the syncytiotrophoblast and extra villous cytotrophoblast cells in severe preeclampsia (202).

PIGF mRNA and protein are localized to the trophoblast bilayer and villous mesenchyme of the human placenta throughout gestation (175). Few studies have explored the placental angiogenic milieu associated with IUGR. Early placental development occurs in an environment of relative hypoxia. Hypoxia promotes angiogenesis and up-regulates vascular endothelial growth factor (VEGF) expression while it down-regulates placental growth factor (PlGF). One study has shown PlGF mRNA to be increased in placentas of IUGR pregnancies compared with gestationally-matched normal placentae (203). Contrasting findings have been described where hypoxia down-regulates placental growth factor, whereas fetal growth restriction up-regulates placenta growth factor expression (203).

A recent study into placental expression of VEGF family mRNA in adverse pregnancy outcomes (75) demonstrated reduced expression of VEGF, PlGF, Flt-1 and KDR in gestational hypertension, preeclampsia as well as small for gestational age fetuses. The selection criteria for the IUGR or SGA in the published studies have not included any physiological parameters such as umbilical artery or uterine artery Doppler studies that may identify placental cause of IUGR rather than constitutionally small fetuses.

KDR expression has been demonstrated in the placenta (181) and is known to be mainly localized to the vascular endothelium. Endothelial cells in the villi with hypoxic changes have demonstrated stronger immunostaining for KDR than did those in normal villi (192). While the expression of sFlt-1 in preeclampsia is well described, less is known about sKDR. A study into plasma KDR concentrations and placental mRNA expression showed that

preeclampsia is associated with reduced plasma concentrations of KDR as well as decreased placental expression of the sKDR mRNA splice variant (204). A previous report on preeclampsia showing no difference in KDR levels did not distinguish between membrane bound and soluble forms (205). Whether or not there is similar variation in IUGR has not been demonstrated.

In summary, while variable results have been demonstrated in multiple studies on the expression of angiogenic factors in the placenta, preeclampsia is generally associated with an increase in VEGF and Flt-1 while the results are not conclusive for PlGF. Hypoxic conditions lead to a reduction in PlGF. Placental expression of angiogenic factors in intrauterine growth restriction is not well categorized.

1.10.4 Soluble angiogenic markers in placental disease preeclampsia

Aberrations in the soluble angiogenic factors have been described in pregnancy complications such as preeclampsia and intrauterine fetal growth restriction.

The last decade has seen a plethora of publications investigating the role of angiogenic factors in placental disease, in particular the VEGF family, and their receptor levels in the maternal circulation. While it is evident that the angiogenic factors and their receptor levels are aberrant in placental disease, whether there are differences between preeclampsia with and without IUGR and isolated IUGR is not clear. It is still not established whether these changes are part of the pathogenesis of placental disease or a reactionary change to the disease process in the placenta.

1.10.4.1 VEGF-A in preeclampsia

Although VEGF plays an important role in placental angiogenesis, the serum or plasma level of VEGF-A has not been a reliable marker for identification of pathological pregnancies. The commercially available ELISA kits measure the free VEGF-A, which is the biologically active form. The measurable levels in the maternal blood have been documented to be below the detection levels of the assay (206-208). The few studies measuring total VEGF have demonstrated elevated VEGF levels in preeclamptic pregnancies as compared to normotensive pregnancies (205, 209-211). These findings suggest that the total circulating VEGF-A may be increased in preeclamptic pregnancies and that the free level is reduced due to binding to the soluble receptor sFlt-1. Soluble Flt-1 binds VEGF-A with a higher affinity than PlGF, resulting in extremely low circulating levels of free VEGF-A (75).

1.10.4.2 soluble fms like tyrosine receptor-1 (sFlt-1) in preeclampsia

Elevated sFlt-1 levels have been shown to be a feature of preeclampsia and are proposed to play a significant role in the pathogenesis of the condition by causing an anti-angiogenic state and endothelial dysfunction in the maternal circulation. Further support for this hypothesis is provided by the fact that the incidence of preeclampsia is increased in mothers carrying Trisomy 13 fetuses (212). The genes for sFlt-1 and Flt-1 are localized to chromosome 13 and maternal circulating levels of sFlt-1 have been demonstrated to be elevated in Trisomy 13 (213).

The first documentation of circulating elevated sFlt-1 in preeclampsia was by Clark et al 1998 (179), followed by Maynard et al in 2003 (155) in a study that demonstrated that placental soluble fms-like tyrosine kinase 1 (sFlt1) is up regulated in preeclampsia, leading to increased systemic levels of sFlt-1 that fall after delivery. Based on these data the increased circulating sFlt1 levels in patients with preeclampsia were suggested to decrease circulating levels of free

VEGF and PlGF and inhibit their biological activities. Considerable evidence have been published on elevated serum sFlt-1 levels in preeclamptic pregnancies both prior to (214, 215) and at the time of clinical diagnosis (155, 178, 205, 215), as well as several reviews on the role of pro and anti-angiogenic factors in preeclampsia (24, 187, 216, 217). In a retrospective study, Levine et al. (215) reported that soluble Flt-1 is elevated in mid-pregnancy in preeclamptic patients 5 weeks before clinical symptoms were observed, and that there is a strong correlation between the level of soluble Flt-1 in maternal serum and the pathological degree of preeclampsia. In a review of 34 studies on soluble markers of preeclampsia, the mean concentrations of sFlt1 and sEng were significantly higher than in normal pregnancy (218, 219).

In preeclamptic women, the sFlt-1 level is observed to be directly (24) proportional to the degree of proteinuria (178), higher in those with earlier onset disease (178, 215), increased disease severity (155, 178) (215) and in those with small-for-gestational-age fetuses (209, 215).

Major sites of placental sFlt-1 expression are degenerative syncytiotrophoblasts known as syncytial knots (179). Whether the excess sFlt-1 is contributed to by other cell types such as macrophages in the placenta or monocytes in the maternal or fetal peripheral circulation has not been explored.

The pathophysiology of up regulation of sFlt-1 in pre-eclampsia and pregnancy complications and whether it is a second order phenomenon is still to be completely understood. It has been postulated that soluble Flt-1 secreted from the trophoblast layer between the maternal and fetal blood vessels plays an important role in creating a barrier of 'VEGF-low' layer between the fetal and maternal sides in normal pregnancy. Unusual stress such as hypoxia in placenta or viral infection may induce abnormal expression of soluble Flt-1, which, in turn, blocks

physiological VEGF in various tissues at maternal side, and induces tissue dysfunction such as proteinuria (96).

Soluble Flt-1 overexpression in rats has been shown to result in the characteristic clinical features of preeclampsia, hypertension, proteinuria and glomerular endotheliosis. The anti-angiogenic state induced by excess placental production of sFlt-1 can be 'rescued' by administering VEGF-A and PlGF (155).

1.10.4.3 Placental growth factor (PlGF) in preeclampsia

Studies into the PlGF levels in normal pregnancy have documented a steady increase in serum PlGF during the first two trimesters, a peak at 29–32 weeks and a decline towards full term (206, 215). Published studies have shown serum PlGF levels to be significantly lower than normal pregnancies in early onset preeclampsia less than 37 weeks compared with late onset preeclampsia, severe disease compared with mild disease and in preeclampsia associated with fetal growth restriction (215, 220). Measured urinary PlGF levels have been shown to have similar profiles to the serum levels in normal and preeclamptic pregnancies (221). Maternal serum PlGF is known to be reciprocal to the levels of sFlt-1 (215).

1.10.4.4 soluble Kinase Domain Receptor (sKDR) in preeclampsia

Several studies have investigated the levels of the soluble form of the KDR receptor (sKDR) in human plasma. Maternal plasma sKDR is reduced in preeclampsia and in growth-restricted pregnancies (222-224) compared to non-pregnant and normal pregnancy levels.

1.10.4.5 soluble Endoglin (sEng) in preeclampsia

Literature to date on Endoglin in pregnancy has shown that the soluble form of Endoglin is elevated in preeclampsia and may play a significant role in the pathogenesis of the disease (188, 223, 225, 226). Placental expression and peripheral levels of sEng are up-regulated in

preeclampsia with the levels correlating with disease severity and falling after delivery. Over-expression of sEng in pregnant rats leads to increased vascular permeability and hypertension without proteinuria. These effects are amplified by co-administration of sFlt-1, leading to severe preeclampsia including the HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome and fetal growth restriction (170). These findings suggest that sEng may act in concert with sFlt-1 to induce dysfunction of the maternal endothelium (26, 218, 227) and severe preeclampsia (170). Although the available data suggest that the placenta is a major source of sEng during pregnancy, other sources such as maternal vasculature or monocytes cannot be ruled out.

A case-control study of healthy nulliparous women showed the levels of sEng to increase gradually with increasing gestation in normal pregnancy and the development of preeclampsia (188). This increase in sEng is associated with an increase in the ratio of sFlt-1: PIGF and the composite biomarker (sFlt-1+ sEng): PIGF has been suggested to be predictive of preeclampsia (188).

1.10.5 Soluble angiogenic markers in small for gestational age (SGA) / intrauterine fetal growth restriction (IUGR)

1.10.5.1 sFlt in SGA / IUGR

The published literature has produced conflicting results on circulating levels of sFlt-1 in mothers of small for gestational age fetuses. Maternal serum sFlt-1 has been described as increased (205, 224) or showing no difference in the first trimester (228), in the second trimester (229) or at term (209) in normotensive women who deliver a SGA infant. A longitudinal study evaluating sFlt-1 at 4 week intervals from the first antenatal clinic visit until delivery has shown no difference in maternal serum sFlt-1 levels in normotensive

women destined to deliver a SGA infant compared with controls (230). The definition of SGA in this study was birth weight <10th centile for gestational age. Doppler studies were not incorporated in the assessment. Four studies have been reported incorporating uterine artery Doppler velocitometry in the assessment of small for gestational age fetuses (224, 231-233) while sparse data is available on angiogenic factor levels in small for gestational age fetuses with abnormal umbilical artery Doppler waveforms (224), a marker of placental insufficiency and vascular disease.

1.10.5.2 PlGF in SGA / IUGR

Published literature is consistent in showing lower PlGF levels in established SGA in normotensive women in the third trimester. The evidence for PlGF in first and second trimester is less consistent with some studies showing lower levels as early as the first trimester of pregnancy compared with gestational age-matched controls (207, 230, 234-236) and others however reporting no significant difference in maternal peripheral (206, 237) or urinary PlGF levels (238). A longitudinal study examining PlGF concentration in pregnant women at <14, 15–19, 21–25, 27–30, 35–38 weeks gestation has shown a reduction in PlGF reaching significantly different levels at 27–30 weeks in normotensive women delivering an SGA infant compared with normotensive controls (206).

1.10.5.3 soluble Kinase Domain Receptor (sKDR) in SGA / IUGR

In published studies, no significant difference in mean plasma sKDR levels was noted between preeclamptic and normotensive women with SGA infants (222). sKDR levels did not vary between normal and abnormal uterine artery Doppler resistance in normotensive SGA pregnancies (222).

1.10.5.4 soluble Endoglin (sEng) in SGA / IUGR

A study into maternal plasma concentrations of soluble Endoglin in pregnancies with intrauterine growth restriction demonstrated that IUGR was characterized by elevated circulating sEng, to a lesser extent than preeclampsia and that there was a strong positive correlation between the sEng and sFlt1 concentrations (239).

1.10.6 Angiogenic factors in the fetal circulation

Few studies have been published on fetal angiogenic factor levels. In the literature to date, several studies by the same authors have explored the differences between clinical groups of preeclampsia and IUGR as well as maternal and cord blood levels of pro and anti-angiogenic factor levels. Umbilical vein sFlt-1 was positively and soluble kinase insert domain receptor (sKDR) negatively correlated with umbilical artery Pulsatility Index (PI) (240). No correlation could be found between umbilical artery serum angiogenic factors and clinical outcome (224, 240). Placental growth factor (PlGF) levels in the umbilical vein were below the detection limit in nearly all samples of IUGR fetuses and lower than in those with preeclampsia. In IUGR, sFlt-1 was increased, and PlGF and sKDR were decreased in serum from the umbilical vein. Additionally, fibroblast growth factor (FGF) was increased in serum from the umbilical vein of women with pregnancies complicated by IUGR (224). In normal and IUGR pregnancies, absolute levels of VEGF were higher, and levels of sFlt-1 and PlGF levels were lower in serum from the umbilical vein and umbilical artery compared with maternal serum (224). The authors concluded that the correlations between maternal and fetal angiogenic growth factor serum levels and Doppler ultrasound indices of uterine and umbilical arteries in preeclampsia and IUGR reflect the severity of the disorders especially for the fetus and that these findings suggest an imbalance of angiogenic and anti-angiogenic factors in IUGR, with formation of an anti-angiogenic state in maternal and, to a lesser extent,

umbilical vein blood. The placenta appears to play a central role in the release of sFlt-1 into maternal and umbilical blood. Umbilical artery blood was unaffected in IUGR, indicating that the fetus does not contribute to changes in angiogenic growth factor concentrations.

In an assessment of umbilical vein serum, amniotic fluid, and maternal serum from preeclamptic and uncomplicated pregnancies that were delivered by caesarean section, low concentrations of soluble Endoglin were found in fetal circulation, which did not differ between preeclampsia and control pregnancies. These results suggested that the fetus appears not to contribute to elevated circulating maternal soluble Endoglin concentrations in preeclampsia (241).

1.10.7 Angiogenic factors and their receptor levels as a predictive test for preeclampsia and intrauterine fetal growth restriction

Recent research has been focused on exploring clinical, ultrasound and laboratory parameters that may lead to early detection or prediction of preeclampsia. These studies have suggested previous history of preeclampsia, pre-existing medical conditions such as renal disease, chronic hypertension, pre-gestational diabetes, multiple pregnancy and anti-phospholipid antibody syndrome as clinical factors, uterine artery Doppler resistance on ultrasound parameters and angiogenic factors sEndoglin, sFlt-1/PlGF ratio, ADAM-12, PAPP-A, Pregnancy Protein13, homocysteine, ADMA, uric acid, leptin and urinary albumin and calcium are other parameters as having potential in the prediction of preeclampsia (21, 242).

Angiogenic factors and their receptor levels have been evaluated for their screening potential for preeclampsia and fetal growth restriction. The results on sFlt-1-1 has been variable with some studies demonstrating no significant difference in maternal serum sFlt-1 levels prior to 20 weeks of gestation in women who develop preeclampsia compared with normal

pregnancies (243), while a few studies have shown a significant difference prior to 20 weeks (244). In a longitudinal study by Levine et al, maternal serum sFlt-1 levels significantly increased 5 weeks prior to the onset of hypertension and proteinuria in women who subsequently developed early onset preeclampsia (215).

In women who subsequently develop preeclampsia, serum PlGF concentrations have been shown to be lower as early as 10–13 weeks of gestation (206, 207, 215, 237, 245-249), with a further reduction in levels 5 weeks prior to the clinical onset of the disease (215). First trimester urinary PlGF levels however have not been shown to be of value in predicting preeclampsia (250). Current evidence suggests that high serum sFlt-1 and low serum PlGF in first and second trimester can distinguish women who subsequently develop preeclampsia from those who remain normotensive throughout pregnancy (244).

The performance of angiogenic factors as biomarkers is improved by using the levels of PlGF in the assessment (235, 249, 251-255) and also using composite markers such as sFlt / PlGF (256-258) or (sFlt-1+ sEng)/ PIGF rather than individual markers (252). However, published literature suggests that VEGF family proteins do not have sufficient power to accurately predict late onset preeclampsia, small for gestational age (SGA) pregnancies or preterm birth (24).

A recent large study evaluated the performance of a combination of maternal clinical and biochemical markers in first trimester including mean arterial pressure, uterine artery pulsatility index, fetal nuchal translucency thickness, pregnancy associated plasma protein A (PAPP-A), free β-hCG, PlGF, placental protein 13 (PP13), disintegrin and metalloprotease (ADAM 12) in the prediction of small for gestational age in the absence of preeclampsia. The detection rates in this study were 73% for SGA requiring delivery before 37 weeks and 46% for those delivering at term (236). Several other studies have evaluated the predictability of a

combination of biomarkers including angiogenic factors in the early prediction of preeclampsia and SGA/IUGR with varying predictability (259).

Based on the published literature, the predictive value of VEGF family angiogenic growth factors appear to be more consistent for preeclampsia than for small for gestational age (SGA) or fetal growth restriction (IUGR). Current data are insufficient to recommend the angiogenic factors in first or second trimester as reliable biomarkers for the prediction of SGA or IUGR.

A recent review provided a summary of studies screening for preeclampsia as well as IUGR (24). The evidence to date on the use of circulating angiogenic factors in the prediction and diagnosis of preeclampsia was recently reviewed (219), with the authors suggesting the use of sFlt-1/PlGF ratio in the diagnosis and the management of preeclampsia. The role of angiogenic factors in fetal growth restriction has not been as well established as in preeclampsia. Variable definitions of small for gestational age and intrauterine fetal growth restriction have led to inconsistent results on the angiogenic factor profile of fetal growth restriction.

1.11 Role of peripheral monocytes in normal pregnancy and placental disease

1.11.1 Human blood monocytes

There is evidence that decidual macrophages play a major role in vascular remodeling in early placentation as well as a role in the immune mechanism responsible for the acceptance of an allogenic fetus by the mother (260). Suboptimal vascular remodeling of spiral arteries is thought to play a key role in the pathogenesis of preeclampsia (261). Monocytes, the peripheral precursor of macrophages have been less studied in the pathogenesis of pregnancy

complications and may play a significant role in pathogenesis of pregnancy complications including preeclampsia and IUGR.

Monocytes are mononuclear cells arising from bone marrow precursors that circulate in blood and eventually migrate into body tissues where they further mature into macrophages and perform multiple functions in the body including roles in homeostasis, immune defense, and tissue repair (262).

1.11.2 Monocyte phenotype and subtypes: classical, intermediate and non-classical

Human monocytes were initially defined on the basis of morphology and cyto-chemistry (monocyte-specific esterase) and later by expression of cell-surface markers such as CD14 and the light scatter properties in flow cytometry (262). Classical monocytes are the cells described by hematologists for a century as monocytes on the basis of size and structure.

With the aid of immunofluorescence and flow cytometry, an additional subset of cells co-expressing CD14 and CD16 antigens and labelled as “non-classical “ monocytes (263) have been identified in human peripheral blood. These CD14+/CD16+ cells account for 2.2% of the mononuclear cells and form about 13% of all cells identified by the monocyte-specific CD14 monoclonal antibody. The CD14+/CD16+ cells can be assigned to the monocyte lineage based on typical morphology, on expression of additional monocyte associated molecules, on the ability to form reactive oxygen intermediates and on the expression of monocyte specific NaF-sensitive esterase. Light scatter analysis reveal lower forward angle and right angle light scatter for the CD14+/CD16+ cells compared with the regular monocytes, and the average cell size is 13.8 and 18.4 microns, respectively. Expression of class II antigens on these "small monocytes" is twofold higher compared with the regular

monocytes. By contrast, the capacity to adhere to plastic surfaces, as well as the ability to phagocytize antibody coated erythrocytes is clearly reduced in the CD14+/CD16+ monocyte subset as compared with the regular monocytes. Hence the CD14+/CD16+ cells represent a new monocyte subset with a distinct functional repertoire. The classical $CD16^{-}$ monocytes and these $CD16^{+}$ cells have been shown to share morphology, cytochemistry, and many cell surface markers (263).

Several research groups have extensively studied the phenotypes and functions of these two groups including whole genome wide analysis (262, 264-268). More recently, it has become evident that further heterogeneity exists in the CD16 positive subtype of monocytes, and an intermediate monocyte phenotype between classical and $CD14^{+}CD16^{+}$ monocyte subsets has been described (269). These are low in numbers, but have been shown to have unique features and expand with cytokine treatment and in inflammation (270). There appears to be a developmental relationship between these cells, changing from classical to intermediate to non-classical in the presence of infection or inflammation (271, 272). This change has been shown with infection or with macrophage colony-stimulating factor (M-CSF) treatment, as an increase first of the intermediate cells followed by an increase of the non-classical $CD14^{+}CD16^{++}$ monocytes (273). The increase in CD14 or CD16 levels indicated as "+" and "++" denotes an expression level that is ~ 10-fold and 100 fold above the isotype control.

Recently a proposed nomenclature of monocyte subtypes by a group of experts in the field was endorsed by the Nomenclature Committee of the International Union of Immunological Societies (262) to facilitate communication and standardization of research into monocytes. The proposed nomenclature for monocytes subdivides human monocytes into 3 subsets classical, intermediate, and non-classical on the basis of the expression of CD14 and the CD16 receptors as listed in Table 1 and Figure 1.6 (262). At this stage the nomenclature does

not include markers of monocyte activation. The use of CD14 and CD16 markers have been published in many studies as being useful in distinguishing the monocyte subtypes. It is recommended to use antibodies directed against the lipopolysaccharide-binding domain for CD14 and those that bind to the Fc-binding domain for CD16 (262). Their use is recommended for determination of monocyte sub-populations (262).

The classical monocytes show high CD14 expression but no CD16 ($CD14^{++}CD16^{-}$), the intermediate monocytes show a high level of CD14 together with low CD16 ($CD14^{++}CD16^{+}$), and the non-classical monocytes express a low level of CD14 together with high CD16 ($CD14^{+}CD16^{++}$). When the intermediate and the non-classical monocytes are not separately defined, then it is proposed to classify them collectively as $CD16^{+}$ monocytes. Gene expression profiling revealing the defining features of the classical, intermediate, and non-classical human monocyte subsets has been published (268).

Human monocyte subtype	**CD14**	**CD16**
Classical	++	-
Intermediate	++	+
Non-classical	+	++

Table 1.2 Human blood monocytes classification (262)

1.11.3 Monocyte subtypes and their in-vivo functions

1.11.3.1 Classical monocytes

CD14++ CD16- monocytes, commonly referred to as the “classical subset are associated with extravasation and inflammation. Once infiltrated into tissues, these monocytes develop into macrophages and help with pathogen clearance and wound healing (274). Classical monocytes are generally described to play an anti-inflammatory role in many disease states (275).

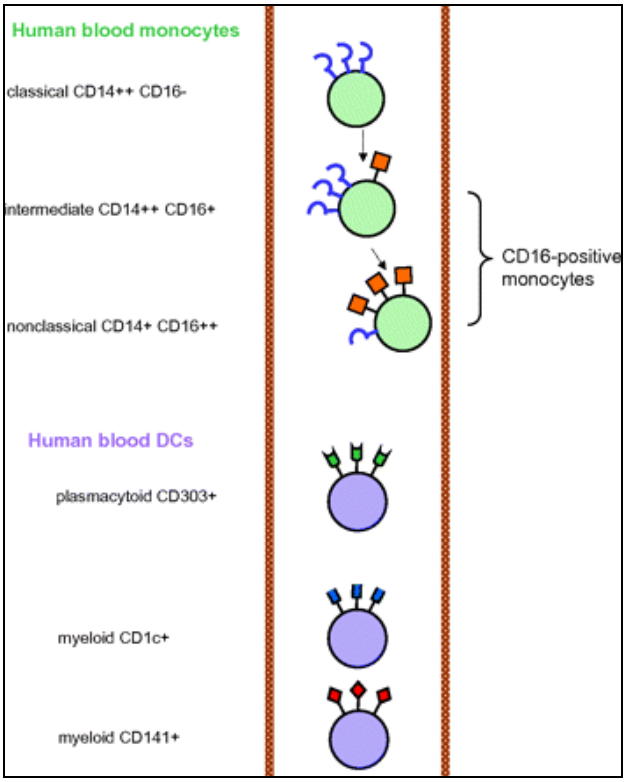

Figure 1.6 Nomenclature of monocytes and dendritic cells (DCs) in blood based on cell surface marker expression. The 6 types of cells are shown with different symbols, which represent the crucial markers of the respective cells. Blue hook indicates CD14; red square flag, CD16; green flag, CD303; blue flag, CD1c; red diamond flag, CD141. A higher number of a given symbol indicates a higher density of a given receptor. The arrows in the upper portion represent the developmental relationship (262).

1.11.3.2 Intermediate monocytes

A recent study explored genetic evidence for a distinct role of $CD14^{++}CD16^{+}$ intermediate monocytes in human immunity (276). The three monocyte subtypes were purified using SuperSAGE in combination with high-throughput sequencing. Unique identifiers of $CD14^{++}CD16^{+}$ monocytes were revealed, delineating these cells from the 2 other monocyte

subsets. Gene Ontology (GO) enrichment analysis suggested diverse immunologic functions, linking $CD14^{++}CD16^{+}$ monocytes to Ag processing and presentation (e.g. *CD74*, *HLA-DR*, *IFI30*, *CTSB*), to inflammation and monocyte activation (eg. *TGFB1*, *AIF1*, *PTPN6*) and to angiogenesis (e.g. *TIE2*, *CD105) (276).*

Intermediate monocytes with CD14++ and CD16+ are gaining increasing attention in the research world as an inflammatory marker. They are low in frequency, have their unique features and expand with cytokine treatment and inflammation (262). The intermediate monocyte subset has been suggested to be a transitional population between the classical and the non- classical subsets and has often been previously grouped together with the non-classical subset in the published literature. The combined non-classical and intermediate subsets are thought to replenish tissue resident macrophages and dendritic cells (277) and have been shown to produce pro-inflammatory cytokines (266, 278). Their pro-inflammatory association was also illustrated by the fact that their number increases during inflammatory conditions, such as during sepsis (271, 278). Their role in HIV-1 infection and atherosclerosis has been recently explored (279, 280).

1.11.3.3 Non-classical monocytes

In vitro experiments have shown that the CD16+ non-classical monocytes are far more mobile than their CD16- relatives with patrolling behaviour (281). Human non-classical monocytes have exhibited crawling behaviour on the endothelium after being adoptively transferred into mice (282). Smooth and mediated movements of CD16+ monocytes *in vitro* through large frontal areas of extended lamellipodium in a seemingly crawling manner have been described (269). This behaviour suggests a surveillance function for the non-classical monocytes, surveying the endothelium for signs of inflammation or damage and poised to transmigrate rapidly (269). Genes associated with cytoskeleton mobility, such as Rho GTPases, RHOC and

RHOF, and several upstream Rho activators and downstream effectors were most highly expressed by the non-classical subset (268, 276) and may explain the highly motile behaviour of the non-classical subset. The non -classical monocytes have been shown to play a pro-inflammatory and pro-fibrogenic role (283).

1.11.4 Markers of monocyte activation into M1 inflammatory and M2 anti-inflammatory subtypes

1.11.4.1 Classification of inflammatory subtypes

Monocytes and macrophages are immune system cells that play an indispensable role in homeostasis and defense. Depending on the environment, monocytes can differentiate into macrophages or dendritic cells. Monocytes and macrophages can be phenotypically polarized by the environment to mount specific functional programs. Polarized activation of cells of the monocyte-macrophage lineage into M1 and M2 cells (Figure 1.7) is an operationally useful, simplified descriptor of the functional plasticity of these cells (284).

Polarized macrophages can be broadly classified in two main groups: classically activated macrophages (M1), whose prototypical activating stimuli are IFN gamma and LPS, and alternatively activated macrophages (M2), further subdivided in M2a (after exposure to IL-4 or IL-13), M2b (immune complexes in combination with IL-1beta or LPS) and M2c (IL-10, TGFβ or glucocorticoids) (285). M1 exhibit potent microbicidal properties and promote strong IL-12-mediated Th1 responses, whilst M2 support Th2-associated effector functions. A transition from pro- inflammatory M1 to anti-inflammatory M2 phenotype is characterized by changes in cell surface marker expression including CD14, CD36, CD163, CD204, CD206, B7-H4 and CD11b, which are distinctive of M2 monocytes and macrophages (286).

M2 polarized macrophages play a role in resolution of inflammation through high endocytic clearance capacities and trophic factor synthesis, accompanied by reduced pro-inflammatory cytokine secretion (285). Differentiation of monocytes into M1 and M2 macrophages play a central role in wound healing (287).

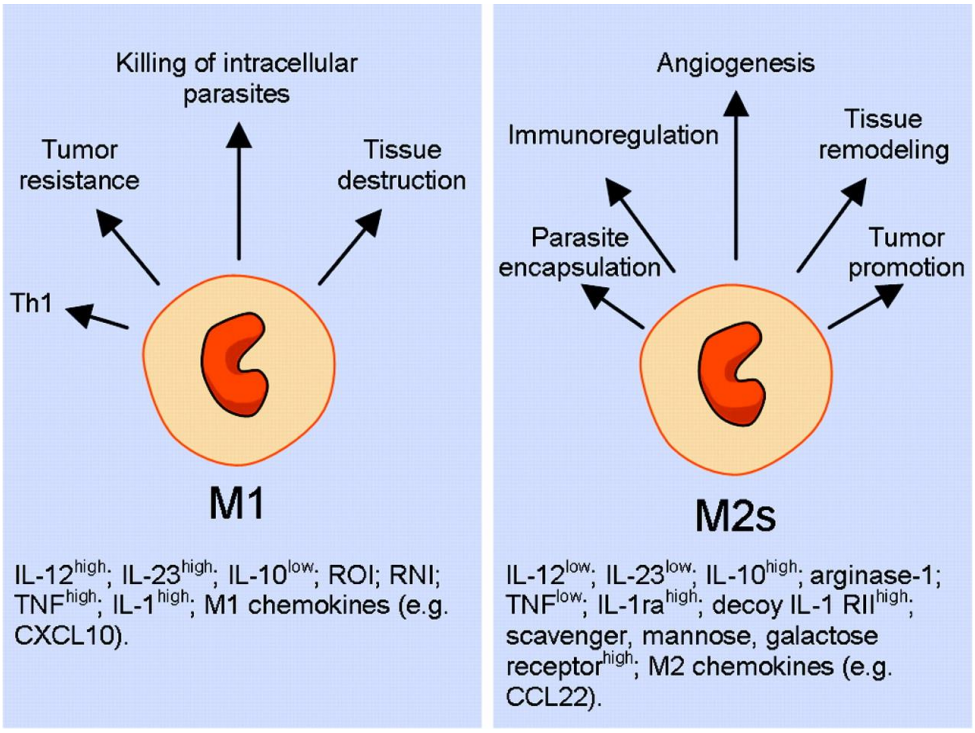

Figure 1.7 Key properties and functions of polarized macrophages. ROI and RNI indicate reactive oxygen and nitrogen intermediates. M2s refer to diverse forms of M2 activation (284).

1.11.4.2 CD86

CD86 is a 75kDa cell surface protein expressed primarily on monocytes, dendritic cells and activated B cells. It plays an important role in co-stimulation of T cells in primary immune responses (288, 289) and is strongly expressed by M1-type macrophages/monocytes.

1.11.4.3 CD163

CD163, a 110-130 kDa transmembrane glycoprotein is a member of the scavenger receptor superfamily known as Scavenger receptor cysteine-rich type 1 protein M130 (M130), haemoglobin scavenger receptor and macrophage-associated antigen. It is a monocyte /macrophage-restricted antigen expressed on tissue macrophages and peripheral blood monocytes. The expression of CD163 on monocytes is up regulated upon cellular activation and reportedly changes on monocytes and macrophages as these cells differentiate. This finding suggests a role for this molecule in the differentiation and/or regulation of monocyte and macrophage function. CD163 is considered part of the M2 anti-inflammatory profile of antigens (290-292).

1.11.5 Monocytes in pregnancy

Pregnancy is associated with a major adaptation of the maternal immune system to accommodate the semi-allogeneic fetus (293, 294). The innate immune system is known to be significantly activated and is thought to be favourable to the newly implanted fetus. An increase in the number of peripheral blood monocytes has been demonstrated as well as an activated phenotype from the second trimester onwards comparable to that seen in systemic sepsis (295, 296). Whether the changes in monocyte number and behaviour during pregnancy are the result of changes in monocyte subsets was explored in a recent study (274). The findings of this study showed that the percentage of combined non-classical and intermediate monocytes is higher during human and rat pregnancies compared to non-pregnant controls. The higher percentage of intermediate monocytes in contrast to non-classical monocytes is responsible for the higher percentage of combined non-classical/intermediate monocytes (274).

Changes in the leukocyte populations in pregnancy have been characterised in previous studies. A study on phenotype and intracellular cytokines of circulating granulocytes, monocytes, and T lymphocytes of pregnant women during pregnancy reported an increased percentage of granulocytes and a decrease in lymphocytes as well as the presence of generalized leukocyte activation (295). In this study the proportion of monocytes was seen to remain stable throughout gestation although a progressive up-regulation of surface markers CD11a, CD54, and CD64 was detected. Monocytes also showed higher production of interleukin (IL)-12 and IL-1beta compared with the non-pregnant state, and granulocytes had greater potential to synthesize IL-8. These changes were particularly marked in late gestation (295). T lymphocytes did not have any characteristics of the activated state and showed decreased IL-6 production. These authors demonstrated that activation of maternal monocytes and granulocytes increases during pregnancy and proposed the idea that pregnancy results in an elevation of the innate immune system and suppression of the adaptive immune system (295).

A recent study of the influence of pregnancy and labour on monocyte subpopulations characterised monocytes by flow cytometry using CD14, CD16 in the peripheral venous blood of non-pregnant, late normal gestation and preterm labour. This study did not demonstrate any difference in the relative proportion of each monocyte subset within the groups (297).

The molecular characteristics of monocytes that were derived from the maternal circulation and the placenta using CD14 immune selection were examined by microarray and real-time reverse transcriptase-polymerase chain reaction for genotype and expression patterns. CD14 monocytes from the maternal blood and the placenta share strong phenotypic and genotypic similarities with an enhanced inflammatory pattern in the placenta, as opposed to the fetal

genotype of the trophoblast cells. The functional traits of the CD14 in blood and placental monocytes suggested that they both contribute to propagation of inflammation at the maternal fetal interface (298).

1.11.6 Effect of preeclampsia and IUGR on monocyte phenotype (subtypes) and function (inflammatory markers)

The leukocytes of healthy pregnant women have been shown to differ substantially and significantly from those of non-pregnant women with increased CD11b, CD14, and CD64 and increased intracellular reactive oxygen species (296). In preeclampsia there was, in addition to these changes, reduced expression of L-selectin and further increases in intracellular reactive oxygen species. These changes were similar but not identical to sepsis. The authors concluded that normal third-trimester pregnancy is characterized by remarkable activation of peripheral blood leukocytes, which is further increased in preeclampsia. This study did not specifically characterize any pregnancy or preeclampsia related changes in monocytes (296).

An increase in peripheral blood monocytes and an altered activation status of these monocytes in preeclampsia as compared to healthy pregnancy has been suggested by previous studies (299-303).

A study into the characterization of monocyte subtypes in pregnancy and preeclampsia found that inflammatory changes associated with preeclampsia showed increased phenotypic and metabolic changes in granulocytes and monocytes by demonstrating a significant increase in mean channel brightness for CD11b on granulocytes and monocytes associated with preeclampsia (274). Basal intracellular reactive oxygen species were increased in monocytes but not in granulocytes (304). It has been suggested that placental syncytiotrophoblast

membrane microparticles (STBM), which are released into the peripheral blood, may contribute to the maternal inflammatory response of monocytes (305).

The inflammatory role of monocytes in preeclampsia has just started to be explored with just a handful of studies documenting a change in the monocyte subsets and inflammatory markers associated with preeclampsia. Monocyte subtypes in isolated intrauterine fetal growth restriction is still to be explored. Whether the difference in the maternal syndrome between PE and isolated IUGR can be explained by a difference in the maternal monocyte activation and secretion of inflammatory markers remains to be explored.

1.11.7 Inflammatory subtypes in pregnancy

Studies assessing the M1/M2 phenotype of peripheral blood monocytes in preeclampsia are few; moreover, studies examining monocyte activation and inflammatory markers in IUGR have not been published. A recently published study evaluated whether the monocyte inflammatory state in preeclampsia might be associated with polarization to either M1 (classically activated) or M2 alternatively activated monocyte subsets (306). The research focused on surface receptors characteristic of M1 activation, such as Toll-like receptor (TLR) 2, TLR4, and CD64, or M2, such as CD163 and CD206 as detected by flow cytometry. Tumour necrosis factor-alpha (TNF-α), interleukin-(IL)-12p40, IL-12p70, and IL-10 were evaluated in the supernatant of monocyte cultures by ELISA. This study demonstrated that expression of M1 markers TLR4 and CD64 by monocytes from preeclamptic women was significantly higher, while the expression of CD163 and CD206 expression was significantly lower compared with normal term pregnant women. Endogenous production of TNF-α, IL-12p40, and IL-12p70 by monocytes was increased, while synthesis of IL-10 was lower in women with preeclampsia. They concluded that monocytes from women with PE are classically activated, producing higher levels of pro-inflammatory cytokines, and express

surface receptors characteristic of the M1 subset. These results provide evidence that the systemic inflammatory environment in preeclampsia may differentiate and polarize these cells to the M1 phenotype (306). Monocyte subsets in this study were characterized as M1 and M2 rather than into subsets of classical, intermediate or non-classical based on CD14 and CD16 expression. Whether or not similar changes were associated with normal pregnancy as compared to non-pregnant females was not explored.

1.11.8 Fetal/Cord blood monocytes in normal and complicated pregnancy

Fetal or cord blood monocyte phenotypes have not been well described. There are no comprehensive data describing the distribution of monocyte subsets in cord blood. A study into cord blood monocyte subsets and Toll like receptor expression in normal pregnancy and pregnancies complicated by parasitic infections described a lower percentage of non-classical (CD14+CD16+) monocytes in cord blood as compared to the maternal circulation(307). The distribution of intermediate monocytes was not mentioned in this study. Another study, dividing monocytes into two groups of classical and non-classical, reported similar frequencies of monocyte subsets in cord blood and adult peripheral blood with concordant expression of CD11c, CD80, CD86, CD163 and HLA-DR (308). Human fetal and adult monocytes have been shown to be functionally distinct in response to cytokines associated with in-utero infection and preterm labour (309). The molecular characteristics of CD14 monocytes that were derived from the maternal circulation and placenta have been shown to be concordant and different to trophoblast cells. Fetal monocytes have not been profiled with respect to preeclampsia or intrauterine fetal growth restriction.

1.12 Angiogenic factor modulation of the immune system

1.12.1 Flt-1 expression on maternal monocytes

Until recently, the expression of Flt-1 has been localized to only two tissues in the body other than trophoblast in pregnancy, namely vascular endothelium and monocytes including monocyte derived mature dendritic cells (310). Flt-1 was initially thought to be almost exclusively expressed on vascular endothelial cells. As an exception, Flt-1 transcript was found to be expressed in human peripheral blood monocytes. The first report of Flt-1 expression external to endothelial cells was the detection of Flt-1 messenger RNA (mRNA) in human peripheral blood monocytes (146, 147). The synthesis of Flt-1 has been recently described by activated platelets binding to monocytes in preeclampsia (311). The strong placental expression of PlGF by trophoblast could contribute to the marked angiogenesis seen in the growing placenta which may be partially mediated by chemo attraction of peripheral blood monocytes (95, 148).

Using monoclonal antibodies against 2 different antigenic epitopes on the Flt-1 extracellular domain, it has been shown that human peripheral blood expresses Flt-1 as a cell surface molecule. KDR was not found to be expressed at detectable levels on monocytes in this study (312). A study by Clauss et al (147) also showed that monocytes, in contrast to endothelium, express only the VEGF receptor Flt-1, but not the second known VEGF receptor, KDR (146), and that Flt-1 also specifically binds PlGF. Both VEGF and PlGF stimulate tissue factor production and chemotaxis in monocytes at equivalent doses, in addition to triggering release of further VEGF (146).

Neutralizing antibodies to the KDR receptor reduce the VEGF-stimulated tissue factor induction in endothelial cells to levels obtained by stimulation with PlGF alone, but neither

affects PlGF-induced tissue factor induction in endothelial cells nor the VEGF-dependent tissue factor production in monocytes. These findings strongly suggest Flt-1 as a functional receptor for VEGF and PlGF on monocytes and endothelial cells and identify this receptor as a mediator of monocyte recruitment and pro-coagulant activity (147).

CD34+cells9 a marker for primitive bone marrow derived progenitor cells) in human cord blood, originally negative for the Flt-1 expression, have been shown to differentiate into Flt-1+ cells with monocyte-macrophage markers after culture in the presence of hematopoietic cytokines (312).

1.12.2 Endoglin expression on maternal monocytes

Endoglin (CD105) is a component of the TGF-β receptor system and acts as a co-receptor for TGF- β1 and TGF- β3 with high affinity (313-315). While Endoglin is primarily expressed on endothelial cells (163) and induces proliferation and migration of these cells, it has also been demonstrated to be present on other tissues including macrophages (165), erythroid precursors (316), syncytiotrophoblast (166), activated monocytes (165), and stromal cells (317). The expression of CD105 is increased in activated endothelium and tissues undergoing angiogenesis such as tumors and in cases of wound healing or inflammation. Soluble Endoglin (sEng) inhibits TGFβ1 binding to its receptor on cell surface, disordering the work of TGFβ1 signal pathway and thus preventing stimulation of endothelial nitric oxide synthase and vessel dilatation (202). The soluble form of Endoglin has been proposed to reduce the bioavailability of TGF-β1, thus inhibiting its signaling pathway (318). Elevated levels of soluble Endoglin levels have been documented in preeclampsia as well as in intrauterine fetal growth restriction (178). Increased levels of Endoglin have been shown to indicate more severe placental disease (189, 230).

1.12.3 Monocyte functions and anti-angiogenic factors

Treatment of human monocytes with vascular endothelial growth factor (VEGF) isolated from tumour cell supernatants was reported to induce monocyte activation and migration. Recombinant human VEGF165, and VEGF121 had a major effect on human monocyte migration (147). Chemotactic activity of VEGF165 was inhibited by a specific antiserum against VEGF, by heat treatment of VEGF165, and by protein kinase inhibitors. Placental growth factor, a heparin-binding growth factor related to VEGF, has also been shown to be chemotactic for monocytes (147).

Barleon et al showed that VEGF and PlGF stimulate the chemotaxis of human monocytes and neutrophils via the Flt-1 receptor *in vitro*. A demonstration of VEGF-mediated monocyte migration even in the presence of KDR-neutralizing polyclonal antibody, suggested that this cell migration signal is mediated by Flt-1 (146). These results strongly suggest that Flt-1 is a novel cell surface marker as well as a biologically functional molecule for monocyte-macrophage lineages.

Investigation into the intracellular signaling pathways mediating the biological functions triggered by Flt-1 in primary monocytes revealed that both PlGF-1 and VEGF-A can activate Flt-1–dependent signaling pathways of PI-3K, p38 kinase, Akt, and ERK1/2 in primary human monocytes, leading to the activation of several intracellular signaling pathways. These pathways are critically involved in primary monocyte chemotaxis (319).

1.12.4 Role of monocytes in pregnancy complications of preeclampsia and IUGR

Peripheral blood mononuclear cells (PBMC) of pregnant women have been shown to be capable of expressing variable amounts of Flt-1 proteins (320). This study suggested that Flt-1 dysregulation in PBMCs of pregnant women resulting in over-expression of sFlt-1 could be

an additional (extra-placental) source of sFlt-1 that contributes to the pathogenesis of preeclampsia. The strong placental expression of PlGF could contribute to the marked angiogenesis seen in the growing placenta which may be partially mediated by chemo-attraction of peripheral blood monocytes (95, 148). While there are sparse data available on monocytes in preeclampsia, there are no published studies exploring peripheral monocyte-derived anti-angiogenic factors in pregnancy complications of fetal growth restriction. Whether the excess circulating sFlt-1 observed in preeclampsia is produced by other cell types such as monocytes/macrophages in the placenta as well as maternal peripheral circulation has not been explored.

1.12.5 Monocyte subtypes and angiogenic factors

The proposed nomenclature for monocytes subdivides human monocytes into 3 subsets on the basis of the expression of CD14 and the CD16 receptors, classical CD14++CD16-. Little is known about the pro and anti-angiogenic factor expression by different subtypes of monocytes. One of the published studies on gene expression on monocyte subsets suggested that surface marker KDR and Endoglin were expressed on intermediate monocytes (321). These genes were apparent only after the threshold criteria in their analysis were lowered. This may be because pro-angiogenesis is not a feature of all intermediate monocytes, and/or that pro-angiogenic monocytes can also be found within non-classical and classical subsets. Only the intermediate subset was able to form cell clusters upon vascular endothelial growth factor (VEGF) stimulation (269).

In one of the few studies on different functional properties of the two monocyte subsets classical (CD14++CD16+) and non-classical (CD14+CD16++), CD16++ monocyte chemotaxis towards the angiogenic ligands VEGF-A and PlGF was reduced compared to CD16- monocytes (322). Flt-1 protein expression was lower in CD16+ monocytes than in

CD16- monocytes. The reduced chemokinesis of CD16+ monocytes was attributed to lower Flt-1 levels, secondary to lower Flt-1 expression (322). The authors concluded that these novel functional differences between CD16- monocytes and CD16+ monocytes may predict different functional roles of both monocyte subsets in vascular repair, arteriogenesis and atherogenesis (322). No data are available for any variation in PE and IUGR.

1.12.6 Fetal circulation /Cord blood monocytes and angiogenic factors expression

There is no published literature on whether fetal/placental circulating monocytes contribute to the anti-angiogenic factor expression.

1.12.7 Monocyte polarization and anti-angiogenic factor expression

The association of monocyte polarization into M1/M2 phenotypes and correlation with anti angiogenic factor expression has not been described in the literature.

1.13 Significance of maternal metabolic syndrome including dyslipidaemia in clinical preeclampsia and fetal growth restriction.

1.13.1 Lipid profile

Lipid profile is a panel of blood tests that serves as an initial broad medical screening tool for abnormalities in lipids such as cholesterol and triglycerides. The results of this test can identify dyslipidemias and help determine the risk of cardiovascular disease. The lipid profile typically includes total cholesterol (TC), high density cholesterol (HDL) and triglycerides (TG). Using these values low density lipoprotein (LDL) and total cholesterol/high density cholesterol ratio (TC/HDL) are calculated.

1.13.1.1. Cholesterol

Cholesterol, an essential component of the cell wall, is a lipid that is mainly synthesized rather than absorbed by the body. In addition to its importance within cells, cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acids and vitamin D (323). Cholesterol is carried in the blood as part of particles called lipoproteins. While there are different types of lipoproteins, the most relevant to cholesterol transport are HDL and LDL.

1.13.1.2 HDL

High-density lipoprotein (HDL) is one of the five major groups of lipoproteins which carry cholesterol in the blood stream. HDL is considered to prevent atheroma forming and is often referred to as good cholesterol. In healthy individuals, about 30% of blood cholesterol is carried by HDL (324).

HDL particles remove fats and cholesterol from cells, including within artery wall atheromas, and transport it back to the liver for excretion or re-utilization. In addition to its cardiovascular effects, HDL has been shown to be a potent inhibitor of inflammation, acting on a number of pathways. HDL inhibits endothelial inflammation in both *in vitro* and *in vivo* models, reducing the expression of key cell adhesion molecules (324). In addition, HDL has been shown to have an effect on the coagulation pathway by inhibiting platelet activation and reducing thrombus formation. By reducing the activation of leukocytes, HDL can inhibit leukocyte recruitment to the endothelium. HDL has been suggested as an anti-inflammatory molecule for a number of diseases (324).

1.13.1.3 LDL

Low-density lipoprotein (LDL) is one of the five major groups of lipoproteins. The majority of cholesterol in the blood is LDL cholesterol, with the proportion varying from person to

person. A study has shown that higher levels of LDL particles are associated with health problems, including cardiovascular disease (325). LDL molecules are often called bad cholesterol as they are responsible for transport of their cholesterol and fat content into artery walls, attract macrophages, and thus promote atherosclerosis (326).

1.13.1.4 Apo lipoprotein AI (Apo1)

As a major component of the high density lipoprotein complex, Apo lipoprotein A-1 has a specific role in lipid metabolism (327). ApoA1 promotes fat efflux, including cholesterol, from tissues to the liver for excretion (327). Apo A-1 is also thought to be a prostacyclin stabilizing factor, and may have an anti-coagulation effect. Apo A1 and Apo E interact to modify triglyceride levels in coronary heart disease patients (327, 328).

1.13.1.5 Apo lipoprotein B

Apo lipoprotein B (ApoB) is the primary Apo lipoprotein of chylomicrons and low density lipoproteins which is responsible for carrying cholesterol to body tissues. It is absolutely required for their formation and on the LDL particle appears to act as a ligand for LDL receptors in various cells throughout the body (329).

1.13.1.6 Triglycerides (TG)

Triglycerides are a commonly measured component of lipid profiles for assessment of cardiovascular risk. A triglyceride is an ester formed by combining glycerol with three fatty acid molecules. Plasma triglycerides are primarily produced by the intestine and liver while dietary triglycerides enter the circulation within chylomicrons. Triglycerides assembled from de-novo synthesized fatty acids and from lipids returning to the liver are secreted in very-low-density lipoprotein (330).

1.13.2 Hyperlipidaemia and the risk of cardiovascular disease

Heart disease remains the most common cause of death in the developed countries (331). Observational epidemiological studies have identified multiple independent risk factors for coronary artery disease, including age, gender, smoking, diabetes, hypertension and dyslipidaemia (331). Dyslipidaemia is causally related to cardiovascular disease (332). Hypercholesterolemia and hyperlipidaemia are strongly associated with cardiovascular disease as they promote atherosclerosis, a precursor to myocardial infarction, stroke, and peripheral vascular disease. The specific markers of dyslipidaemia include individual and combinations of HDL, LDL, Apo lipoprotein B and triglycerides.

The role of low-density lipoprotein (LDL) particles in the development of atherosclerosis and cardiovascular disease is well documented (333). Reducing the high levels of LDL using treatments such as HMG-CoA reductase inhibitors (statins) has reduced the risk of cardiovascular disease and is now considered part of standard clinical management. Data from several studies have demonstrated that although this risk reduction is demonstrable when LDL is at high levels, the protective benefit is lower when the LDL is at moderate or low levels (334, 335).

Since the Framingham Heart Study (336), elevated levels of HDL cholesterol have been recognized as an independent protective factor against coronary heart disease. In a re-analysis of the data from several studies including the Framingham Heart Study (337) on hyperlipidaemia and cardiovascular risk, Gordan et al (338) found that a 1mg/dl (0.026 mmol) increment in HDL was associated with a significant coronary heart disease risk decrement of 2% in men and 3% in women as well as a significant decrease in cardiovascular disease mortality rates of 3.7% in men and 4.7% in women. The 95% confidence intervals for these decrements in coronary heart and cardiovascular disease risk were in the range of 1.9-2.9%.

ApoB has been documented to be predictive of ischemic cardiovascular disease in the general population (339). There is considerable evidence that levels of ApoB may be a better indicator of risk of heart disease risk than total cholesterol or LDL (340). However, primarily for historic reasons, cholesterol, and more specifically LDL, remains the primary lipid test clinically in use for the risk factor of atherosclerosis. A panel of 30 international experts concluded that CVD risk is more directly related to the circulating atherogenic LDL quantity than to cholesterol content and advocated using Apo B as a therapeutic target in managing patients on lipid lowering therapy (341).

Some studies have suggested that ApoB and the Apo B/Apo A1 ratios are thought to be a better marker of risk of vascular disease and a better guide to the adequacy of statin treatment than any cholesterol index (342). Several studies including the INTERHEART study found that the ApoB / ApoA1 ratio is more effective at predicting heart attack risk in patients who had had an acute myocardial infarction, than either the ApoB or ApoA1 measure alone (343). A recent large study concluded that the non-fasting Apo B/Apo A1 ratio was superior to any of the cholesterol ratios for estimation of the risk of acute myocardial infarction in all ethnic groups, in both sexes, and at all ages (343).

Hyperlipidaemia is an established risk factor for coronary heart disease in both men and women. Raised triglycerides are a component of the metabolic syndrome and are strongly associated with future risk of diabetes as well as cardiovascular disease (344, 345). Published epidemiological studies on hypertriglyceridemia and cardiovascular disease support the view that triglycerides are an independently associated risk factor (332) and are thought to form a component of cardiovascular risk above that delineated by low density lipoprotein (LDL) cholesterol. Elevated TG has been suggested as an explanation for residual cardiovascular risk even after statin therapy (346).

In summary, HDL and its major protein Apolipoprotein AI are thought to be protective against atherosclerosis through the ability to mediate reverse cholesterol transport, while elevated ApoB, LDL and Triglycerides have been described as leading to a higher risk of cardiovascular disease. Published studies to date suggest that LDL, ApoB/ApoA1 and TC/HDL are useful markers for screening for increased cardiovascular disease risk.

1.13.3. Lipid profile in normal pregnancy

1.13.3.1 Maternal lipid profile in normal pregnancy

Several authors have examined the changes in lipid and lipoprotein profile in pregnancy. Pregnancy related increase in serum triglyceride and, to a lesser extent, of cholesterol (347-350) has been documented. An increase in the levels of Apo lipoproteins AI and B in mid pregnancy was first reported by Hilman et al (351). The findings were later confirmed by other studies such as (352). Pregnancy changes and reference values have been described on a study of 719 healthy pregnant women across all 3 trimesters showed that all lipids and apolipoproteins were significantly elevated in pregnancy (350). The most prominent change in this study was a 2.7-fold triglyceride increase in the third trimester. In second trimester of pregnancy, ApoB levels increased by 56%, total cholesterol by 43%, low-density lipoprotein (LDL) cholesterol by 36% and Apolipoprotein A1 by 32%, high-density lipoprotein (HDL) cholesterol rose maximally (25%). The data on elevated HDL levels in pregnancy have been more controversial with some studies failing to detect any change (353, 354). The atherogenic indices TC/HDL, ApoB/ApoA1 and LDL/HDL have been shown to be reduced in first trimester and elevated in third trimester (350). Further studies into normal pregnancy changes have described elevated triglycerides, total HDL and LDL cholesterol levels along with

corresponding Apo lipoprotein (355, 356). None of the lipoprotein components have been correlated with the age and parity of the pregnant women (350).

1.13.3.2 Fetal lipid profile normal pregnancy

Lipid profile of the fetal and placental circulations in normal pregnancy have only been mentioned as part of investigation into IUGR (48, 357) and have not been well characterized.

1.13.3.3 Lipid profile in the postnatal period

Both cholesterol and triglyceride concentrations have been shown to decrease significantly within 24 hours of delivery and this has been reflected in all lipoproteins(347). While triglyceride levels continued to decrease rapidly returning to non-pregnant levels during the puerperium, LDL remain elevated for at least six to seven weeks post-partum. The pregnancy related increase in lipids and triglyceride levels have been shown to reverse earlier and completely during lactation, leading to a lower long term cardiovascular risk (358).

1.13.4 Lipid profile in pregnancy complications of preeclampsia and intrauterine fetal growth restriction

1.13.4.1 Maternal Lipid profile in preeclampsia

Early pregnancy dyslipidaemia is associated with an increased risk of preeclampsia (359-361). Increased serum triglyceride levels in early pregnancy before 20 weeks are associated with preeclampsia (359). Diffuse endothelial dysfunction, secondary to oxidative stress, is an important pathological feature of preeclampsia (33). Elevated oxidized-LDL, particularly in conjunction with elevated triglycerides, appears to be a risk factor of preeclampsia. Oxidative conversion of low density lipoproteins (LDL) to oxidized-LDL is considered an important

step in transforming macrophages into lipid-laden foam cells destined to develop into early atherosclerotic lesions (362). Levels of Apolipoprotein A were found to be elevated in severe preeclamptic women (363) while very-low-density lipoproteins were also increased in pregnancies complicated by hypertension or preeclampsia (347, 363).

Conflicting data have been published on lipid profiles in preeclampsia. Preeclamptic women have been shown to exhibit, in the third trimester and puerperium, higher mean serum TG concentration and lower high density lipoprotein (HDL) cholesterol and Apolipoprotein AI levels compared with healthy pregnant women in third trimester of pregnancy (364). LDL-mean particle diameter (LDL-MPD) and LDL cholesterol-Apo lipoprotein B ratio were also significantly reduced in the pathologic group. Another published study has not confirmed these findings, documenting that triglycerides, total cholesterol, LDL, HDL, and LDL cholesterol did not differ significantly between preeclamptic women and pregnant controls (365). ApoA1 and ApoB levels have also been described to not be different in normal and preeclamptic patients (366).

Peripheral monocytes and tissue macrophages are known to have a role in dyslipidaemia and atherosclerosis (367). A role for the lipid status and metabolic milieu of the mother has been proposed as a mechanism for the clinically diverse presentations of preeclampsia and fetal growth restriction in the mother with similar placental disease (65).

1.13.4.2. Maternal Lipid profile in IUGR

The lipid profiles in pregnancies complicated by IUGR without PE have not been well characterized. Lipid profile in mothers with insulin resistance and IUGR in the fetus has been noted to have lower total TC and LDL levels when compared with normal pregnancy controls (368). In one of the few studies on lipid profile in IUGR, a cross-sectional study of lipid and

lipoprotein concentrations in the third trimester, from normal pregnancies, and those complicated by IUGR without preeclampsia found no significant differences in the median concentrations of triglyceride, high-density lipoprotein, and very-low-density lipoprotein 1 (VLDL1), between cases and controls. The TC, LDL, VLDL2 and intermediate density lipoprotein levels were significantly lower. Because VLDL2 and intermediate-density lipoprotein are the synthetic precursors to LDL in the circulation, their significantly lower concentrations may imply a failure of appropriate LDL synthesis in IUGR pregnancies. LDL measurements in the mother were proposed by the authors as a screening test for pregnancies at risk for IUGR (369).

1.13.4.3 Fetal lipid profile in PE and IUGR

While little literature is available on the fetal lipid profile in PE, the lipid status in cord blood and neonates of IUGR pregnancies has been documented.

Fetal / umbilical cord blood serum lipids and low-density lipoprotein (LDL) concentrations were measured in IUGR and constitutionally small for gestational age neonates and compared with those of healthy, adequate for gestational age, born neonates. Fetal high-density lipoprotein cholesterol (HDL) and total cholesterol (TC) concentrations were found to be significantly lower in the IUGR compared to the normal and SGA groups (357). In a comparison between asymmetrical and symmetrical fetal growth restriction, the serum triglyceride levels with respect to controls were observed to be elevated in the asymmetrical IUGR groups. The authors concluded that newborns with intrauterine growth retardation have higher triglyceride levels than normal term newborns. SGA at birth has been associated with cardiovascular disease in late life (48). The elevated TG may play a role. Cholesterol levels

have not been shown to be influenced by birth weight and gestation while triglyceride values increased with prematurity and growth retardation in a south east population (370).

A comparison of circulating Apolipoprotein A1 and B concentrations in fetal umbilical plasma samples obtained at diagnostic cordocentesis of growth-retarded compared with normal fetuses found that while there were no differences in median plasma Apolipoprotein AI concentrations between the groups, plasma Apolipoprotein B levels and the ApoB/A1 ratio were significantly higher in growth-retarded fetuses. This observation may reflect the link between low birth weight and adult onset atherosclerosis (371). Apolipoproteins are under genetic control and present a genetic risk for changes in the metabolism of cholesterol, coagulation, and cardiovascular disease in adulthood (372). Maternal smoking during pregnancy has marked effects on lipid metabolism in the fetus leading to lower HDL, ApoA1, higher TC/TG and ApoB/ApoA1 ratios (373).

1.14 Overview and Significance

It is clear that establishment and maintenance of adequate placental angiogenesis and placental circulation is critical for normal fetal growth and development. Placental vascular disease as defined by abnormal umbilical artery Doppler studies underlies pregnancy complications such as intrauterine growth restriction and preeclampsia.

To date most of the studies on angiogenic factors have investigated aberrations in levels of angiogenic factors in maternal blood. Whether aberrations arise from the fetal circulation or the placenta is less well documented.

With the elucidation of aberrations in the pathophysiology of angiogenic factors, the pathogenesis of placental vascular disease can be better understood. Better understanding of the pathogenesis of placental disease may lead to the development of diagnostic tests for early detection of these clinically significant disease processes.

A collection of studies into the pathogenesis of preeclampsia and intrauterine fetal growth restriction are presented, focusing on factors involved in the angiogenesis and maintenance of the microcirculation of the placental villi as well as maternal factors such as circulating monocytes and lipid status. Previous literature on angiogenic factors has used various definitions for PE and IUGR, leading to inclusion of different types and stages of preeclampsia as well as small for gestational age fetuses including normal constitionally small fetuses in the patient selections. Automated analysis of placental expression of angiogenic factors in PE and IUGR has not been published. The results of this work will identify and compare the localisation of angiogenic factors and their receptors in the human placenta and levels in the maternal peripheral and fetal cord blood between normal pregnancy and pregnancies complicated by preeclampsia and fetal growth restriction. An analysis of placental staining using automated image analysis will be developed and described as an objective method to reduce interobserver variability in assessing immunohistochemical staining.

The role of maternal and fetal monocytes in preeclampsia has just started to be explored in the literature and there are currently no studies published on IUGR. This study will also aim to clarify the contribution of maternal peripheral monocytes to the angiogenic profile of the normal and complicated pregnancy as well as investigate whether monocyte phenotype and polarization of monocytes/macrophages into inflammatory vs healing phenotypes play a role in the pathogenesis of placental vascular disease. The literature is sparse on the maternal

monocyte contribution to the circulating sFlt-1 and Endoglin levels. There are no data published on fetal monocyte sFlt-1 and Endoglin expression. While the lipid status in preeclampsia has been previously explored in the literature, the role of maternal and fetal lipid status in IUGR is not clearly defined. A pilot study into the maternal and fetal lipid status in PE, IUGR and PE+IUGR will be presented.

Better understanding of the pathogenesis of placental disease may lead to the development of diagnostic tests for early detection of placental disease. Manipulation of regulators of angiogenesis may provide novel and powerful methods to prevent or reverse the progression of the disease, reducing perinatal morbidity and mortality associated with placental disease and ensure positive outcomes for most pregnancies. Identifying and screening for maternal risk factors may lead to preventative strategies for preeclampsia and intrauterine fetal growth restriction during pregnancy as well as long term cardiovascular diseases in the mother.

1.15: Hypotheses

1. Aberrant expression of placental angiogenic factors is associated with placental vascular disease and its clinical manifestations such as fetal intrauterine growth restriction (IUGR) and preeclampsia (Chapter 3).

2. Maternal peripheral and fetal umbilical arterial blood levels of angiogenic factors and their receptors are deranged in fetal intrauterine growth restriction (IUGR) and preeclampsia as compared to normal pregnancy (Chapter 4).

3. There is a difference in the monocyte phenotype in preeclampsia compared to normal pregnancy and pregnancy complicated by fetal growth restriction (Chapter 5).

4. Inflammatory marker expression on monocytes shows a pro-inflammatory phenotype in PE but not in IUGR (Chapter 5).

5. The monocyte phenotypes will be different with increasing gestation in normal pregnancy (Chapter 5).

6. There will be a difference in the cord blood monocyte subset distribution between pregnancies complicated by preeclampsia and fetal growth restriction as compared to normal pregnancy (Chapter 6).

7. There will be a difference between the cord blood monocyte subset distribution between maternal and fetal circulations (Chapter 6).

8. The monocyte expression of Flt-1 and Endoglin will change with increasing gestation in normal pregnancy (Chapter 7).

9. There will be a difference in the expression of membrane bound anti-angiogenic factors Flt-1 and Endoglin in different monocyte subtypes (Chapter 7).

10. Abnormalities in soluble anti-angiogenic factors such as sFlt-1 and sEndoglin found in maternal plasma of mothers with preeclampsia and /or fetal growth restriction are contributed to by maternal circulating monocytes and/or fetal circulating monocytes (Chapter 7).

11. There will be a difference in the monocyte expression of membrane bound anti-angiogenic factors Flt-1 and Endoglin in preeclampsia as compared to the normal pregnancies and pregnancies complicated by fetal growth restriction (Chapter 8).

12. The inflammatory M1 monocyte phenotype is associated with an increased expression of Flt-1 and Endoglin.

13. The maternal and fetal lipid profile differs between the clinical groups of normal pregnancy, preeclampsia, intrauterine fetal growth restriction and preeclampsia with IUGR.

METHODS

Chapter 2 – Materials and Methods

2.1 Ethics approval

The collection and use of the samples for this research was approved by two separate ethics committee submissions.

1. Human Research Ethics committee of the Western Sydney Area Health Service approval number HREC2005/10/4.14 (2219) and the University of Sydney.

2. Human Research Ethics committee of the Western Sydney Local Health District: Approval number HREC/13/WMEAD/117 (3706).

All women provided written informed consent before the collection of samples. All samples were collected between January 2006 and May 2014.

2.2 Summary of research

Several prospective case control studies were performed, investigating maternal, fetal and placental angiogenic factor expression, monocyte subsets and lipid profiles in normal pregnancies and pregnancies complicated by preeclampsia and intrauterine fetal growth restriction.

The research included in this thesis was performed in two stages of sample collection and experiments. The placental work and maternal circulating angiogenic factors presented were performed under a separate ethics application and a time scale (sample collection 2005-2008) to the later chapters on monocyte work (sample collection 2013-2014).

Stage 1: 2006-2011

Experiment 1: Localisation and placental expression of angiogenic factors and their receptors in normal and complicated pregnancies using immunohistochemistry (Results Chapter 3).

Experiment 2: Expression of angiogenic factors and their receptors in the maternal peripheral circulation and fetal cord blood in normal and complicated pregnancies (Results Chapter 4).

Stage 2: 2012-2014

Experiment 3: Characterization of maternal and fetal monocyte phenotype and polarization in preeclampsia and intrauterine fetal growth restriction (Results Chapter 5).

Distribution of cord blood monocyte subtypes in preeclampsia and intrauterine fetal growth restriction (Results Chapter 6).

Experiment 4: Anti-angiogenic factor expression by maternal and fetal monocytes

(Results Chapter 7).

Experiment 5: Maternal and fetal lipid profiles in preeclampsia and intrauterine fetal growth restriction (Results Chapter 8).

2.3 Patient selection

2.3.1 Participant groups

Participants were recruited from four clinical groups of pregnant patients over 16 years of age, between 24-41 weeks of gestation, delivering a singleton pregnancy at a major tertiary centre, Westmead Hospital in Sydney Australia.

1. Normal pregnancy (Normal)

2. Pregnancies complicated by preeclampsia (PE)

3. Pregnancies complicated by intrauterine fetal growth restriction (IUGR)

4. Pregnancies complicated by preeclampsia and intrauterine fetal growth restriction (PE+IUGR)

2.3.2 Selection criteria

Gestational age was calculated using last menstrual period (LMP) dates and confirmed by antenatal ultrasound. The confirmed expected date of confinement (EDC) and gestation from the hospital records were used for the study. The hospital has a protocol on deciding on the final EDC by correlation of the (LMP) dates and the first trimester ultrasound. When there is a discrepancy of > 4 days between LMP and ultrasound dates, the ultrasound dates were used for the EDC to manage the pregnancy.

Normal pregnant women were enrolled from either the delivery suite or the antenatal clinic at Westmead Hospital. A patient was considered to have a normal pregnancy if she met the following criteria: (1) no medical, obstetrical, or surgical complications, (2) delivery of a normal term (≥37 weeks) infant whose birth weight was over the 10^{th} percentile for gestational age.

Since the research was commenced in 2005, preeclampsia was defined according to the 2001 guidelines of the International Society for the Study of Hypertension in Pregnancy / Australian Society for the Study of Hypertension in pregnancy (ISSHP / ASSHP): diastolic blood pressure of 90 mmHg or more on two or more consecutive occasions more than 4 hours apart and proteinuria of more than 300mg /24 hours or proteinuria as spot urine

protein/creatinine ratio ≥ 30mg /mmol (20). All patients included in stages 1 and 2 of the research study satisfied the ISSHP / ASSHP 2001 criteria for preeclampsia. The 2001 ISSHP/ASSHP definition of hypertension and proteinuria were continued throughout the research as it was thought appropriate to continue the same definition of preeclampsia since the focus of the research was to define the maternal, fetal and placental factors that lead to the development of the maternal syndrome of preeclampsia with and without the fetal syndrome of intrauterine fetal growth restriction.

The recent 2014 release of the ISSHP and SOMANZ guidelines is acknowledged (21) and included in the literature review. The ISSHP /SOMANZ statements were released after the presented research was conducted.

Intrauterine fetal growth restriction was defined as birth weight less than 10^{th} centile (374) with umbilical artery Doppler Systolic / Diastolic ratio or Resistance Index >95^{th} centile for gestation (375) or in the presence of abnormal waveforms (absent or reversed end-diastolic velocities). The elevated resistance of the umbilical artery Doppler was included in the selection criteria to ensure the study of placental IUGR rather than constitutionally small fetuses or IUGR secondary to other causes such as intrauterine infection. The birth weight centiles were incorporated into the study by the fact that all neonates delivered to the IUGR or PE+ IUGR groups were <10^{th} centile for gestation, while all normal pregnancy and PE only groups delivered a neonate with birth weight ≥ 10th centile for gestation. All ultrasound assessments were performed by trained and qualified staff using General Electric (GE) Voluson 730 or GE Voluson E8 ultrasound equipment.

Patients satisfying the criteria for inclusion were recruited from the antenatal clinic, obstetric ultrasound service and from the inpatient wards. The samples were collected prior to

established labour and prior to caesarean section in elective caesarean section to eliminate any bias due to the method of delivery.

2.3.3 Urinary protein excretion

The first stage of the study (sample collection 2005-2008) used a 24 hour urinary collection and a value of protein excretion of > 300mg /24 hours was used in the definition of preeclampsia (results presented in Chapter 3 and 4) as this was the hospital clinical practice at the time.

The second stage of the study (sample collection 2013-2014) used spot urinary protein /creatinine ratio of ≥ 30mg/mmol as the definition of proteinuria in the diagnosis of preeclampsia. The change was due to a change in practice protocols at the hospital from 24 hour urinary collection to spot urine testing for quantification of proteinuria.

2.3.4 Exclusion criteria

Patients with pre-existing hypertension, renal disease with pre-existing proteinuria, pre-existing diabetes, gestational diabetes and multiple pregnancies were excluded from the study.

Patients with any overt signs or evidence of bacterial or virus infections (chorioamnionitis, upper respiratory tract infections, urinary tract infection, known active Hepatitis A, B, C or HIV infection) were excluded from the study.

2.3.5 Umbilical artery Doppler resistance

Umbilical artery Doppler resistance (Figure 2.1) has been well described as a physiological measure of placental function (14, 15, 376). In a comparison of the screening efficiency of the different indices, the sensitivity was not shown to be significantly different although the

specificity was higher with Pulsatility index (377). All patients with PE, PE+IUGR or IUGR underwent antenatal ultrasound examination after 24 weeks of gestation and within 7 days of delivery. The frequency of ultrasound was determined by the clinical guidelines of the hospital and the presence of preeclampsia or IUGR, with biometry evaluated fortnightly and elevated resistance of the umbilical artery Doppler resistance re-assessed on a weekly basis.

Index	**Abbreviation**	**Description**
S / D ratio	SD	Peak Systolic velocity/ End Diastolic velocity
Pulsatility Index	PI	(Peak Systolic velocity-End Diastolic velocity) / time averaged maximum velocity
Resistance (Resistive) Index	RI	(Peak Systolic velocity-End Diastolic velocity) / Peak Systolic velocity

Table 2.1 Description of indices used for evaluation of umbilical artery Doppler resistance.

2.3.6 Data collection

De-identified demographic information of the mother and neonate as well as details of diagnosis, ultrasound findings and clinical outcomes were extracted from the hospital patient files and ultrasound records. The demographic data for the pregnancies recruited into the study were personally collected by the investigator on a data collection form to eliminate errors associated with a hospital database and cross checked with the obstetric clinical database (Obstetrix) at Westmead Hospital. Data storage was in a secure research facility at the hospital.

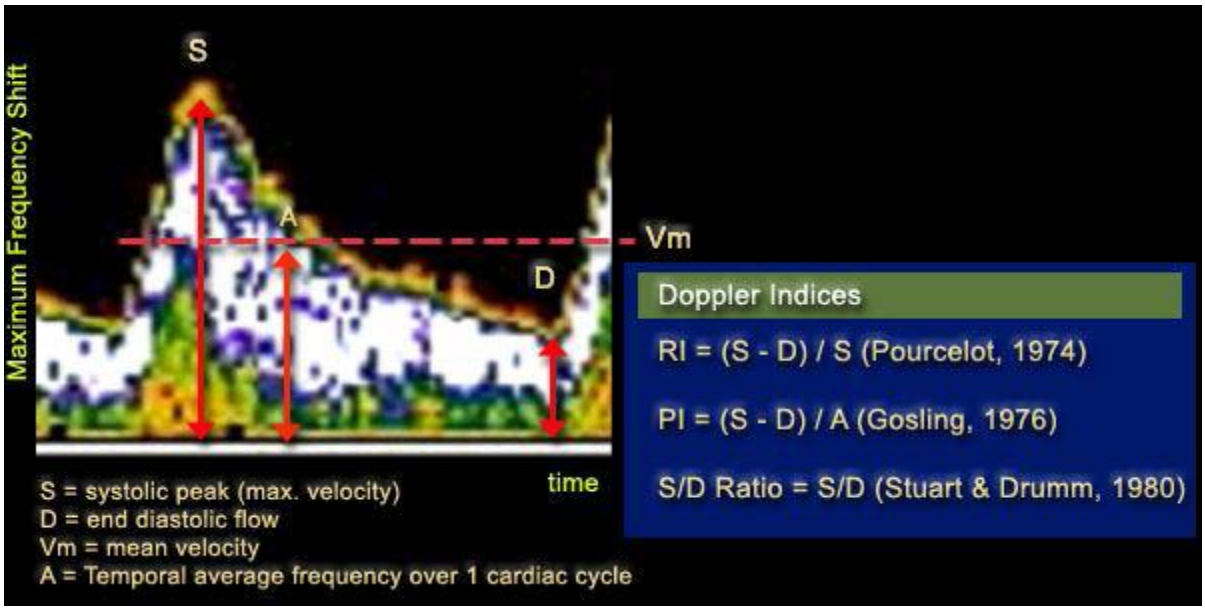

Figure 2.1 Commonly used umbilical artery Doppler Indices and their calculation.

RI = Resistance index (also called resistive index or Pourcelot's index); Systolic/diastolic (S/D) ratio, sometimes called the A/B ratio and Pulsatility index (PI). These indices are all based on the maximum Doppler shift waveform (378).

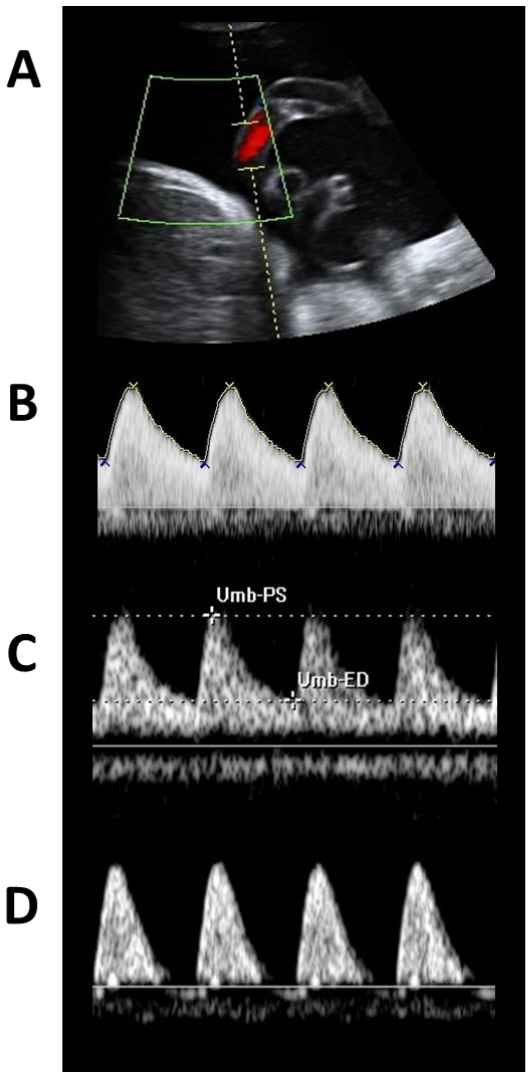

Figure 2.2 Umbilical artery Doppler waveform evaluation was used to assess placental function. Elevated systolic /diastolic ratio or Resistance index was used as an indicator of significant placental insufficiency including loss of villi (15, 375). A: Doppler sampling technique for recording umbilical artery Doppler waveform. B: Normal S/D ratio and resistance Index. C: Elevated Systolic/Diastolic ratio or Resistance index. D: Absent end diastolic flow indicating severe placental disease.

2.4 Immunohistochemistry

2.4.1 Placental sample collection and processing

A prospective cohort study was undertaken into the placental expression and localization of angiogenic factors and receptors of the vascular endothelial growth factor family. Placentas were collected at delivery from all consented patients. Four representative 1cm x 1cm placental blocks were selected randomly from each placenta, 2cm away from the placental margin. Areas of obvious infarction were avoided. The tissues were fixed in 10% neutral buffered Paraformaldehyde for 18 hours prior to embedding in paraffin. Although the heterogeneous nature of the placenta is well known, placental immunohistochemistry on representative samples was chosen due to the focus of interest being to describe the expression of angiogenic factors and their receptors with respect to the histologic type of tissue. Similar techniques and number of samples have been described in previous published literature (379). Placental weights were not available for all pregnancies. Placental weights of the normal pregnancies were not recorded.

2.4.2 Placental Morphologic Criteria

The hematoxylin and eosin stained slides were reviewed by an independent blinded perinatal pathologist. The placental morphological characteristics were classified using accepted pathological features of preeclampsia and fetal growth restriction such as increased syncytio-trophoblastic knots, villous hypercapillarization, increased perivillous fibrin deposition, villous infarction, shrinkage of villi, vascular obliteration and villous fibrosis.

2.4.3 Quantification of number of villi

The number of villi per high power field (magnification x 400) was recorded for five randomly selected fields from each placental sample by an independent perinatal pathologist. The morphologically normal villi in a placental sample containing villous infarction were considered peri-infarction villi.

2.4.4 Placental immunohistochemical staining for angiogenic factors and their receptors

The overall goal was to achieve optimal specific staining accompanied by minimal interference from background staining. The placental expression of VEGF, PlGF, Flt-1 and KDR were examined using commercially available polyclonal antibodies (Table 2.2) on formalin fixed, paraffin embedded serial tissue sections prepared for immunohistochemistry by standard methods.

2.4.4.1 Optimization

Primary antibody dilutions were optimized using manual immunohistochemical staining methods using Santa Cruz biotechnology Immunocruz staining systems. Manually optimized antibody dilutions were used to assess the localization and expression of VEGF, PlGF, Flt-1 and KDR.

2.4.4.2 Staining Method

The staining procedure was performed using the rabbit ImmunoCruz staining system sc-2051 (Santa Cruz, California, USA). The antibodies and the dilutions used are listed in Table 2.2.

Antibody	Host	Source	Dilution	Incubation time
anti-VEGF antibody (sc-152)	Rabbit polyclonal	Santa Cruz Biotechnology, California, USA	1:100	2 hours
Anti-PlGF antibody (sc-1880)	Goat polyclonal	Santa Cruz Biotechnology, California, USA	1:50	2 hours
anti-Flt-1 antibody (sc-316)	Rabbit polyclonal	Santa Cruz Biotechnology, California, USA	1:100	2 hours
Anti-KDR antibody (sc-19530)	Goat polyclonal	Santa Cruz Biotechnology, California, USA	1:50	2 hours

Table 2.2 Summary of primary antibodies used for immunostaining.

Five micron sections were cut from formalin fixed tissue embedded in paraffin blocks and mounted on silane coated slides for immunohistochemistry. To remove possible variation arising from manual staining techniques, an automated immunostainer (Discovery XT, Ventana Medical Systems, Tucson, Arizona, USA) was used with standard staining techniques. The samples were incubated with blocking serum to reduce non-specific reactions (20 min), and incubated with the primary antibody at 37^0 Celsius. The slides were incubated with secondary antibody for 20 min at 37^0C. The sections were counterstained with Mayer's Hematoxylin. Immunoreactivity was localized with *3,3'-diaminobenzide* (DAB) map kit with an incubation time of 30 minutes. All sections were stained in one of two batches to minimize

inter-batch variations. A Haematoxylin and Eosin (H&E) stain was performed on a consecutive paraffin section for all samples.

2.4.4.3 Controls

Tissue sections stained with secondary antibody only were used as negative controls.

2.4.5. Semi-quantitative (manual) analysis of immunohistochemical staining intensity

Quantification of immunoreactivity was accomplished using an immunohistochemical scoring system. Five randomly selected high power fields (magnification x 400) from each placental sample were examined by two independent observers (one blinded to clinical group) for VEGF and Flt-1 staining and scored for localization, tissue types and intensity in colour. Staining intensity was rated on a scale of 0 to 3, with 0 = negative; 1 = weak staining; 2 = moderate staining, and 3 = strong staining. The mean of the 5 fields was taken as the staining intensity score for each sample. Localization of staining was recorded for trophoblast, vascular endothelium, stromal cells and Hofbauer cells (defined in Chapter 1 section 1.2.3).

The slides stained with PlGF and KDR were not assessed with the semi-quantitative method due to the low overall staining intensity levels. This was based on the results of comparison between the semi-quantitative (manual) and automated quantitative staining assessments for VEGF and Flt-1 that suggested a correlation between the methods for moderate and strong staining, with a poor correlation for weak staining.

To assess the inter-observer variability in semi-quantitative scoring of immunostaining, two observers manually analyzed and scored VEGF immunostaining of 120 slides from 30 placentas. Observer 2 was blinded to the clinical study groups.

2.4.6 Analysis of placental angiogenic factor expression using Aperio Scanscope digital image analysis

2.4.6.1 Digital image analysis of immunohistochemical staining.

Traditionally, immunohistochemical staining characteristics have been presented as positive or negative staining. Semi-quantitative manual reading of immunohistochemistry staining with semi-quantitative scoring systems were used to compare staining characteristics between normal and pathological pregnancies including studies of angiogenic factors. While digital image analysis techniques have been used in two studies to evaluate placental morphological characteristics (379, 380) in preeclampsia and intrauterine fetal growth restriction, to date no studies have been published using digital imaging techniques for the analysis of placental angiogenic factor expression. Digital image analysis were used in this study and reproducibility of the results was assessed.

2.4.6.2 Digitization of images

Four placental samples from each placenta were analyzed for each of the angiogenic factors and receptors VEGF, PlGF, Flt-1 and KDR. A total of 120 individual slides for each of the biomarkers were digitized using the Aperio Scanscope CS microscope (Aperio Technologies, Vista, CA, Version 6.25) with a 20x objective magnification and a digital image generated.

2.4.6.3 Quantification of immunohistochemical staining

Quantitation of percentage of cells that were immunopositive (DAB, brown colour) was accomplished using Aperio Image scope reader software v11.2.0.780, a free software image viewer.

To numerically analyze the immunohistochemical staining, the digitized images were read using the Positive pixel count algorithm for quantitative analysis. The region of interest (ROI)

on each of the virtual slides was set as the whole tissue sample to minimize any selection bias in choosing more or less stained sections for analysis.

Using the positive pixel count algorithm, the intensity of membrane specific staining was used to calculate the staining intensity and percent target labeled for each sample by digitally analyzing the color intensity. A color markup image for each slide was obtained based on membrane staining intensity. The output was viewed as determinations of staining intensity ranging from 0 to 3 to correlate with conventional manual scoring methods (0, negative; and 3, strong staining) and statistical analyses were performed using the mean of these values.

2.4.7 Positive pixel count algorithm (Aperio technologies version 9.1) for quantification of staining

The Positive Pixel Count algorithm was used to quantify the amount of brown (DAB) stain present in the scanned slide images.

For pixels that satisfy the colour specification, the algorithm counted the number and intensity sum in each intensity range. Intensity (Red + Green + Blue)/3) is the measure of brightness of the pixel and is the average of the given three colours. Intensity ranges from zero (black) to 255 (bright white), so that a large intensity value means that the pixel is brighter. Intensity is the opposite of density. Intensity is proportional to the amount of light transmitted through the slide, while density is proportional to the amount of light that is blocked by the stained tissue (Figure 2.3). It is worth noting that using this positive pixel algorithm, the stronger staining pixels will have a lower value due to lower intensity (brightness) of the pixel while the higher numbers indicate lower staining.

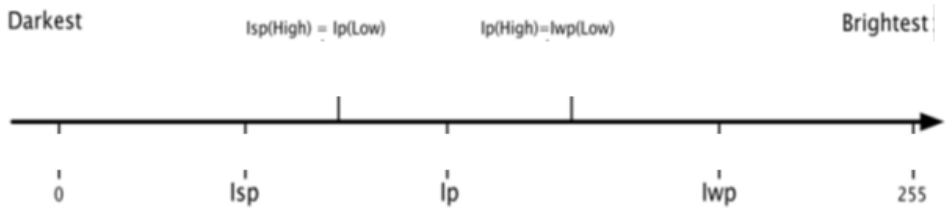

Figure 2.3 Intensity ranges as assigned by the Aperio positive pixel algorithm (381).

Isp = strong positive intensity. Ip = positive intensity. Iwp = weak positive intensity.

The algorithm's pre-configured set of default input parameters for brown colour quantification in the three intensity ranges were used for the quantification of the current slides. A pre-programmed algorithm for analysis of IHC staining consisted of the following: hue value of 0.1 (consistent with recognition of brown pixels), hue width of 0.5 and colour saturation of 0.04. These parameters allowed for consistent identification of brown pixels (positive immunoperoxidase signal) and consistent exclusion of pixels containing other colours.

The pseudo colour markup image generated by the program corresponded to the desired colour and intensity ranges observed with the naked eye using a light microscope (Table 2.3 and Figure 2.4). The thresholds used to define each intensity category are listed in Table 2. Pixels which stained, but did not fall into the positive colour specification, were considered negative stained pixels. These pixels were also counted by the algorithm, so that the fraction of positive to total stained pixels could be determined.

Intensity range	Intensity range	Colour on mark-up image	Semi-quantitative levels
No tissue	NA	White	NA
Negative	>220	Blue	negative
Weak-Positive Intensity	175-220	Yellow	1
Moderate Positive Intensity	100-175	Orange	2
Strong-Positive Intensity	<100	Red	3

Table 2.3 Intensity thresholds and colour as assigned by the positive pixel algorithm.

Colour coding of the three intensities were used to ensure that the three thresholds correlated with the colour intensity as viewed through the light microscope. Different colors indicate the intensity level of positive pixels (red = strong, orange = moderate, yellow = low). Black arrows = syncytiotrophoblast, Red arrow = vascular endothelium.

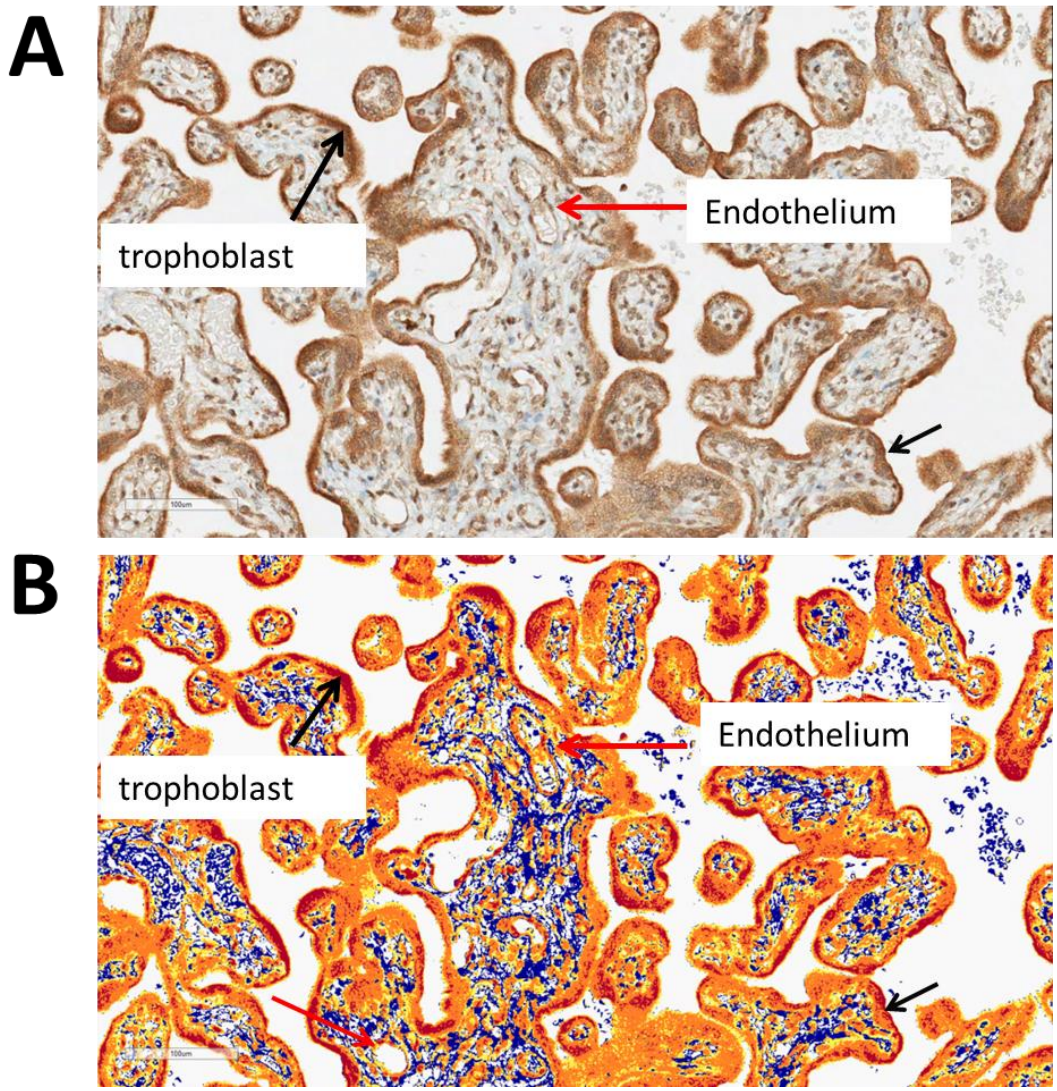

Figure 2.4 The image scope markup image showing the correlation between the manual read of intensity (A) and the automatic analysis using the Positive Pixel count algorithm set thresholds (B). Magnification x 200

The analysis of immuno-histochemical staining of digitized images achieved using the Aperio Scanscope CS microscope and Aperio Image scope reader software has been shown to correlate with manual reading of slides via photomicroscope in several different tissues (382, 383). The percentage of strongly positive /3+ pixels was used to compare pathologist's scores with Aperio algorithm results. Satisfactory digital images were obtained from all immunohistochemically stained slides. No cases were excluded from the analysis.

2.5 Enzyme-linked immunosorbent assay (ELISA) for measurement of circulating angiogenic factor levels

2.5.1 Methods and population

A prospective cross-sectional case control study was conducted. A total of 84 patients between 24-40 weeks of gestation, delivering at Westmead Hospital during the period 2005 - 2007 were recruited into four clinical groups normal pregnancy, preeclampsia, preeclampsia with IUGR and IUGR.

2.5.2 Sample Collection

Maternal venipuncture was performed and blood collected within 24 hours prior to delivery for all of the pregnancies delivered during the study and during the antenatal clinic visits for the normal controls less than 37 weeks from uncomplicated pregnancies. Umbilical artery cord blood samples were collected at delivery. All maternal and cord blood collections were performed manually with 10-20ml syringes. The umbilical cord was wiped down with an alcohol wipe prior to sample collection to avoid maternal cell contamination. The same technique was used for all blood sample collections. Blood samples were collected into tubes containing EDTA. The samples were left at room temperature for 30 minutes, centrifuged 10 minutes at 1000g and plasma stored at −70°C. The samples were collected prior to established labour in laboring women and prior to caesarean section to eliminate any bias due to the method of delivery.

2.5.3 Measurement of circulating pro and anti-angiogenic factor levels using ELISA

The concentrations of plasma free VEGF, PlGF, sFlt-1, sKDR and sEndoglin were measured using enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, US), performed according to the manufacturer's instructions.

These assays used the quantitative sandwich immunoassay technique. Recombinant human VEGF, Flt-1, PlGF, sKDR and sEng standards and maternal plasma specimens were diluted in 0.1% PBS and incubated in duplicate wells for 2 hours in 96-well plates pre-coated with monoclonal capture antibody directed specifically to the biomarkers VEGF, sFlt-1, PlGF, sKDR or sEng. During this incubation, the immobilized antibodies in the plate bound the marker being tested which was present in the standards and samples. The wells were then washed three times in 0.05% PBS and incubated with a secondary antibody against VEGF, sFlt-1, P1GF, sKDR, or sEng conjugated to horseradish peroxidase for an additional 2 hours. After an incubation period, the assay plates were washed again three times to remove unbound antibody-enzyme reagent. With the addition of a substrate solution (tetramethylbenzidine), colour developed in the assay plates proportionally to the amount of the angiogenic factor bound in the initial step. The plates were read with a BioRad microplate reader (model 680) and optical density was determined at 450 nm. BioRad Immunowash model 1575 was used for plate washing.

All assays were done in duplicate, and the protein levels were calculated using a standard curve derived from known concentrations of the respective recombinant proteins (see Figure 2.4 A and B) for each set of data. Correction wave length was set at 570nm. The antibodies used, minimal detectable levels, inter and intra assay coefficients of variation have been listed in Table 2.4.

The measurable free plasma VEGF levels were noted to be less than the sensitivity level of the commercial assay in the optimization steps of the study using normal and pathological pregnancies. Further measurement of the VEGF levels was not undertaken of the study samples as this was unlikely to produce any interpretable results. The R&D Systems ELISAs for serum sFlt-1, PlGF and KDR have been previously validated. Correlations between serum and plasma levels of angiogenic factors have also been done and have been found to be parallel with the plasma levels marginally lower than serum levels. Plasma levels rather than serum are recommended (209, 384).

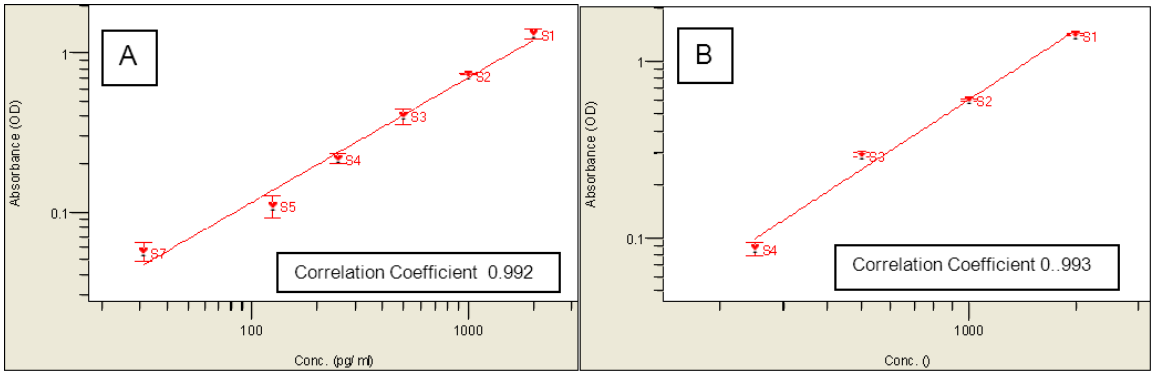

Figure 2.5 Examples of standard curves generated for sFlt-1 using known concentration controls.

A 11.10.2007: Regression type- linear; Correlation Coefficient 0.992. B 13.10.2007 Standard Curve for sFlt-1 13.10.2007; Correlation Coefficient 0.993. A correlation coefficient of > 0.09 was considered as an acceptable result for the assay.

Antibody for Immunoassay	Company	Minimum detectable levels (from product sheet)	Inter-assay precision (coefficient of variation %)	Intra-assay precision (coefficient of variation%)
Human VEGF Immunoassay DVE00	R&D Systems USA	9.0pg/mL	6.2-8.8	6.7-5.1
Human PlGF Immunoassay DPG00	R&D Systems USA	7.0pg/mL	10.9-11.8	3.6-7.0
Human sFlt-1 Immunoassay DVR100B	R&D Systems USA	3.35pg/mL	6.0-9.2	1.7-4.0
Human sKDR Immunoassay DVR200	R&D Systems USA	4.6pg/mL	6.9-7.0	2.9-4.2
Human sEng Immunoassay DNDG00	R&D Systems USA	0.007ng/mL	6.3-6.7	2.8-3.0

Table 2.4 The antibodies used in ELISA, minimal detectable levels, inter and intra assay coefficients of variation.

2.6 Flow cytometry for characterization of maternal and fetal monocyte phenotype, monocyte polarization and angiogenic factor expression in preeclampsia and intrauterine fetal growth restriction.

2.6.1. Methods and population

A prospective cross sectional case control study was conducted. Pregnant women between 24-40 weeks of gestation, delivering at Westmead Hospital during the period 2013 -2014 were recruited and classified into four clinical groups of normal pregnancy, preeclampsia, intrauterine fetal growth restriction (IUGR) and preeclampsia with IUGR.

2.6.2. Sample collection

For each sample from a pathological pregnancy at gestational age between 26-40 weeks, two maternal venous samples from normal pregnancy were collected as controls. Patients also had cord blood umbilical vein samples collected at delivery where possible.

For each pregnancy in the study, the following samples were collected with consent from the mother:

- 7 ml of peripheral venous blood from the mother
- 7 ml of cord blood from the umbilical artery at delivery

Preterm normal pregnancy samples were collected antenatally. The term normal pregnancy and the maternal samples for pathological pregnancies were collected antenatally within 7 days prior to delivery. Not all corresponding fetal cord blood samples could be collected due to several reasons including inadequate blood volumes in the umbilical cord of fetal growth

restricted fetuses, two cases of fetal demise after maternal blood collection due to severe fetal growth restriction, one case delivering in another hospital and emergency delivery after hours.

Patient numbers were limited by technical difficulties associated with cord blood processing, time constraints (each sample requiring approximately 12 hours for collection, preparation and flow cytometric and analysis) as well as the limitations of a single operator study**.** A number of cord blood samples were clotted and adequate monocyte numbers could not able to be harvested for flow cytometry.

2.6.3. Sample preparation

The maternal venous blood and fetal cord blood from the umbilical vein were collected using sterile tubes containing an EDTA salt as the anticoagulant. The samples were kept at room temperature (18 – 25°C) prior to analysis. The samples were analyzed within 2 hours of venipuncture.

Before cell staining for flow cytometry, an aliquot (500 µl) of the blood samples was used to establish total blood leukocyte numbers. Leukocytes were counted using a microcell counter (model Sysmex X1-1800c, Roche diagnostics, Australia Pty Ltd) at the Institute of Clinical Pathology and Medical research (ICPMR), Westmead Hospital.

2.6.4.1 Staining of whole blood for expression of monocyte markers using flow cytometry

Flow cytometry was performed on whole blood samples. Staining was performed for expression of surface markers - CD14 used for identification of monocytes, CD16 for classification into monocyte subtypes and CD86 and CD163 for classification into phenotypes M1 and M2.

2.6.4 Flow cytometry

Monocyte phenotype assessment and cell marker profile was assessed by flow cytometry using a BD Canto II flow cytometer and Flow Jo software version 10.6 (Tree star, Inc., Ashland, OR, USA). Two flow cytometry protocols using a multicolour fluorescence minus one principle and a three colour protocol were used for data acquisition.

Cell processing first involved specific staining of monocytes by incubation of whole blood aliquots with cell surface antibodies. This was followed by BD FAC lysing Solution Optilyse C for lysing red blood cells and fixation. Any unbound antibody was removed by two wash steps using PBS as described in the protocols (Figures 2.11 and 2.12). Standard operating procedures were followed to minimize variations in results due to sample preparation and analysis. All steps were performed at room temperature. Data on the stained samples was acquired on the Flow cytometer within 6 hours of preparation to minimize the inter-assay variation.

2.6.5 Isotype controls

The positive data set for each antibody was identified by using fluorescence minus one protocol with the recommended isotype control for each antibody fluorochrome colour and volume and overlying its image on the histogram. To minimize variations, isotype controls were used with the same dilutions as the marker of interest.

2.6.6. Monocyte gate

Using FACS Canto II flow cytometer, 20000 events in the P1/monocyte gate, defined as cells with respective side scatter (SSC) and CD14 staining characteristics, were acquired. Corresponding levels of CD86, CD163 expression were recorded on the cells obtained from

the CD14 cell gate. The results are expressed as mean fluorescence intensity (MFI) in CD14+ cells analyzed.

2.6.7 Optimization steps in developing staining protocols

The staining process was optimized for antibody concentrations, staining time, wash steps and use of mouse block to reduce non-specific staining. Some of the optimization steps are presented in the following Figures 2.6-2.10. The antibodies and the concentration used (2.5uL-20uL) are indicated in the legend within each figure.

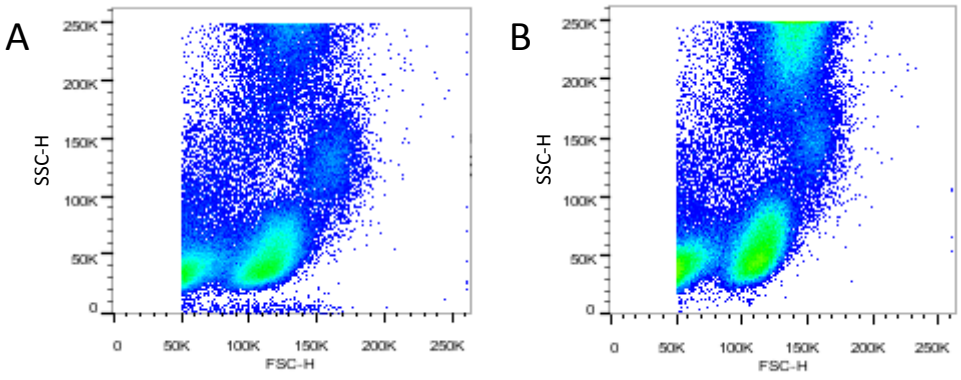

Figure 2.6 The effect of one cycle of washing on the differentiation of cell types on flow cytometry.

A: With wash cycle. B: Without wash cycle. With the inclusion of a wash cycle, a small difference was noted in reducing the amount of debris.

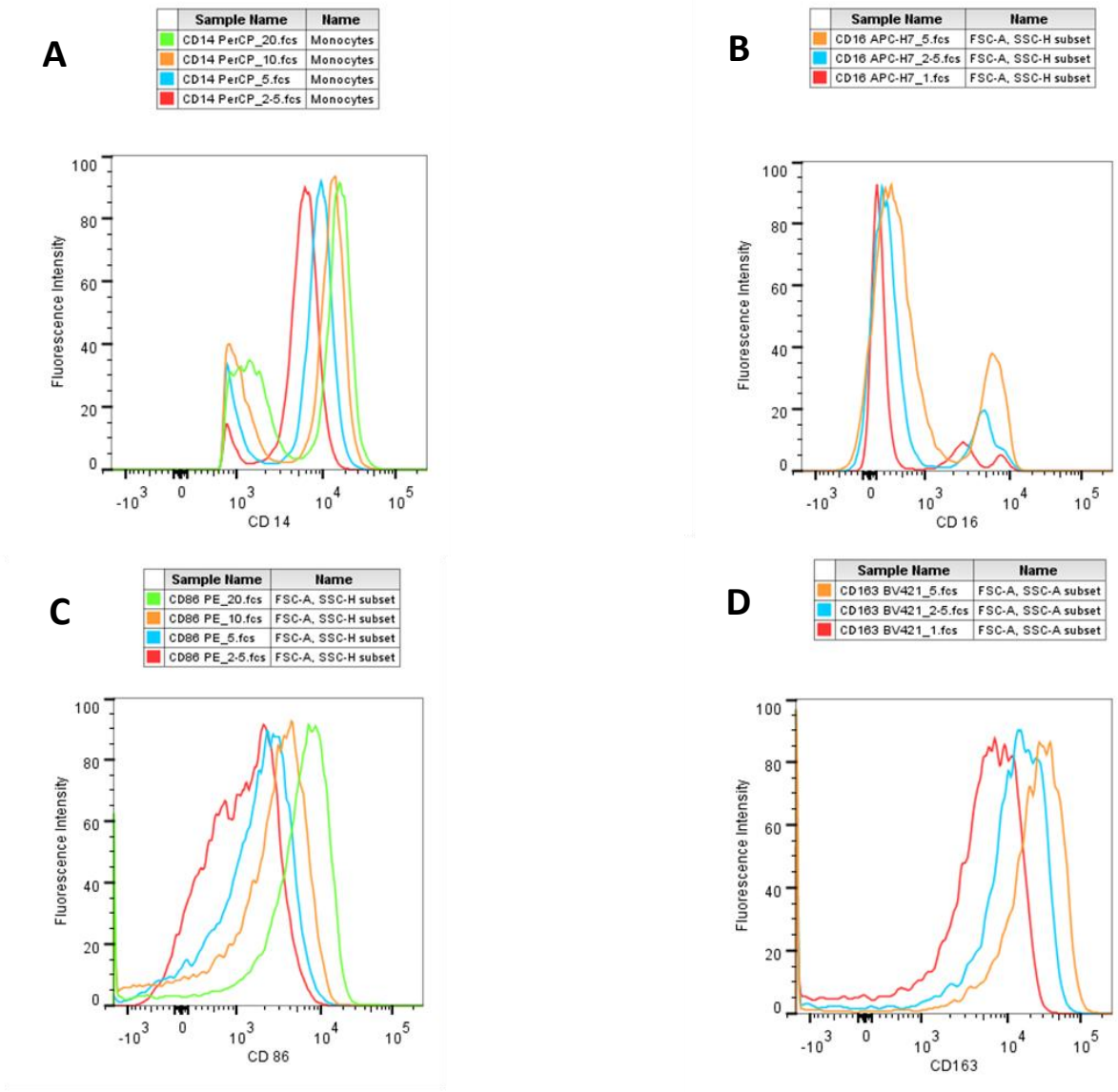

Figure 2.7 Optimization steps for titration of monocyte surface receptor antibodies.

A = CD14, B: CD16, C: CD86 and D: CD163. The tested concentrations all resulted in histograms appropriate for analysis (as presented here) and different to the isotype controls (results not presented). The smallest concentration for each marker was used for testing of the samples.

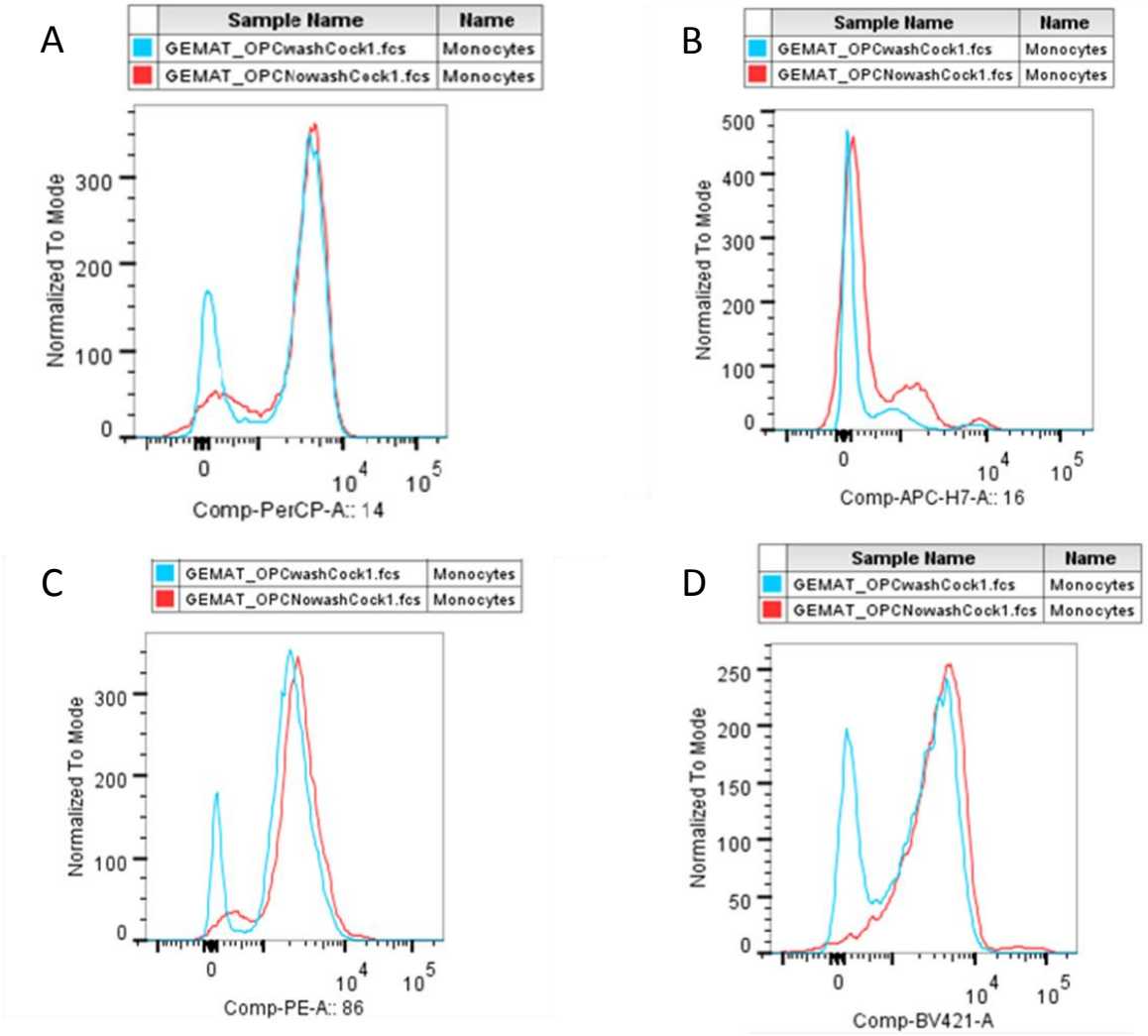

Figure 2.8 Effect of wash step on the fluorescence intensity of monocyte surface receptor antibodies.

A: CD14, B: CD16, C: CD86 and D: CD163. Red line = No wash step. Blue line = With wash step. No notable difference seen in the fluorescence intensity of the given antibodies with washing, as evident by the overlapping histograms. The separate peaks in the washstep (blue line) likely represents a separate cell type other than monocytes.

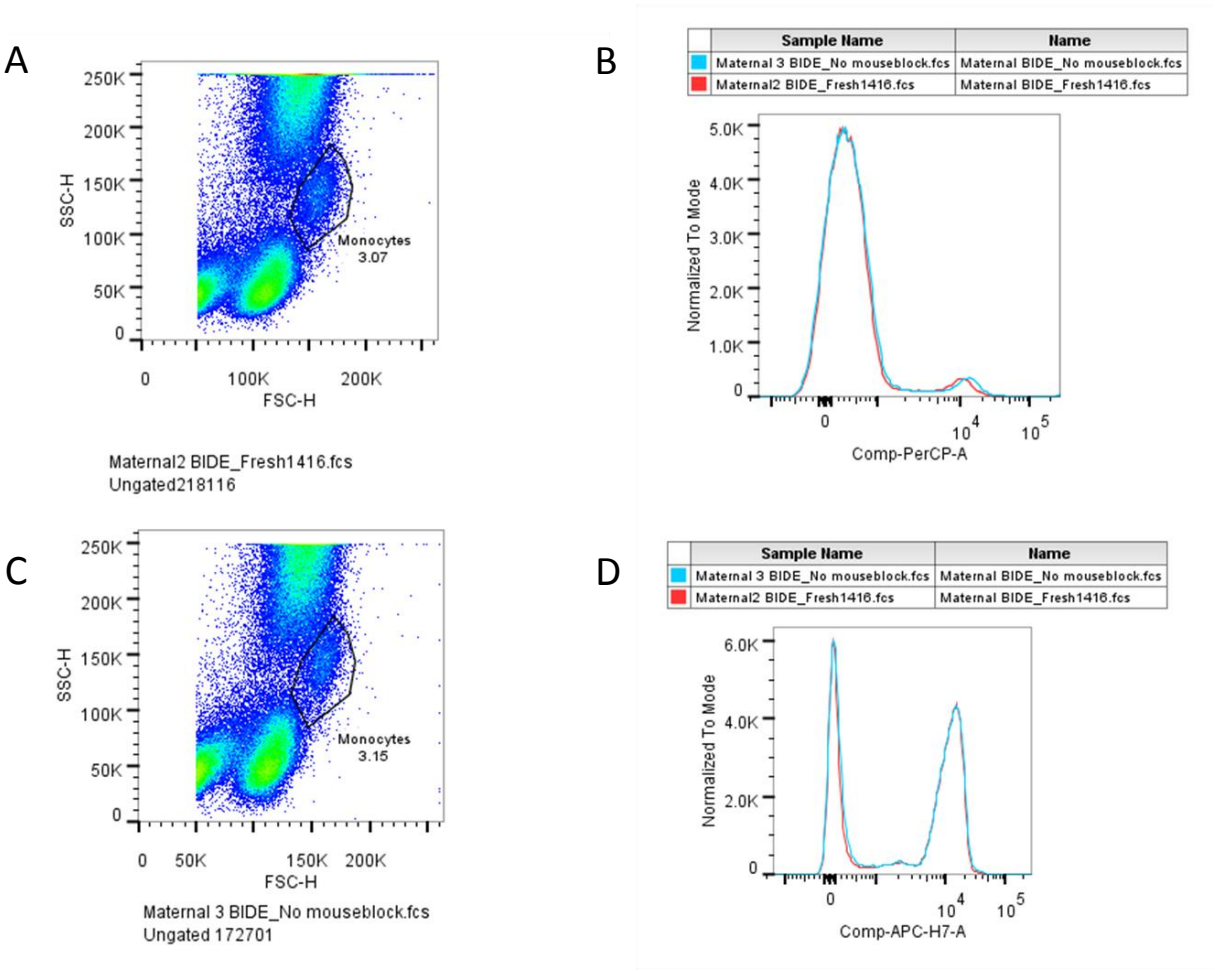

Figure 2.9 CD14 (Per-CP-A) and CD16 (APC-HT-A) with and without mouse block.

A: flow cytometry image showing separation of cell types with mouse block; B: Mean fluorescence Intensity of CD14 with mouse block; C: flow cytometry image showing separation of cell types without mouse block; D: Fluorescence Intensity of CD16 without mouse block. Red line = isotype control. Blue line = CD14 and CD16. No difference observed in the staining characteristics.

A difference in the fluorescent intensity was observed with the wash step in Flt-1 staining with the MFI reduced by the wash step. The difference was less marked with Endoglin. These findings may be due to the attachment of soluble Flt-1 to the cell surface, which is washed away with the wash steps (Figure 2.9). A decision was made to include two wash steps in the

staining protocols as the study was specifically focusing on cell surface expression of Flt-1 and Endoglin.

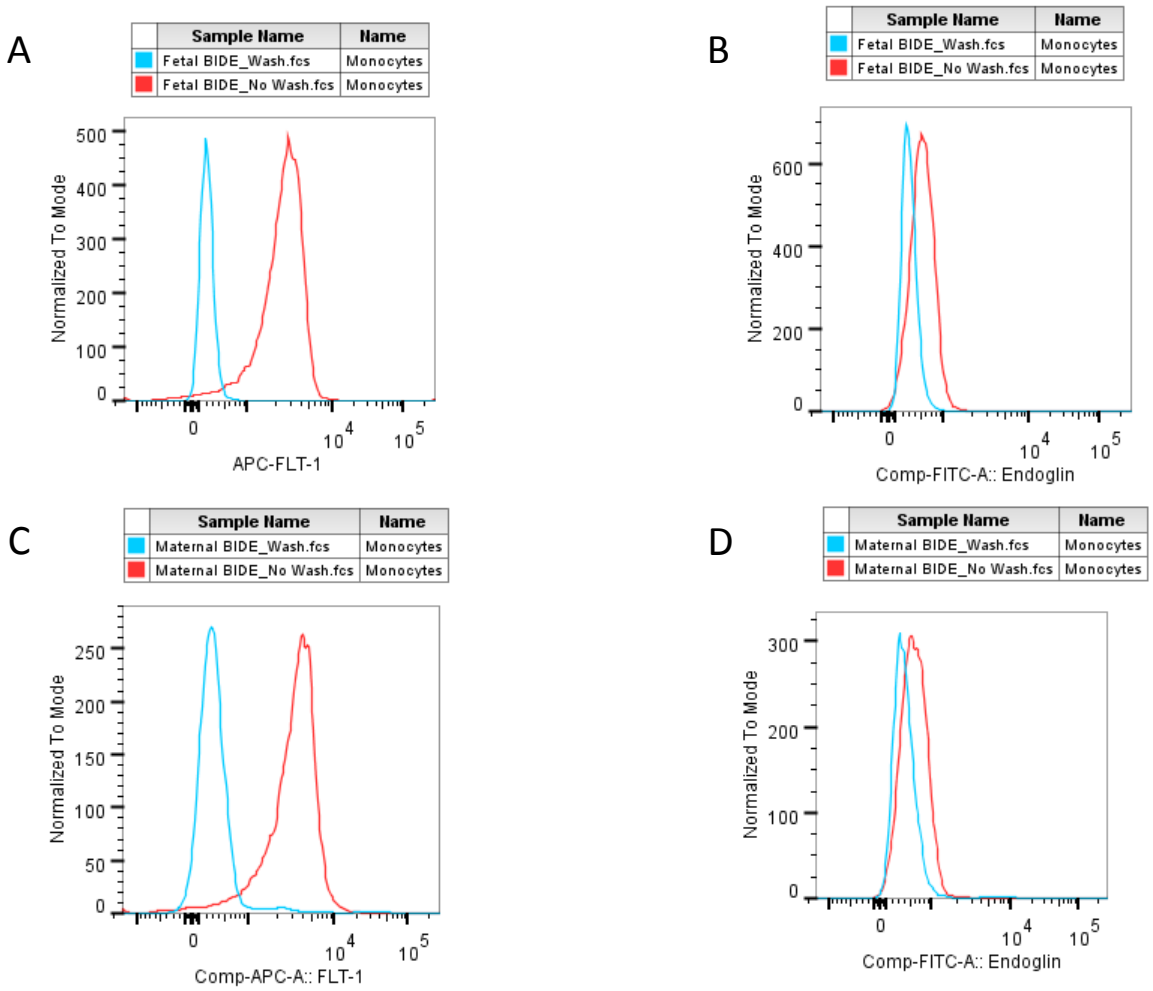

Figure 2.10 Effect of wash step on the fluorescence intensity of monocyte surface receptors Flt-1 and Endoglin on maternal peripheral and fetal cord blood.

A: Fetal monocytes stained with Flt-1. B: Fetal monocytes stained with Endoglin. C: Maternal monocytes stained with Flt-1. D: Maternal monocytes stained with Endoglin. Blue = isotype control. Red line = Antibody. A difference in the fluorescent intensity was observed with the wash step in Flt-1 staining, possibly due to washing of soluble Flt-1 attached to the

cell surface. The difference was less marked with Endoglin. A wash step was included in the protocol since the aim of the study was to test the surface marker expression.

2.6.8 Optilyse B vs Optilyse C for red cell lysis and fixation of cells

OptiLyse C Lysing Solution is an erythrolytic reagent intended for the lysis of red blood cells in the preparation of biological samples for flow cytometry analysis after staining of leukocytes with fluorescent antibodies. Immunostaining followed by whole blood lysis is the preferred method for providing flow cytometric results. The biological sample containing red blood cells for lysis is incubated in the presence of the OptiLyse C solution, which results in the lysis of red blood cells accompanied by the fixation of leucocytes.

Both Optilyse B (100ul) recommended for BD FACS machines and Optilyse C (300ul) recommended for Beckman Coulter flow cytometers were tested in the sample preparation and the optimization steps. Optilyse C was found to have a superior performance with lysing of the red blood cells resulting in a reduced amount of debris detected through the flow cytometer. Although Optilyse C is compatible with a “No wash” technique of lysing and fixation of the cells, the use of two wash steps in the sample preparation to remove excess antibodies produced superior results.

2.6.9 Flow Cytometry Protocol 1 -Multicolour Flow Cytometry for expression of monocyte markers

Antibody	Company and Catalogue No	Colour	Band pass filter	Purpose	Clone	Antibody volume
CD14	BD(340585)	PerCP	670LP	Monocyte marker	MϕP9	2.5ul
CD16	BD A(560195)	APC-H7	780/60	Monocyte subtype marker	3G8	2.5ul
CD86	BD (555658)	PE	585/42	M1 marker	2331(FUN-1)	2.5ul
CD163	BD (562643)	BV421	450/50	M2c marker	GHI/61	1ul
FLT-1 (VEGFR1)	R&D (FAB321A)	APC	660/20	Flt-1 Rc	49560	5ul
Endoglin CD105	BD (561443)	FITC	530/30	Endoglin Rc CD105	266	5ul
Mouse IgG1 isotype	R&D (IC002A)	APC	660/20	Isotype control for Flt-1 Rc	11711	5ul
Mouse IgG1,k	BD (555749)	PE	585/42	Isotype control for CD86	MOPC-21	2.5ul
Mouse IgG1,k	BD (62438)	BV421	450/50	Isotype control for CD163	X40	1ul

Table 2.5 Multicolour flow cytometry protocol antibody selection

2.6.9.1 Antibody selection

A multicolour flow cytometry protocol was developed using six colours and a fluorescence minus one technique (Table 2.5 and Figure 2.11). Cells were washed and incubated with the following monoclonal antibodies according to the manufacturer's instructions. Fluorescence compensation was achieved with compensation beads.

2.6.10 Flow Cytometry Protocol – Three colour protocol for expression of monocyte markers

2.6.10.1 Antibody selection

A three colour flow cytometry protocol was developed using identifying colours for CD14 and CD16 and same phycoerythryn colour for the antibodies of interest CD86, CD163, Flt-1 and Endoglin (CD105) (Table 2.6, Table 2.7 and Figure 2.12). Cells were washed and incubated with the following monoclonal antibodies according to the manufacturer's instructions. The antibodies and the volume used are described in Table 2.6. The voltages used on Flow cytometer are listed in Table 2.7.

Multicolour FACS protocol Monocytes in pregnancy:

1. Do WBC count on blood and dilute blood with PBS to a density of 5x10^6/mL (5/WBC of ml blood + add up to 1ml of PBS)
2. Add antibodies to tubes.

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
CD14 -PerCP	2.5µL	2.5µL	2.5µL	2.5µL	2.5µL
CD16 – APC-H7	2.5µL	2.5µL	2.5µL	2.5µL	2.5µL
CD163 – BV421	1 µL	1µL	1µL	1µL	1µL
CD86 - PE	2.5µL	2.5µL	2.5µL	2.5µL	2.5µL
Endoglin FITC	5µL	5µL	5µL	0	5µL
FLT-1 APC	5µL	5µL	5µL	5µL	0
Iso – BV 421	0	0.5µL	0	0	0
Iso - PE	0	0	1µL	0	0
Iso - FITC	0	0	0	5µL	0
Iso – APC	0	0	0	0	5µL

3. Add 100ul aliquot to tubes Vortex and incubate in the dark for **20mins** at RT in dark
4. Add 300µL of Optilyse C lysing solution (to lyse the RBC and Fix the white blood cells). Vortex gently each tube before moving on to next.
5. Incubate for 10mins at RT in the dark.
6. Add 500ml of PBS, leave for 10 minutes

Figure 2.11 Multicolour FACS protocol for Monocytes in pregnancy - Staining technique of whole blood for flow cytometry.

Antibody	Company and Catalogue No	Colour	Bandpass filter	Purpose	Clone	Optimised volume of Antibody
CD14	BD560349	V450 purple	450	Monocyte subtype	MϕP9	2.5ul for $1x10^6$ cells or 100ul
CD16	Ab 140477	APC	660/20	Monocyte subtype	3G8	2.5ul for $1x10^6$ cells or 100ul
Mouse IgG1,k	BD (555749)	PE	585/42	Isotype control for CD105, CD309 (KDR), CD86, CD163	MOPC-21	5ul
Mouse IgG1isotype	R&D (IC002P)	PE	585/42	Isotype control for Flt-1	11711	10ul
CD86	BD 555658	PE	585/42	M1	2331 (FUN-1)	5ul
CD163	BD 556018	PE	585/42	M2	GHI/61	5ul
Flt-1 (VEGFR1)	R&D (FAB321A)	PE	585/42	Flt-1 Receptor	49560	10ul
Endoglin CD105	BD (561443)	PE	585/42	Endoglin Receptor	266	5ul
KDR (VEGFR2) CD309	BD(560872)	PE	585/42	KDR Receptor	89106	5ul

Table 2.6 Three colour flow cytometry protocol: Antibody selection.

Three colour Monocytes in pregnancy FACS protocol Do WBC count on blood and dilute blood with PBS to a density of 5x10^6/mL (5/WBC of ml blood + add up to 1ml of PBS)

1. Add antibodies to tubes.
    a. Prepare CD14 and CD16 cocktail: 20 ul of CD14 (V450 BD560349 2.5ul per tube x 8) and 20ul of CD16 (APC ab140477 2.5ul per tube x 8). Dilute with 40ul PBS, allocate 10ul of the solution to each tube

	V450	APC	PE	Comment
1	CD14 2.5ul	CD16 2.5ul	FLT 5ul	Washx2
2	CD14 2.5ul	CD16 2.5ul	Endoglin 5ul	Washx2
3	CD14 2.5ul	CD16 2.5ul	CD86 5ul	Washx2
4	CD14 2.5ul	CD16 2.5ul	CD163 5ul	Washx2
5	CD14 2.5ul	CD16 2.5ul	BD PE Isotype 5ul	Washx2
6	CD14 2.5ul	CD16 2.5ul	R&D PE Isotype 5ul	Washx2

2. Add 100ul aliquot to 4 tubes Vortex and incubate in the dark for **30mins** at RT in dark
3. Add 300µL of Optilyse C lysing solution (to lyse the RBC and Fix the white blood cells). Vortex gently each tube before moving on to next.
4. Incubate for 10mins at RT in the dark.
5. Add 500ml of PBS, leave for 10 minutes
6. Wash step 1 Add 3ml of PBS. Centrifuge for 5 minutes at 500g, aspirate

Remove the supernatant with vacuum aspiration

7. Wash step 2 :Add 3ml of PBS

    Centrifuge for 5 minutes at 500g, aspirate

Remove the supernatant with vacuum aspiration

8. Resuspend in 500ul of PBS.
9. Analyse by flow cytometry. Count 15000 events in P1 (approximate monocyte gate)

Figure 2.12 Three colour FACS protocol for monocytes in pregnancy - Staining technique of whole blood for flow cytometry.

2.6.10.2 Voltage settings on BD Canto II FACS

Laser	Voltage
FSC	360
SSC	450
PE	454
APC	517
V450	412

Table 2.7 Voltage setting on BD Canto II FACS for three colour FACS protocol are listed.

2.6.11 Comparison between three colour and multicolour flow cytometry protocols

A comparison was made between the multicolour protocol using a different colour and wavelength for each variable antibody being tested as compared to the three colour protocol using the same colour (Phycoerythryn) for all the variables being tested.

The benefit of the multicolour protocol was that the effects of different variables on each other could be tested in the same sample. The negative was that CD16 antibody was not stable over 24 hours and that a larger volume of antibody was required to stain the samples.

The three colour protocol was easier to execute and analyze as well as using a significantly lower volume of antibodies. A decision was made to continue with the three colour protocol for all the study samples and stain some representative samples of each group with the multicolour protocol.

2.6. 12 Flow Jo analysis and data collection.

2.6.12.1 Gating strategy

Flow Jo software version 10.6 (Tree star, Inc., Ashland, OR, USA) was used to gate the cells and analyse the expression of cell surface markers. In the gating strategy used for analysis, single cells were first selected using forward (FSC) and side scatter (SSC) plots (Figure 2.13). Analysis of the different physical properties of the forward scatter was used to distinguish between granulocytes, monocytes and lymphocytes and differentiate from cellular contaminants and debris. Subsequently the monocytes were selected from the live cells in the forward/side scatter. CD14 positive cells were selected to exclude contamination with natural killer cells. The selected CD14+ monocytes were then plotted against CD16 (Figure 2.14). Three subsets were identified: Classical (CD14++CD16-) intermediate (CD14++CD16+) and non-classical (CD14+CD16++) (Figure 2.15). The percentages of the three populations within the monocyte subtype population were calculated. By definition, the intermediate and classical monocyte subsets possess the same levels of CD14. Hence, the end point of CD14 expression by the classical monocytes was used as a set point to segregate between the intermediate and non-classical subsets, as depicted in Figure 2.13.

Surface expression of markers CD86 and CD163 and their increase in mean fluorescent intensity (MFI) over that of the isotype control was recorded for total monocytes as well as the different monocyte subtype populations.

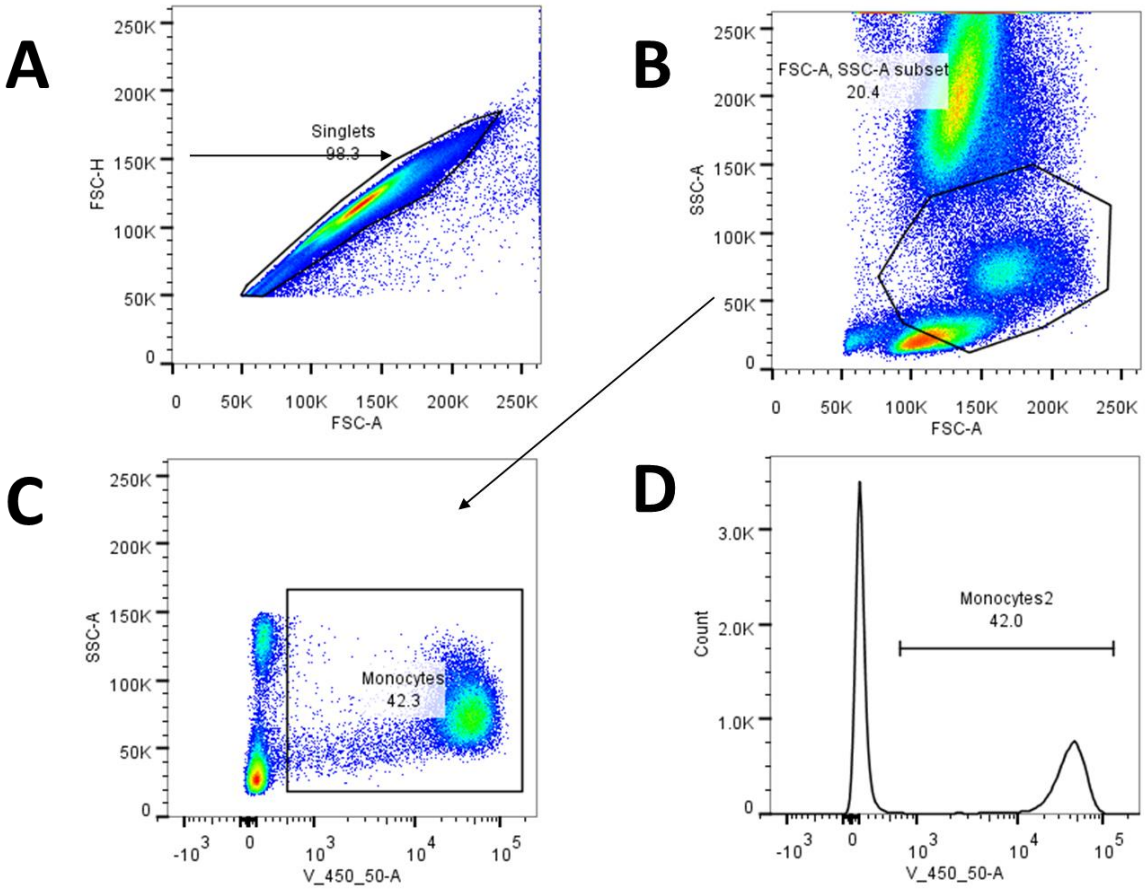

Figure 2.13 Gating strategies for selection of CD14 monocytes.

A: Analysis of the different physical properties of the forward scatter was used to distinguish between granulocytes, monocytes and lymphocytes and differentiate from cellular contaminants and debris. B: The monocytes were selected from the live cells in the forward/side scatter plot. C and D: CD14 positive cells were selected to exclude contamination with natural killer cells.

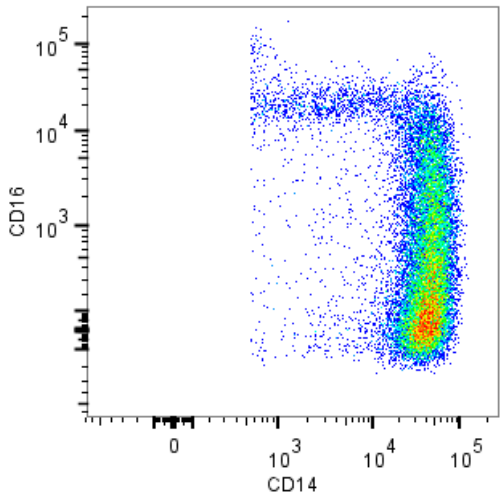

Figure 2.14 Gating strategies of the three monocyte subsets based on relative CD14 and CD16 expression.

Flow cytometry dot plot showing the distribution of classical, intermediate and non classical monocyte subsets.

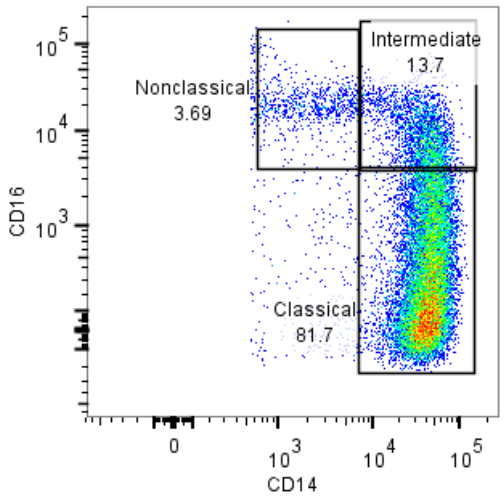

Figure 2.15 Flow cytometric gating strategy for monocyte subsets in human peripheral whole blood.

Classical monocytes express high levels of CD14 but no CD16; intermediate monocytes express high levels of CD14 and low CD16, while non-classical monocytes express low CD14 but high CD16.

2.6.12.2 Mean Fluorescence Intensity (MFI)

A single parameter histogram was used for evaluating the total number of cells in each sample that stained for the marker of interest (Figure 2.14and 2.15). Mean fluorescence intensity and percentage of total cells that express the marker were calculated for each marker of interest CD86, CD163, Flt-1, Endoglin and KDR using Flow Jo software and recorded separately for total monocytes, classical monocytes, intermediate monocytes and non classical monocytes. The results of the isotype control MFI was deducted from the sample MFI. The following Figures 2.16 - 2.19 demonstrate establishment of positive staining for CD86, CD163, Flt-1 and Endoglin.

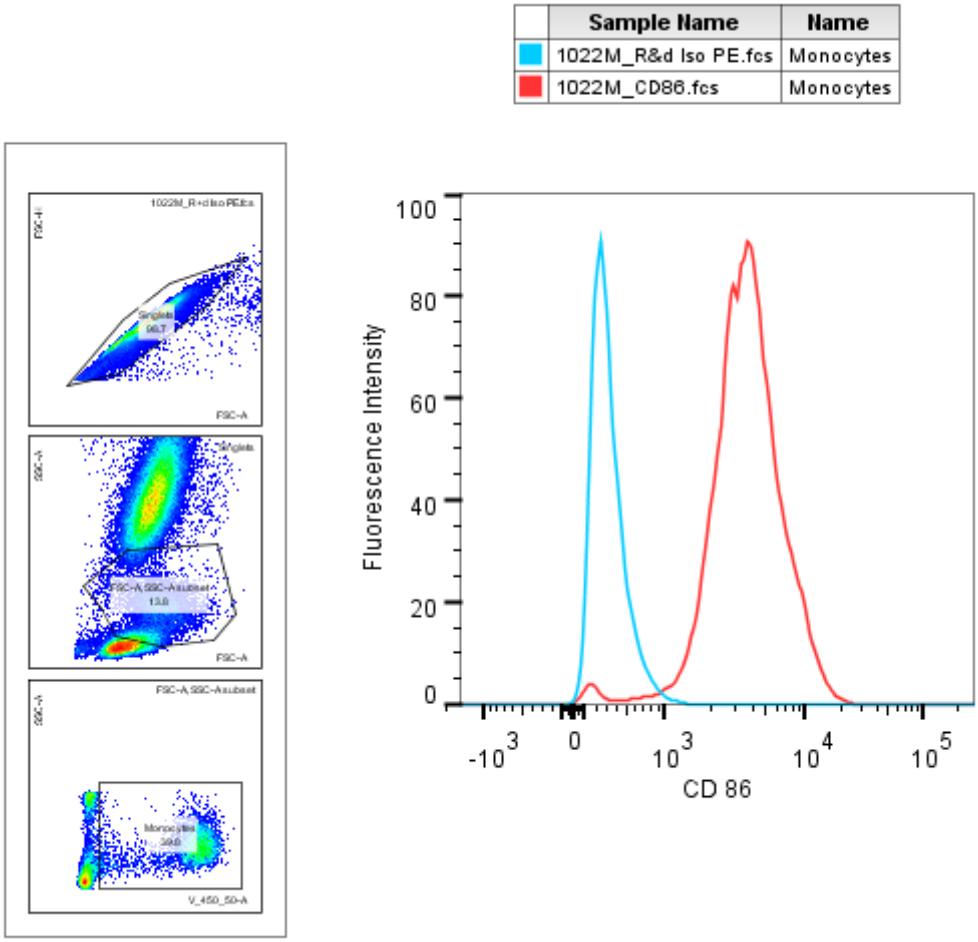

Figure 2.16 Comparison of Mean Fluorescence Intensity between CD86 (red line) and its isotype control (blue line) in normal pregnancy.

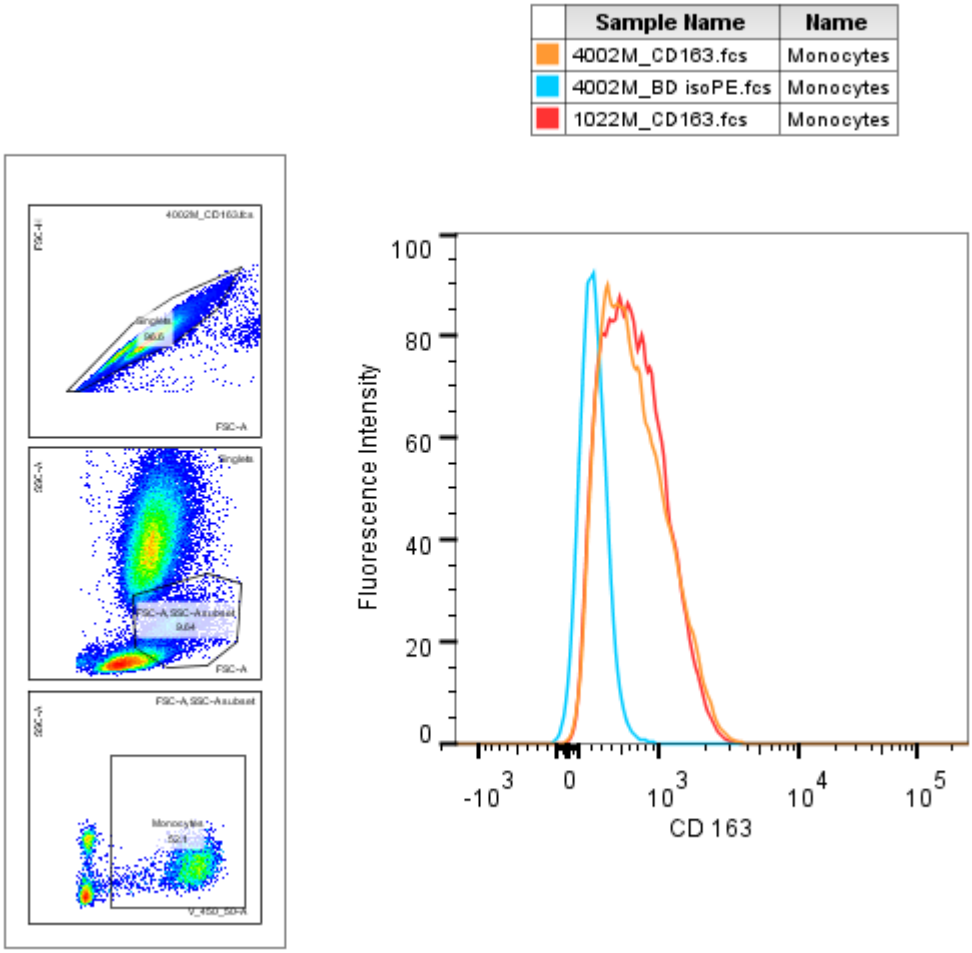

Figure 2.17 Comparison of Mean Fluorescence Intensity between CD163 in normal pregnancy (red line), CD163 in PE+IUGR (orange line) and their isotype control (blue line).

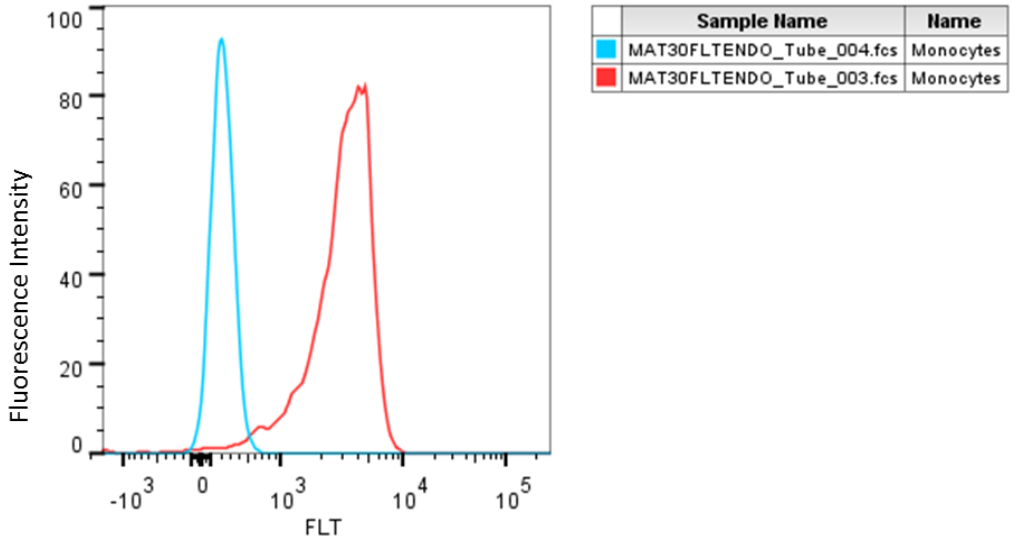

Figure 2.18 Comparison of Mean Fluorescence Intensity between Flt-1 (red line) and its isotype control (blue line) in normal pregnancy at 30 weeks of gestation.

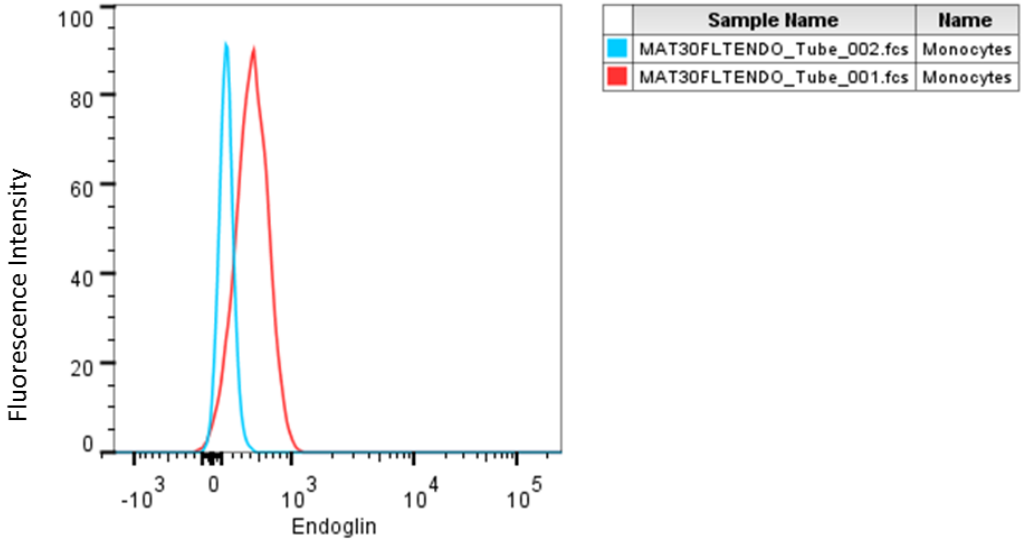

Figure 2.19 Comparison of Mean Fluorescence Intensity between Endoglin (red line) and its isotype control (blue line) in normal pregnancy at 30 weeks of gestation.

2.7 Serum and plasma assays for measurement of cholesterol and lipid profiles

A prospective cross-sectional case control study was conducted. The maternal peripheral venous blood was collected antenatally prior to delivery from 52 pregnant women between 24-40 weeks of gestation, delivering at Westmead Hospital during the period 2013 -2014. Fetal cord blood from the umbilical vein was collected at the time of delivery from 30 of these pregnancies. Participants were recruited from four clinical groups of normal pregnancy, preeclampsia, intrauterine fetal growth restriction and a combination of PE+IUGR as described in section 2.3.1. Peripheral blood was collected from all consented patients by standard venipuncture techniques. Testing was performed at the Institute of Clinical Pathology and Medical research (ICPMR), Westmead Hospital.

The lipid profile including triglycerides (TG), total cholesterol (TC), High density lipoprotein (HDL), Low density lipoprotein (LDL), Apo lipoprotein A1 (ApoA1) and Apo lipoprotein B (ApoB) of each donor was tested and recorded. The ratios TC/HDL, ApoB/ApoA1 were calculated.

Serum was analyzed for cholesterol and triglyceride using enzymatic analysis (Siemens Dimensions Vista system). Apo lipoproteins A1 and B were assayed by immunoturbidimetry (Abbott Diagnostic Architect C4000 Ci 4100). A summary of the techniques and commercial kits used for the assays are listed in Table 2.08. The estimation of LDL cholesterol was done in the clinical laboratories using Friedewald equation to make the lipid profile cost effective (385). Specimens with triglyceride > 4-5 mmol/l were also used in the analysis as maternal triglycerides in pregnancy are generally over the 4.6 mmol/L range. The fasting time for the sample collection ranges from 6 hours to 12 hours. While the TG assays are generally

performed after a 10 hour fasting state, there is evidence non-fasting and postprandial triglycerides >2.5mmol/L can be considered parallel to the fasting 2.0mmol/L in assessing the risk of cardiovascular disease (386).

Variable	Method/Assay	
Total Cholesterol (TC)	Siemens Dimensions Vista system CHOL	Automated enzymatic assays K1027
High density cholesterol (HDL)	Siemens Dimensions Vista system	Automated enzymatic assays K3048A
Low density cholesterol (LDL)	Calculated from TC, HDL and TG	TC-HDL- TG/2.2
Triglycerides(TG)	Siemens Dimensions Vista system	Automated enzymatic assays
Apolipoprotein A1 (ApoA1)	Abbott Diagnostic Architect C4000	Automated immuno-turbidimetric assay
Apo lipoprotein B (ApoB)	Abbott Diagnostic Architect C4000	Automated immuno-turbidimetric assay

Table 2.8 List of markers in the lipid profile and the commercial assays used for testing the samples.

The venous blood collected was allocated last for the lipid profile testing. This resulted in a smaller number of samples available for analysis, especially in the fetal cord blood as the volume available was limited. Several cord blood samples were not able to be processed due to clotting. The serum lipid levels have been documented to be significantly different in the pregnant population as compared to the non-pregnant women. Table 2.9 lists the reference ranges for pregnant population.

Variable	Reference range	5th, 50th and 95th centile values for the pregnant population
TC mmol/L	3.0-5.5	1.35, 7.38, 9.83
HDL mmol/L	≥1.0	1.04, 1.63, 2.46
LDL mmol/L	≤3.5	2.56, 4.31, 6.48
TG mmol/L	≤2.0	1.4, 2.63, 4.68
ApoA1g/L	1.10-1.89	1.42, 2.0, 2.61
ApoBg/L	0.59-1.32	0.89, 1.32, 1.92

Table 2.9 The reference ranges for serum and plasma lipids for non pregnant population as listed in the commercial assays and the 5th, 50th and 95th centile values in third trimester of pregnancy as published by Piechota et al (350).

2.8 Statistical analysis

2.8.1 Sample size

The sample numbers for the experiments 1 and 2 were selected after reviewing similar published studies. For maternal and fetal soluble markers, most published studies in this field have used 50-150 total patients to identify a significant difference in the blood markers. The experiments 3-4 on monocytes in pregnancy complications is a new area of study where sample size cannot be accurately defined without preliminary data. As such the numbers were based on calculations by collaborators of this study who are investigating similar monocyte markers in other areas of study. The current studies were performed as preliminary experiments with a significance level of 0.05, without using Bonferroni corrections, to identify associations to be studied at a future date. Due to technical issues such as immediate clotting of fetal blood and inadequate sample volumes, all collected samples could not be analysed for all aspects of the study.

2.8.2 Statistical software

The statistical analyses for all results included in this thesis were performed by a senior hospital medical statistician. The statistical software packages SPSS for windows Version 21 and SPLUS version 8 were used to analysing the data. Two-tailed tests with a 5% significance level were used throughout.

2.8.3 Comparison of tested variables between clinical groups

The studies presented in this research are exploratory in nature with the aim of identifying further areas of research and hypothesis generating rather than to prove a definite association regarding a clinical condition.

Continuous demographic and outcome variables were tested for departure from normality using Shapiro-Wilks tests. Where no significant departure was detected such as demographic data, parametric analyses were conducted and data summarized using mean ± standard deviations. Where significant departure from normality was detected, non-parametric analyses were conducted and data summarized using median, upper and lower quartiles.

One way analysis of variance or Kruskal-Wallis non parametric equivalent was used to test for differences between the clinical groups. Where statistically significant different heterogeneity was detected, multiple pairwise comparisons were used to examine differences between particular clinical groups. Bonferroni correction was applied to these multiple pairwise comparisons for placental and circulating angiogenic factor level analyses in Stage 1 of the research (Experiments 1 and 2, chapters 3 and 4).

Due to the exploratory nature of the experiments 3 and 4, further corrections were not performed in the analysis of the results in chapters 5, 6, and 7, as the levels of these corrections may potentially hide any significant trends that would benefit from further study.

2.8.4 Comparison of maternal and fetal samples

Paired t tests were used to compare the plasma levels in maternal and fetal samples.

2.8.5 Presentation of data

Box plots were used to illustrate the distribution of continuous variables by patient group. Data are summarized as median and interquartile range unless specified otherwise. Scatterplots were used to illustrate the association between continuous variables. Receiver operating characteristic (ROC) curves were used to evaluate the predictive value and

graphically present the sensitivity and specificity of a biological marker in predicting the presence of the pregnancy complications preeclampsia and IUGR.

Data were adjusted for maternal age and parity using a general linear model where clinical group was fitted as a four level factor and maternal age and parity as continuous covariants of the clinical groups.

2.8.6 Correlation between two variables tested

We used Spearman rank correlation to quantify the extent of the association between variables. The rank correlations and associated p values have been given when significant correlation were noted.

2.8.7 Evaluation of tested variables as potential biomarkers

In experiment 2, receiver operating characteristic graphs were generated to evaluate an individual marker or ratio of markers in the maternal circulation as a predictor or identifying marker that distinguishes normal pregnancy from pathological pregnancies with preeclampsia or intrauterine fetal growth restriction. The areas under the ROC curves were used to quantify the global performance of each individual marker or their ratios in terms of predicting normal versus complicated pregnancies affected by preeclampsia and/or intrauterine fetal growth restriction.

2.8.8 Immunohistochemistry

2.8.8.1 Statistical analysis of semi-quantitative methods

Four samples were analyzed from each placenta. The mean immunostaining data categorized into three levels of 1, 2 and 3 for each variable was used in repeated measures analysis of variance to investigate the effects of the intensity of VEGF staining characteristics and the

clinical group. For the three pathological groups, a linear mixed effects model was used to investigate the effect of villous infarction on the staining characteristics.

2.8.8.2 Analysis of digital image analysis

The median value of the four samples was used as representative of each placenta. Median and inter-quartiles were used to describe the representative results of four samples from each placenta as the digital analysis of staining allocate a range of intensities into one staining category such as weak, moderate and strong. Kruskal Wallis non-parametric analysis of variance was used to test for homogeneity across the four clinical groups for each of the variables VEGF, PlGF, Flt-1 and KDR. Mann-Whitney tests were used for pairwise comparisons between normal pregnancy and each of the clinical groups as well as between each of the pathological groups.

2.8.8.3 Correlation between observers in semi-quantitative analysis and digital image analysis of staining intensity.

The Bland-Altman plots were used to investigate the inter-observer variation in semi-quantitative analysis of staining intensity. The Spearman rank correlation was used to quantify the extent of the association between the manual semi-quantitative reading and digital image analysis of the staining intensity for each of the clinical groups. The kappa correlation coefficient was also calculated as a measure of the association.

2.8.9 ELISA

Maternal and fetal levels of angiogenic factors PlGF, sFlt-1, KDR and sEng were log transformed prior to analysis in order to stabilise the variance. The log transformed variables were approximately normally distributed within each clinical group. ANOVA was used to test for heterogeneity between the clinical groups. If this was observed, then pairwise multiple

comparisons with Bonferroni corrections were used to test for the differences between specific clinical groups.

Spearman's rank correlation was used to quantify the level of association between gestational age and each of the variables of interest. Receiver operating characteristic (ROC) graphs and area under the curve (AUC) were used to quantify the performance of each individual marker and their ratios in predicting normal versus pathologic pregnancies affected by PE and/or IUGR.

2.8.10 Flow cytometry

Kruskal Wallis non-parametric analysis of variance was used to test for homogeneity across the four clinical groups for each of the variables percentage of monocytes, percentage of monocyte subtypes, mean fluorescence intensity of CD86, CD163, Flt-1 and Endoglin. Where heterogeneity was identified, Mann-Whitney tests were used for pairwise comparisons between normal pregnancy and each of the clinical groups as well as between each of the pathological groups. The Spearman rank correlation was used to quantify the extent of the association between monocyte surface Flt-1, Endoglin expression and gestational age in normal pregnancies. Data were also analyzed for any association between Flt-1, Endoglin expression and monocyte subtype as well as monocyte polarization and pro-inflammatory status as defined by CD86/CD163 ratio.

The sample sizes were similar to those previously published M1/M2 marker expression on placental macrophages in preeclampsia (387).

2.8.11 Serum and plasma lipid profiles

Kruskal Wallis non-parametric analysis of variance was used to test for homogeneity across the four clinical groups for each of the variables, TC, HDL, LDL, TC/HDL, Apo lipoprotein A1, Apo lipoprotein B and ApoB/ApoA1 ratio. Where heterogeneity was demonstrated, Mann-Whitney tests were used for pairwise comparisons between normal pregnancy and each of the clinical groups as well as between each of the pathological groups. The Spearman rank correlation was used to quantify the extent of the pairwise association between each of the variables TC, HDL, LDL, TC/HDL, Apo lipoprotein A1, Apo lipoprotein B and ApoB/ApoA1 ratio and gestational age.

RESULTS

Chapter 3 – Localisation and placental expression of angiogenic factors and their receptors in normal and complicated pregnancies using immunohistochemistry

3.1 Summary

Introduction: Angiogenic factors VEGF and PlGF as well as their receptors Flt-1 and KDR play a major role in the angiogenesis of the placenta. Aberrations in the expression of these factors may lead to pregnancy complications.

Aims: Localization, quantification and comparison of VEGF, PlGF, Flt-1 and KDR in the placentas of normal pregnancy and pregnancy complications of PE, IUGR and PE+IUGR.

Methods:

A prospective cross-sectional case control study was conducted. A total of 30 pregnant women between 24-40 weeks of gestation, were recruited and classified into four clinical groups. Representative placental samples from each group were immunohistochemically stained for VEGF, PlGF, Flt-1 and KDR. The VEGF staining characteristics of the placental biopsies were analyzed according to the presence or absence of villous infarctions in the sample. Analysis was performed using semi-quantitative methods and digital image analysis in identifying staining characteristic in the placenta.

Results:

A decreased number of placental villi were noted in both PE and IUGR compared to the normal term placenta. The cumulative effects of PE and IUGR appear to lead to a more significant loss of villous architecture than either condition alone. VEGF, Flt-1, and PlGF

were mainly expressed in the syncytiotrophoblast layer of the placenta, while KDR expression was found in the endothelial layer.

Overall VEGF and Flt-1 were strongly expressed in normal, preeclamptic and IUGR placentas. The intensity of VEGF staining was reduced in the areas of significant pathology such as villous infarction compared to the non-infarcted areas. One of the most important findings in the present study is significantly reduced expression of PlGF and KDR in the placentas from pregnancies complicated by IUGR compared to normal and preeclamptic pregnancies.

Conclusion: The results of this study suggest compensatory villous regeneration in non-infarcted areas of the placenta and that changes in VEGF and Flt-1 expression may be a consequence rather than the cause of placental vascular disease and preeclampsia. The lack of placental PlGF and KDR may lead to the development of intrauterine fetal growth restriction and may explain the loss of vasculature and villous architecture in IUGR.

3.2 Introduction

Angiogenic factors VEGF and PlGF as well as their receptors Flt-1 and KDR play a major role in the angiogenesis of the placenta. Aberrations in the expression of these factors may lead to pregnancy complications. While variable results have been demonstrated in multiple studies on the expression of angiogenic factors in the placenta, preeclampsia is suggested to be associated with an increase in VEGF and Flt-1 while the results are not conclusive for PlGF (215, 219). Placental expression of angiogenic factors in intrauterine growth restriction is not well categorized.

3.3 Aims:

1. Localization of VEGF family angiogenic factors and their receptors in human placental tissue.

2. Identify any difference in expression of pro-angiogenic factors VEGF and PlGF as well as their receptors Flt-1 and KDR between normal pregnancy and pregnancies complicated by preeclampsia and/or IUGR.

3. Correlate the clinical and histopathological findings with pro and anti-angiogenic factor expression in an attempt to understand the pathogenesis of placental disease in preeclampsia and IUGR.

4. Measure angiogenic and anti-angiogenic factor expression using digital pathology tools and correlate with semi-quantitative methods

3.4 Methods

A prospective cross-sectional case control study was conducted. A total of 30 pregnant women between 24-40 weeks of gestation, were recruited from four clinical groups as described in Chapter 2.3.

3.4.1 Placental H&E and immunohistochemical staining

Representative placental samples from each group were H&E and immunohistochemically stained for VEGF, PlGF, Flt-1 and KDR using methods described in Chapter 2.4.4. The H&E stained slides were analysed for morphological characteristics. Placental morphology including characteristic and semiquantitative staining analysis were performed by a qualified pathologist and two research scientists as described in Chapter 2.4.2-2.4.3. The VEGF staining characteristics of the placental biopsies were analyzed according to the presence or absence of villous infarctions in the sample.

3.4.2 Staining characteristics

For manual semi-quantitative staining, a scoring system from 0-3 was used, with 3 indicating strong staining. The same slides were scanned by Aperio ScanScope scanner. Image analysis was performed with Aperio Positive Pixel Count Algorithm to quantify the proportion of antigen-positive pixels in the area analyzed. The evaluation of staining was performed using semi-quantitative methods (Chapter 2.4.4) and digital image analysis (Chapter 4.4.5) to identify staining characteristic in the placenta.

The percentage of immune-reactive cells and the intensity as evaluated by automatic Aperio digital image analysis software were recorded for each of the antibodies staining for VEGF, PlGF, Flt-1 and KDR. Stronger staining of an antibody (darker brown pixels) was associated with a lower intensity score in the positive pixel algorithm. Please refer to Chapter 2.4.6

section for detailed explanation of the positive pixel algorithm and chapter 2.8.8 for the analysis of results.

3.4.3 Statistics

Demographic data and semi-quatitative analysis data are presented as mean ± standard deviations. Automatic digital analysis data are summarized as median and interquartile range unless specified otherwise (Please refer to Chapter 2.8.8).

3.5 Results

3.5.1 Maternal and neonatal demographic data and clinical characteristics of the study population

The demographic data and clinical characteristics of the four clinical groups are given in Table 3.1. Results are presented for 120 placental samples from 30 pregnancies. No statistically significant difference was seen in maternal age between the clinical groups.

The pregnancies from the study groups delivered earlier and had lower birth weights as compared to term normal controls. The mean gestational age at delivery and birth weight in the control group were significantly different to the pathological groups but were comparable between the pathological groups. The majority of the pregnancies were delivered by caesarean section (n = 28) with the exception of two patients with isolated IUGR.

Clinical groups	Normal Pregnancy (n = 5)	PE (n = 9)	IUGR (n = 10)	PE + IUGR (n = 6)
Maternal age (years)	27.8	27.2	30.0	31.8
Gestation at delivery (weeks)	38.7(0.4)	31.7(1.2)*	32.4(1.0)*	33.1(1.6)*
Birth weight (g)	3478(329)	1784(230)*	1860(183)*	1588(271)*
Placental weight (g)	470(117)	350(62)	247(42)*	249(34)*
Primigravidae (n)	1	7	2	7
Current Smokers(n)	0	2	1	1
Caesarean sections (n)	5	9	8	6
Maternal Corticosteroid treatment prior to delivery	N/A	8	4	6

Table 3.1: Maternal and fetal demographic data and clinical characteristics of the study population. Results are presented as mean ± SD for each continuous variable unless otherwise specified. * Significantly different to normal pregnancies $p < 0.05$.

3.5.2 Morphological characteristics of the placenta

Comparative morphological study of the placentas was carried out by an independent pathologist. The results are displayed in Table 3.2.

Approximately 50% of the placental biopsies in the three pathological groups had areas of villous infarction as well as widespread syncytial knots, loss of villi, and fibrin deposition. Villous infarction was not detected in the placental samples from the control group. Fifty

percent of term placentas had occasional syncytial knots (Table 3.2). A striking feature in all the three pathological groups was the prominent presence of extra-villous trophoblast in the stem villi with infiltration into the areas of villous infarctions. The morphological changes associated with preeclampsia and IUGR are demonstrated in Figures 3.1 and 3.2.

Placental morphology	Normal Pregnancy (n = 5)	PE (n = 9)	IUGR (n = 10)	PE+IUGR (n = 6)
No of placental biopsies	20	36	40	24
Mean number of villi per high power field (Magnification x 400)	12.7 (SD 0.53)	11.3 * (SD 0.39)	10.4* (SD 0.37)	9.3* (SD 0.49)
Placental biopsies with loss of villi	0/20 (0%)	16/36(44%)*	19/40 (47.5)*	11/24 (45%)*
Loss of villi	0/20	18/36*	34/40*	12/24*
Hypovascular villi	0/20	1/36	7/40*	3/24*
Syncytial knots	10/20	34/36*	40/40*	22/24*
Intervillositis	0/20	0/20	4/40	0/24
Perivillous fibrin	4/20	20/36	32/40*	18/24
Large infarcts	0/20	2/36	3/40	6/24*

Table 3.2: Placental morphological characteristics by clinical group.

Placental biopsies stained with Haematoxylin and Eosin were examined for known pathological features of preeclampsia and intrauterine fetal growth restriction. The number of villi per high power field mean ± standard deviation and sections with infarcted villi were also documented. * Significantly different to normal pregnancies, $p < 0.05$.

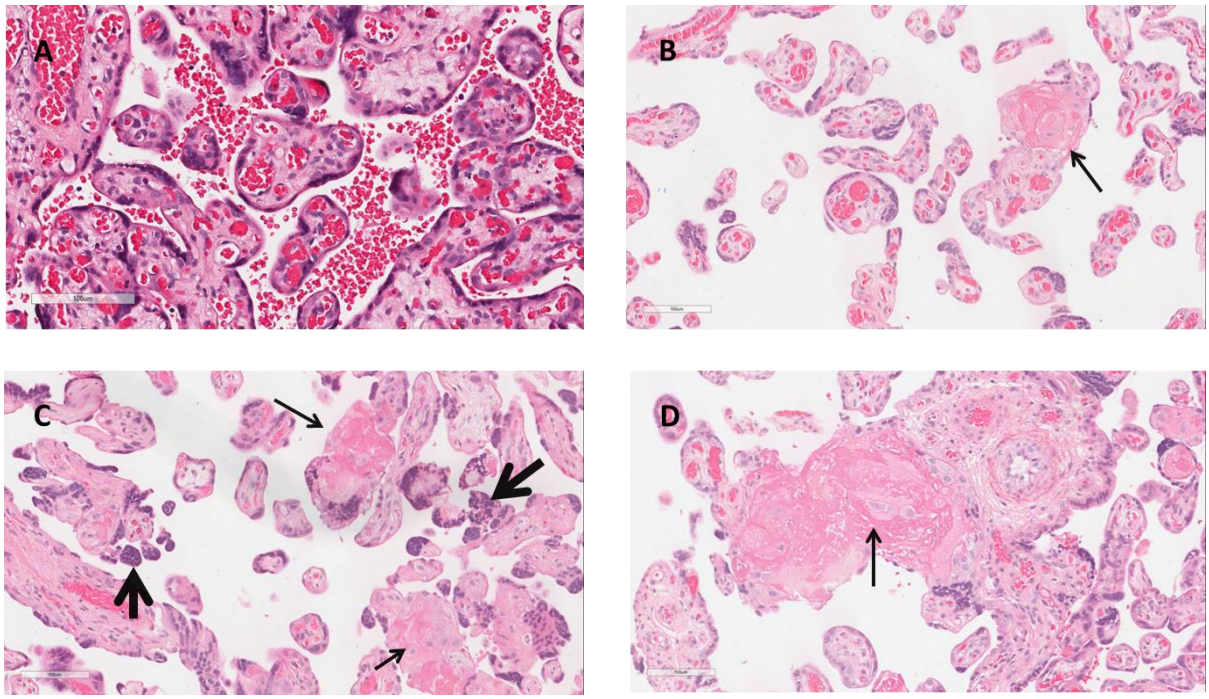

Figure 3.1 Morphological changes associated with preeclampsia and IUGR.

A: Normal placenta, B: Intrauterine fetal growth restriction, C: Preeclampsia, D: Preeclampsia and intrauterine fetal growth restriction. Thin arrow: Hypovascular villi and fetal thrombotic vasculopathy. Thick arrow: Syncytial knotting. Sections B, C and D demonstrate loss of villus density. Magnification x 200.

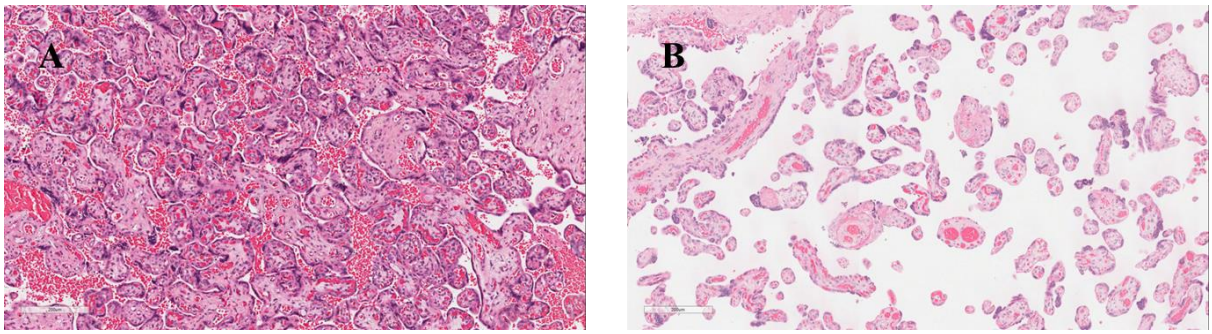

Figure 3.2 Loss of villi associated with preeclampsia and intrauterine fetal growth restriction (B) compared to normal term placenta (A). Magnification x 50.

3.5.3 Density of terminal villi

The normal pregnancy group, as expected showed normal term placental morphology, vasculature and villous architecture (Table 3.2). Placentas from pregnancies with IUGR as defined in the methods section 2.3.3 with high resistance umbilical artery Doppler waveforms had a significant reduction in the mean number of villi per high power field ($p < 0.001$) in comparison to the normal group (Figures 3.2 and 3.3).

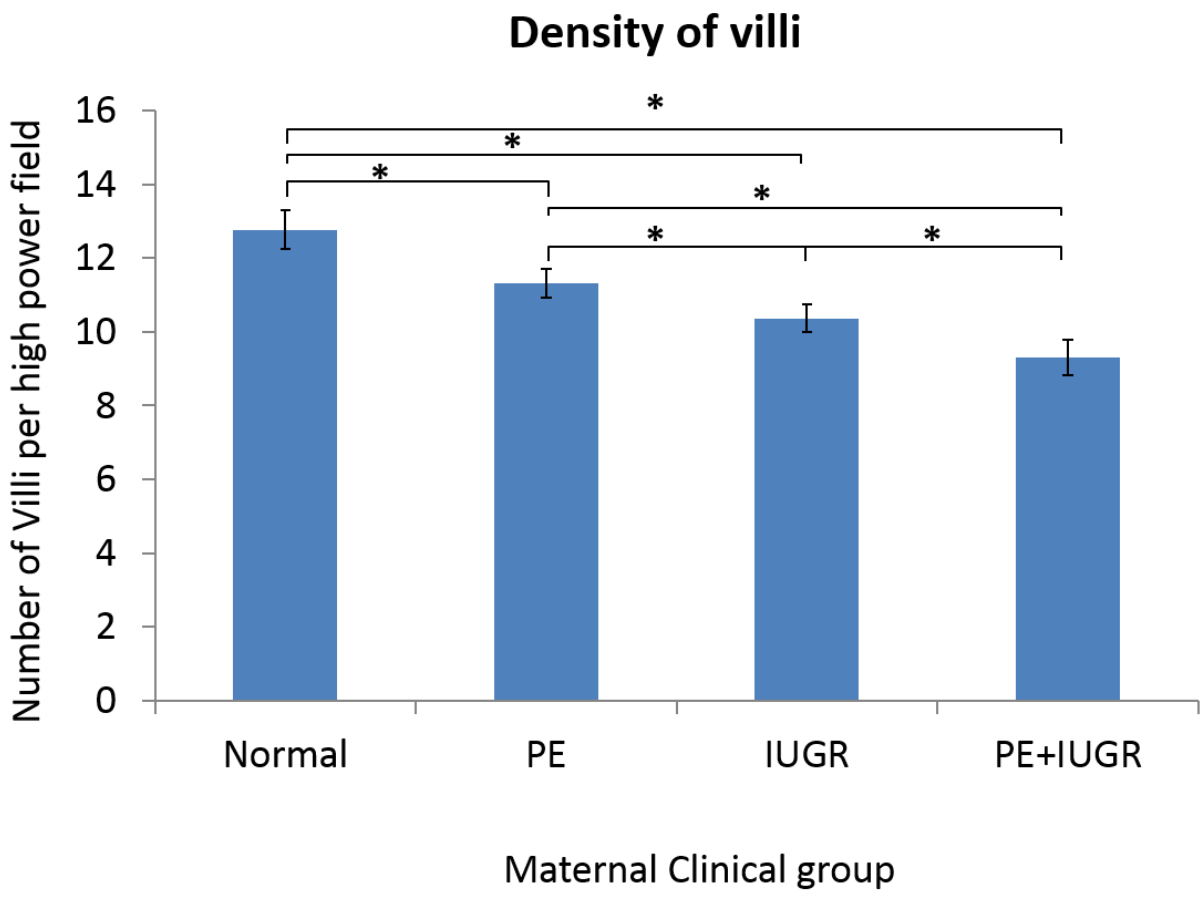

Figure 3.3 Number of villi per high power field (x400) in placental biopsies represented as mean ± SE. Preeclampsia and IUGR had significantly fewer number of villi than normal placenta. The combination of PE and IUGR appear to have a cumulative effect in reducing the number of villi. * Significantly different to normal pregnancies $p < 0.05$.

3.5.4 Angiogenic factors and their receptors: Analysis of Immunostaining intensity

A total of 120 formalin-fixed placental tissue specimens from four clinical groups were analysed manually for intensity of staining characteristics. The immunolocalisation of the VEGF staining in different tissue types was similar in all clinical groups. Immunostaining for VEGF was detected in syncytiotrophoblast, cytotrophoblast, extra villous trophoblast, endothelium, and Hofbauer cells (Figure 3.4) while the Flt-1 immunoreactivity was mainly syncytiotrophoblast, cytotrophoblast and Hofbauer cells. The average VEGF staining intensity score was correlated with the clinical groups and did not demonstrate any differences between the groups. Immunohistochemical staining intensity for VEGF (Figure 3.5) and Flt-1 in syncytiotrophoblast, endothelium, stroma and Hofbauer cells were scored separately (Figures 3.6 and 3.7). No staining was evident in negative control sections. Increased staining for KDR was demonstrated in the endothelial cells. Strong PlGF immunoreactivity was localized to the syncytiotrophoblast and endothelium with minor staining of the villous stroma. A feature of the pathological placentas from PE and IUGR, was the prominent presence of extra villous trophoblast in the stem villi and areas of villous infarction (Figure 3.4). These cells demonstrated intense immunoreactivity for VEGF as well as its Flt-1 receptor. Comparative images for angiogenic factors VEGF and PlGF as well as their receptors Flt-1 and KDR are presented in Figure 3.8 for the four clinical groups.

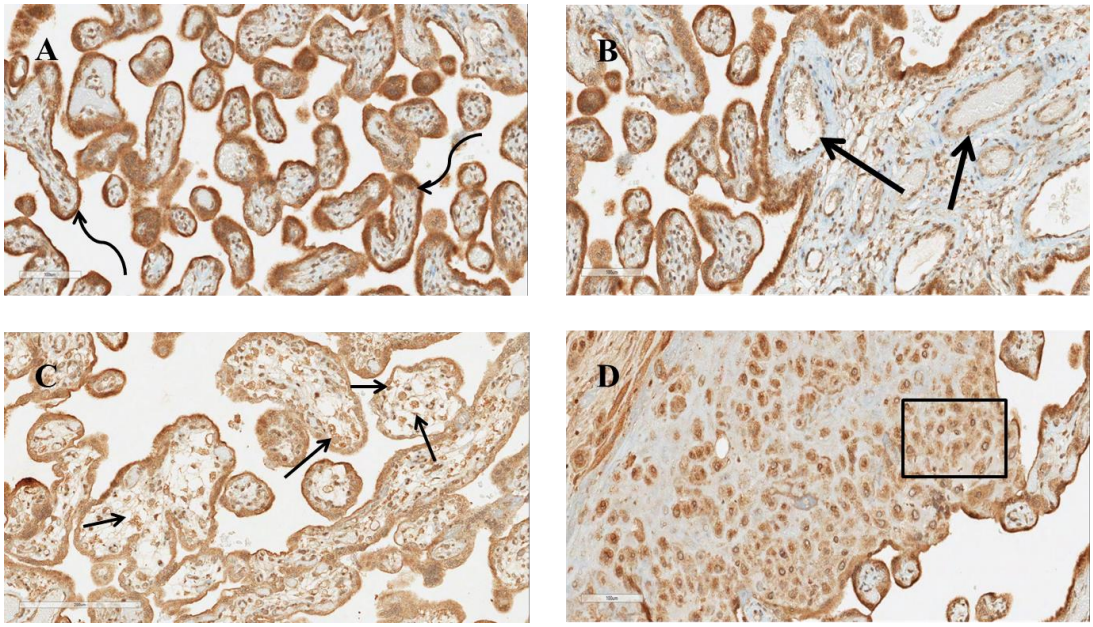

Figure 3.4 VEGF immunostaining in preeclamptic placentas.

A: syncytiotrophoblast (curved arrow), B Vascular endothelium (thick arrow), C: Hofbauer cells (smaller arrow), D: Extra villous trophoblast (rectangle) demonstrated intense VEGF immunoreactivity in the pathological clinical groups. Magnification x 200.

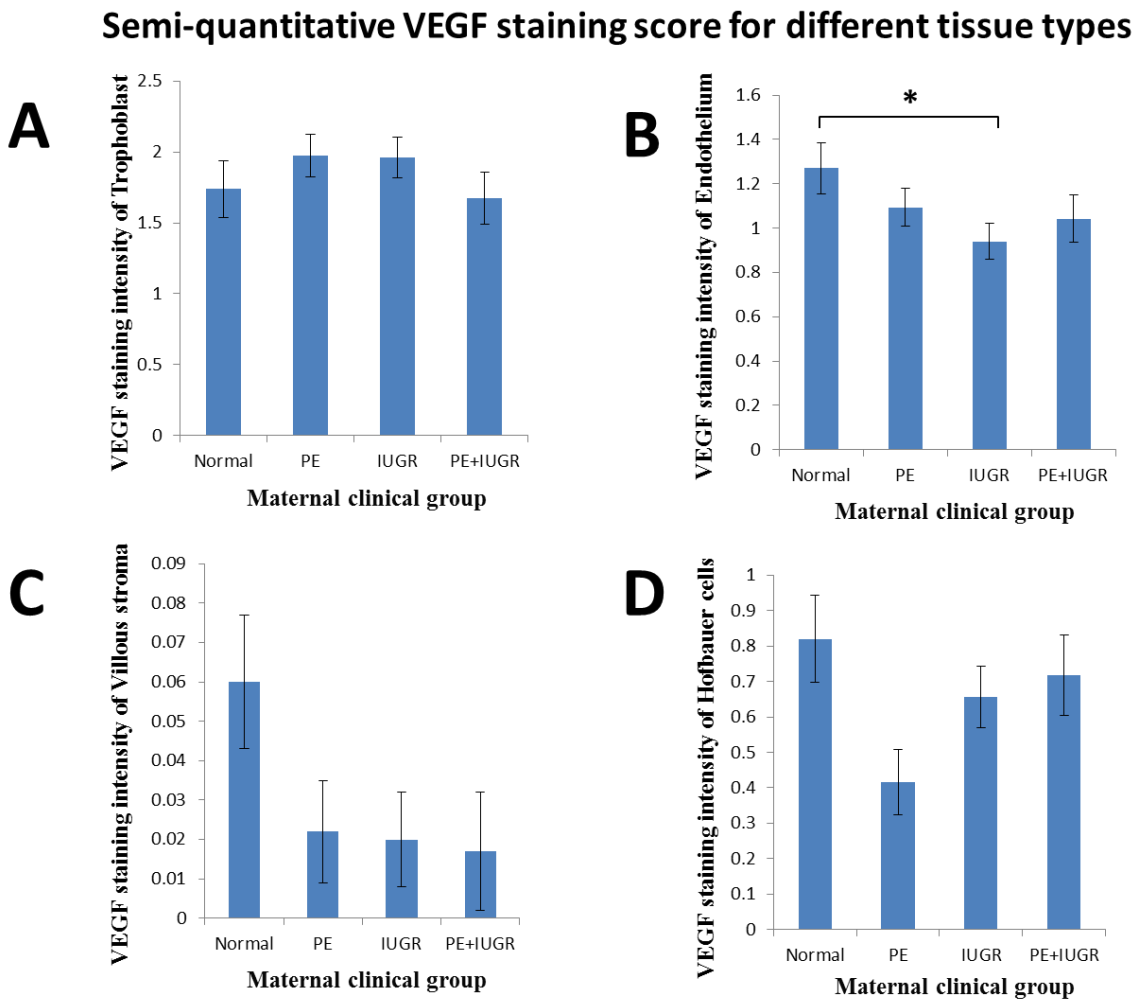

Figure 3.5 VEGF Staining Intensity of Trophoblast (A), vascular endothelium (B), villous stroma (C) and Hofbauer cells (D). Results presented as mean ± SD.

No significant difference seen between clinical groups for staining of trophoblast, villous stroma and Hofbauer cells. For VEGF staining of vascular endothelium, a statistically significant difference was noted between normal pregnancy and IUGR.

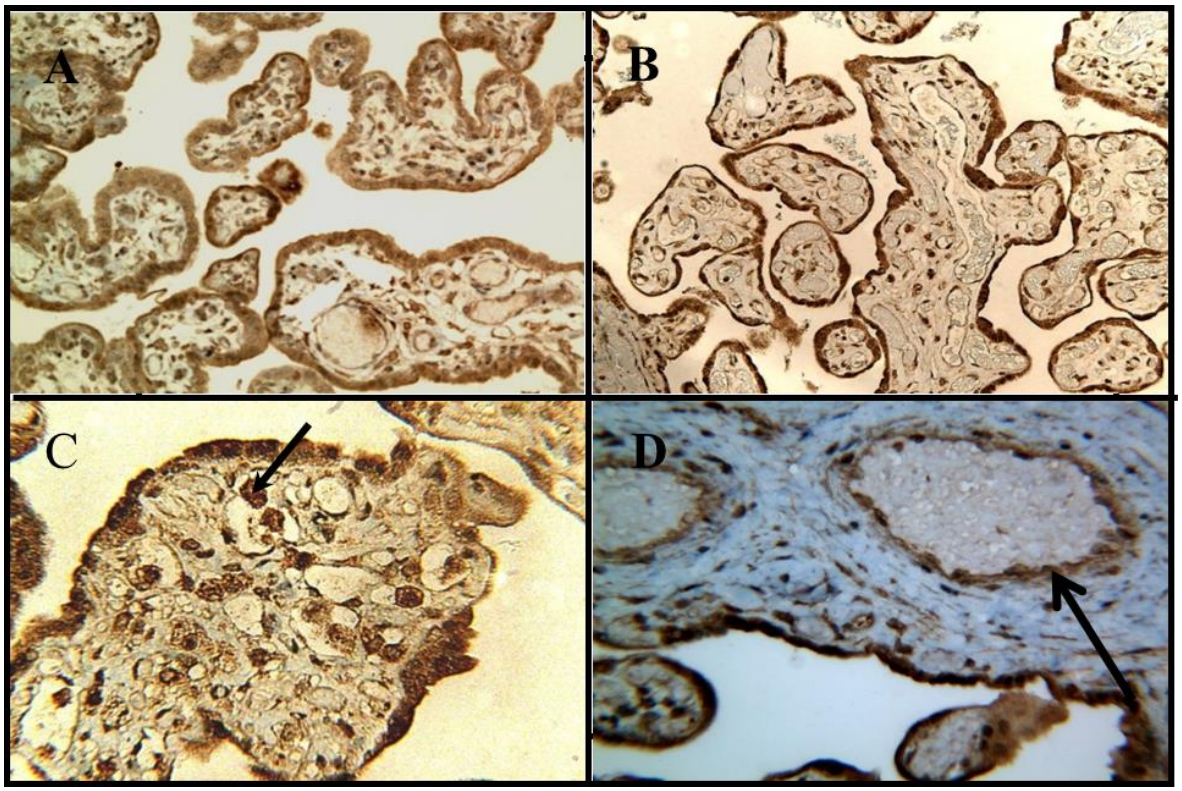

Figure 3.6 Immunolocalization of Flt-1 in the placenta.

A: Normal term placenta x 200 B: Preeclampsia x 200, C: Isolated IUGR x 400 D: endothelial staining isolated IUGR x 400. Small arrow = intense staining of Hofbauer cells. Large arrow = staining of endothelial cells.

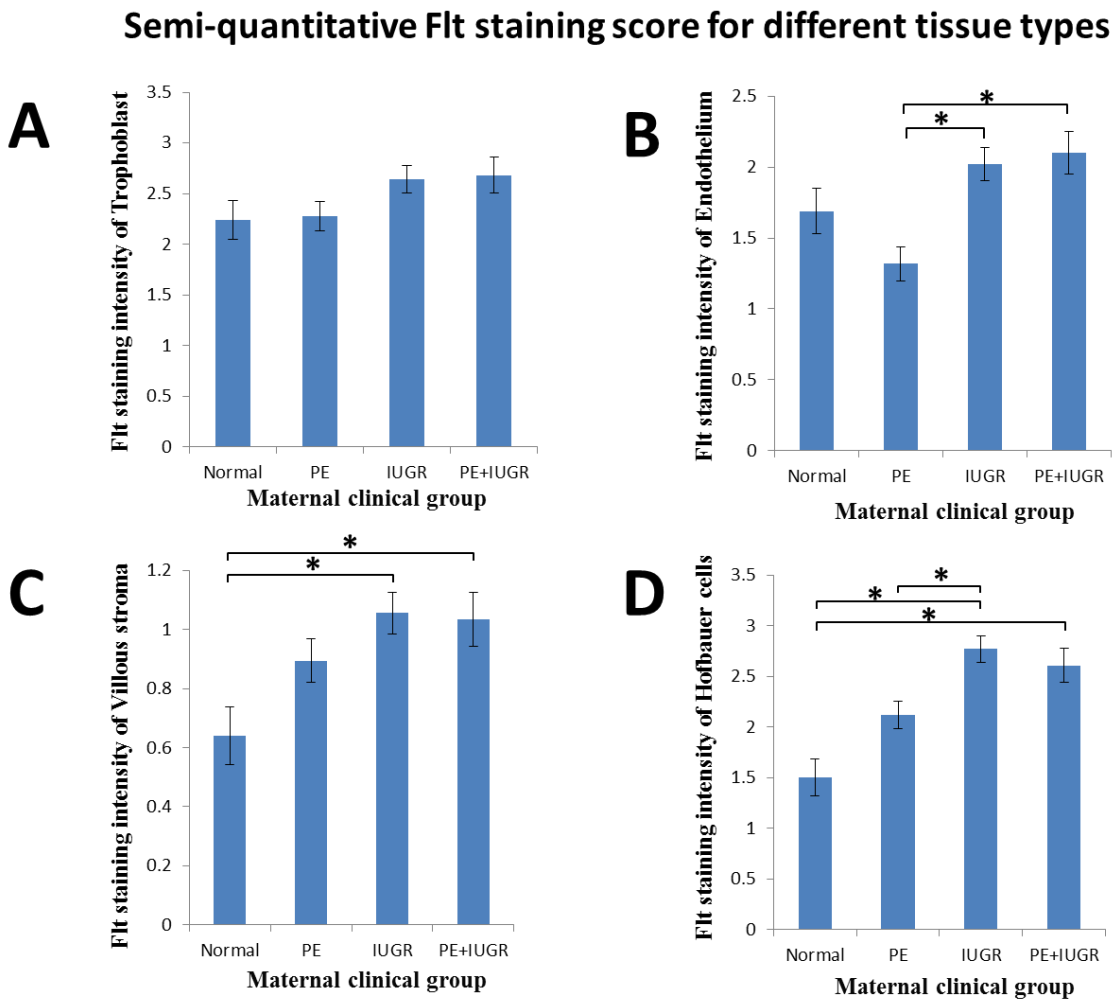

Figure 3.7 Flt Staining Intensity.

A: Trophoblast, B: Vascular endothelium, C: Villous stroma, D: Hofbauer cells. Results presented as mean ± SD. No significant difference seen between clinical groups for staining of trophoblast. IUGR and PE+IUGR groups showed increased staining of endothelium, villous stroma and Hofbauer cells.

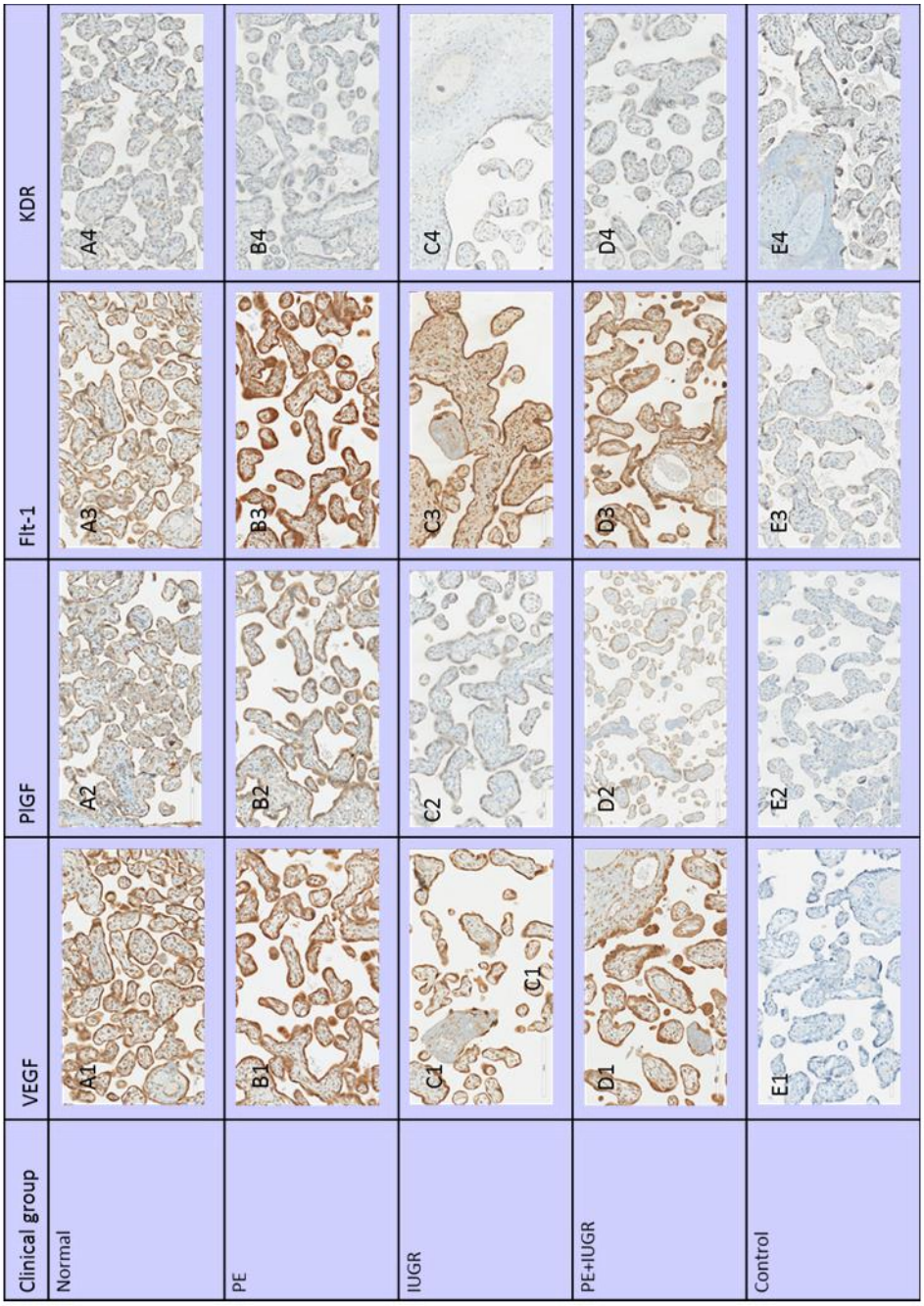

Figure 3.8 Comparative immunolocalisation and expression of VEGF, PlGF, Flt-1 and KDR in placental villous structures. Representative sections of placenta from normal pregnancy (A1-A4) and pregnancies complicated by PE (B1-B4), IUGR (C1-C4) and PE+IUGR (D1-D4). Images E1-4 represents negative controls for VEGF, PlGF, Flt-1 and KDR.

3.5.5 Association of villous infarction with the VEGF and Flt-1 staining Intensity

The Intensity scores for VEGF staining were further analyzed according to presence or absence of villous infarction within the same placental biopsy. The VEGF staining intensity of syncytiotrophoblast was found to vary with the proximity of villous infarction within a placental sample (Figure 3.9). Morphologically normal villi within a placental sample with villous infarction (peri-infarction), displayed a significantly reduced VEGF staining intensity of trophoblast (Figure 3.10) as compared to the villi of placental sections without the presence of villous infarctions ($p = 0.0001$). This reduced VEGF expression in the peri-infarction syncytiotrophoblast was evident irrespective of the clinical group and was statistically significant for preeclampsia ($p = 0.0006$), preeclampsia with IUGR ($p = 0.0076$) as well as the IUGR only groups ($p = 0.0001$). Villous infarctions in placental samples were also associated with a significantly reduced staining of villous vascular endothelium in IUGR but no difference was noted in the Flt-1 expression of trophoblast.

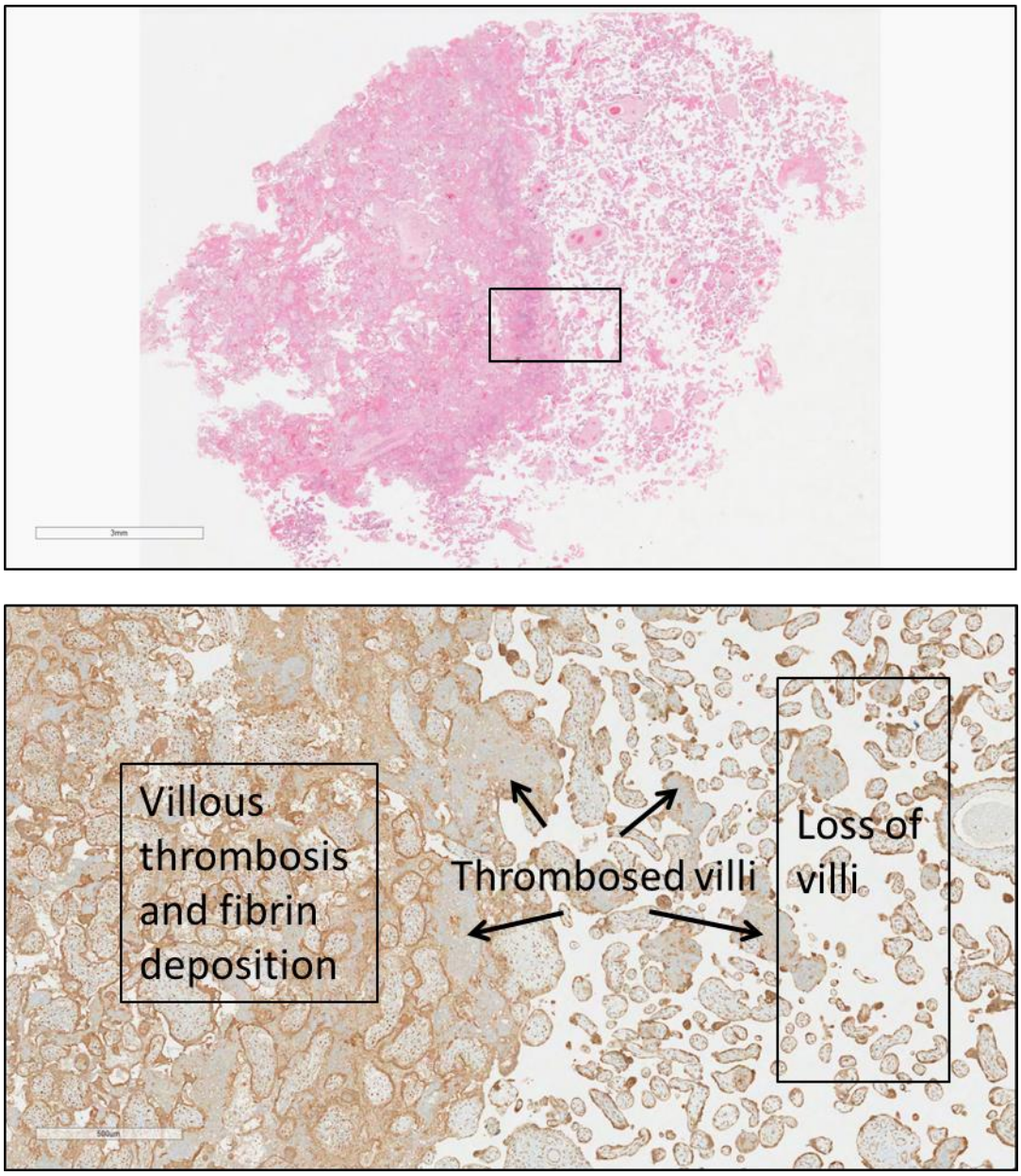

Figure 3.9 H&E stained placental biopsy demonstrating area of villous thrombosis, infarction, hypovascular villi and resultant loss of villi. Increased fibrin deposition noted. Arrows point to infarcted villi.

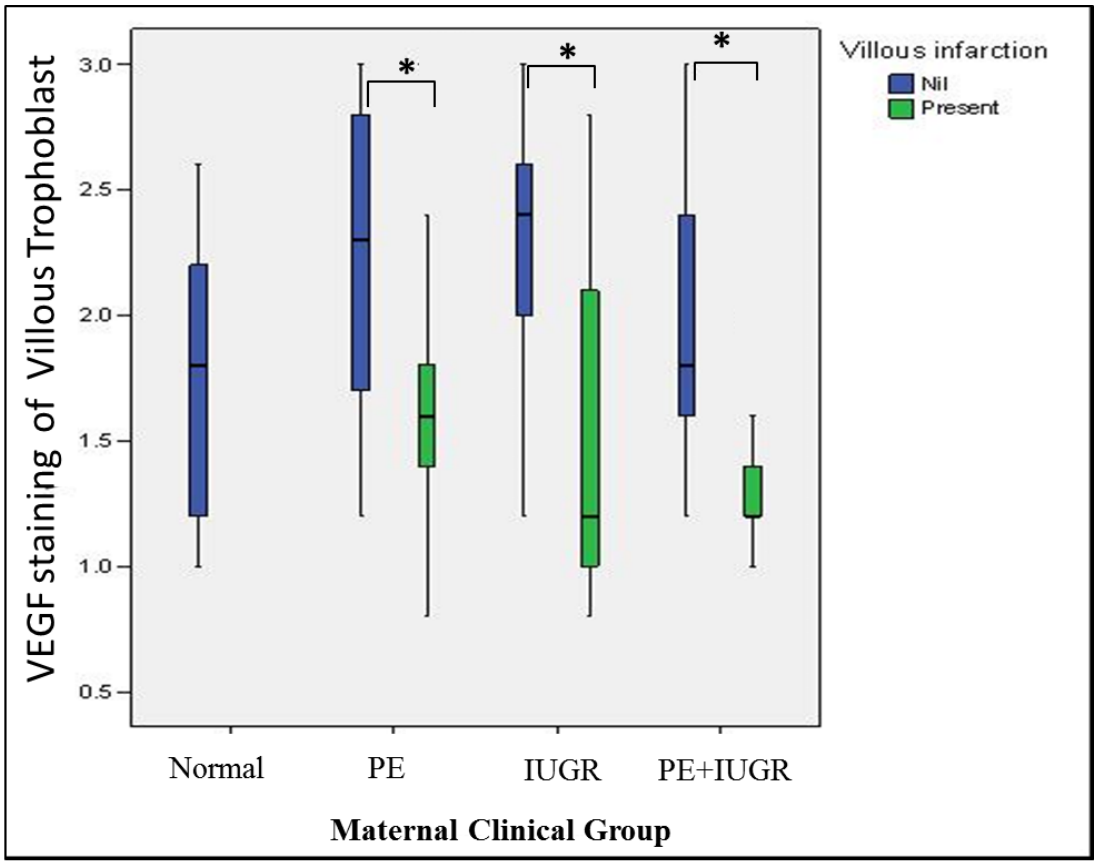

Figure 3.10 VEGF staining of trophoblast in the presence and absence of villous infarction in the placental biopsy. The median and interquartile staining scores are shown for each clinical group. The placental biopsies from normal pregnancies did not show evidence of villous infarction. The placental biopsies of the pathological pregnancies are divided into average VEGF staining intensity score of placental biopsies with no evidence of villous infarction (the box plots in blue) and the average VEGF staining intensity score of placental biopsies with evidence of villous infarction (box plots in green). The VEGF staining intensity scores were significantly lower in the placental biopsies with infarction as compared to without infarction in PE, IUGR and PE+IUGR. No difference noted between pathological groups. * = $p < 0.001$.

3.5.6 Placental VEGF: Quantitative analysis of immunohistochemical staining.

VEGF immune staining characteristic for positive pixel counts and staining intensity in all clinical groups are presented in Table 3.3 and Figures 3.11-3.13. Stronger staining of an antibody (darker brown pixels) was associated with a lower intensity score in the positive pixel algorithm. Please refer to Chapter 2 section 2.4.7 for detailed explanation of the positive pixel algorithm.

Summary of results for percentage of positive placental tissue and intensity for each marker are presented in Figures 3.11 and 3.12.

Growth Factor	Clinical Group	Average Area(mm^2) Analyzed	Average % of positive cells	Distribution of intensity of positive cells %		
				Weak	**Moderate**	**Strong**
VEGF	Normal	17.82	69.76	21.72	37.69	10.35
	PE	34.45	74.52	30.57	39.05	4.90
	IUGR	25.46	75.2	27.91	36.7	10.59
	PE+IUGR	25.33	71.34	25.46	37.31	8.57
PlGF	Normal	23.38	57.11	23.38	30.19	3.54
	PE	31.42	68.70	31.42	35.4	1.88
	IUGR	10.49	15.76	10.49	5.23	0.04
	PE+IUGR	12.00	20.07	12.00	8.00	0.07
Flt	Normal	22.01	76.52	26.17	39.91	10.44
	PE	40.10	77.66	27.85	43.98	5.83
	IUGR	30.52	82.23	33.88	42.01	6.34
	PE+IUGR	31.18	80.24	33.06	42.22	4.96
KDR	Normal	18.68	13.77	6.53	6.91	0.33
	PE	31.08	6.06	3.96	1.72	0.38
	IUGR	18.58	8.11	4.87	3.20	0.04
	PE+IUGR	22.2	11.85	7.93	3.86	0.06

Table 3.3 The percentage of immune-reactive cells and the intensity as evaluated by automatic Aperio digital image analysis software were recorded for each of the antibodies staining for VEGF, PlGF, Flt-1 and KDR.

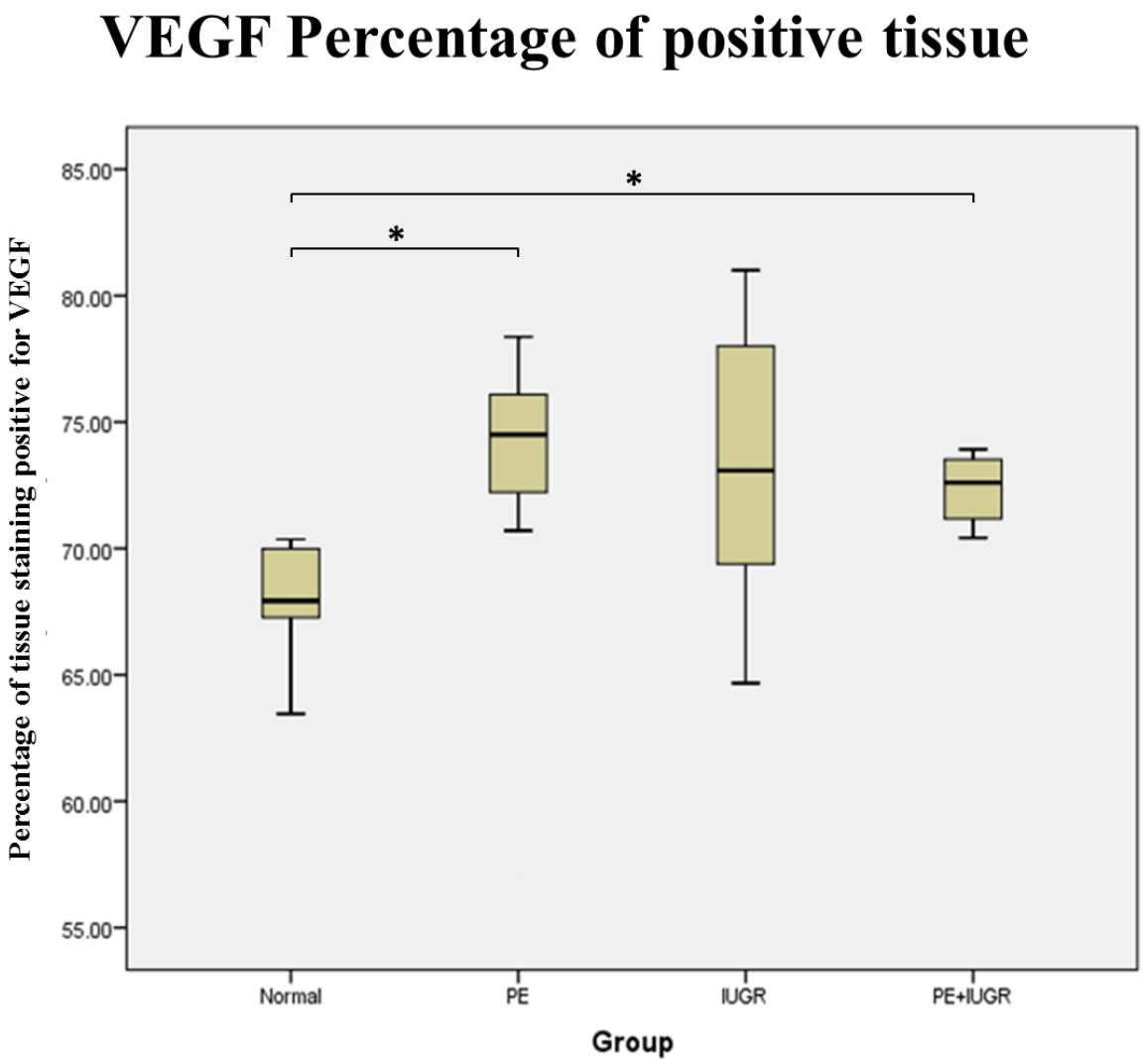

Figure 3.11 VEGF immunostaining: Percentage of positive staining pixels over total pixels.

Over 65% of tissue stained positive for VEGF in all clinical groups. Statistically significant differences were seen between Normal pregnancy and PE ($p = 0.019$) and between Normal pregnancy and PE+IUGR ($p = 0.004$). No significant difference was noted between the pathological groups.

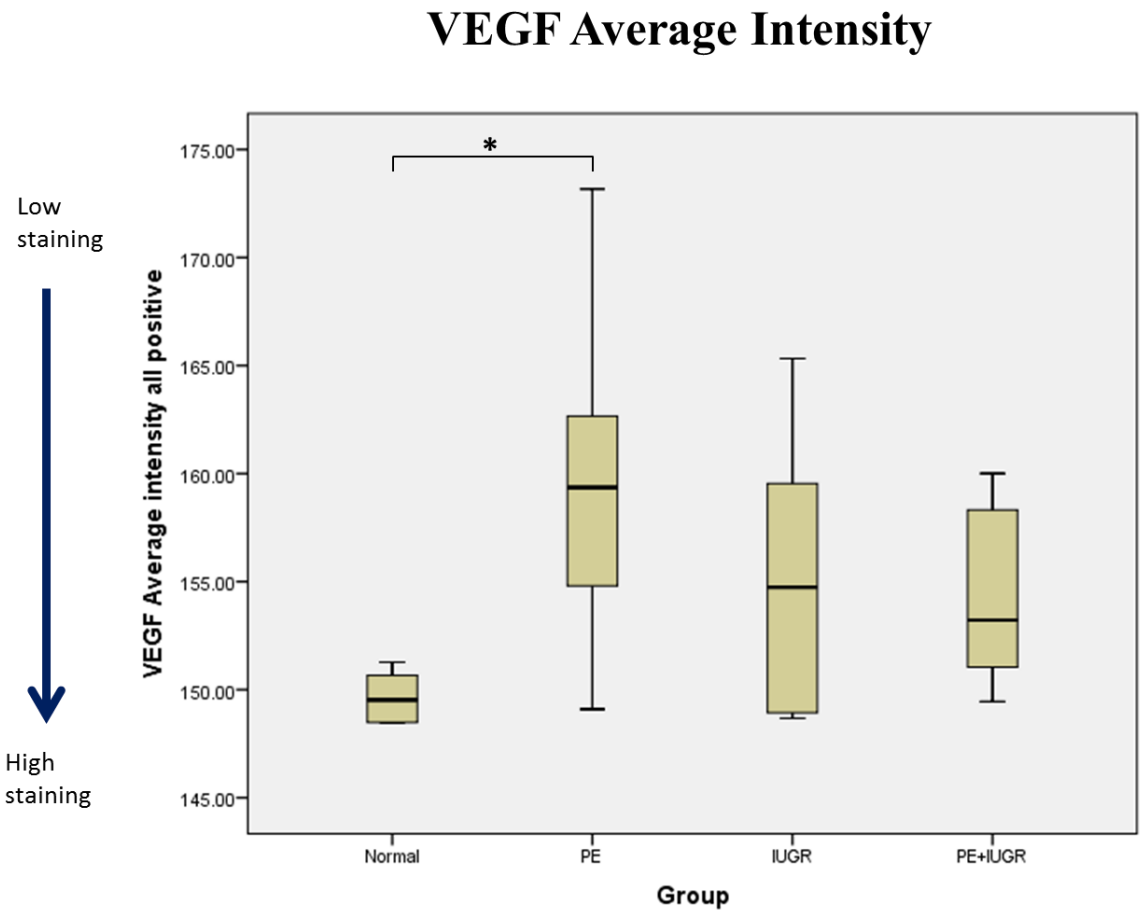

Figure 3.12 VEGF average staining intensity for all positively stained pixels.

All the clinical groups demonstrated an average VEGF staining intensity in the moderate range (100-175) with some variation in the levels in the intensity between the groups. Normal pregnancy placentas had lower intensity (higher staining) compared to preeclampsia. ($p = 0.007$). No significant difference between Normal vs. IUGR ($p = 0.055$) and Normal vs. PE+IUGR ($p = 0.052$). No difference was seen between the pathological groups. Arrow indicates that higher intensity = lower staining.

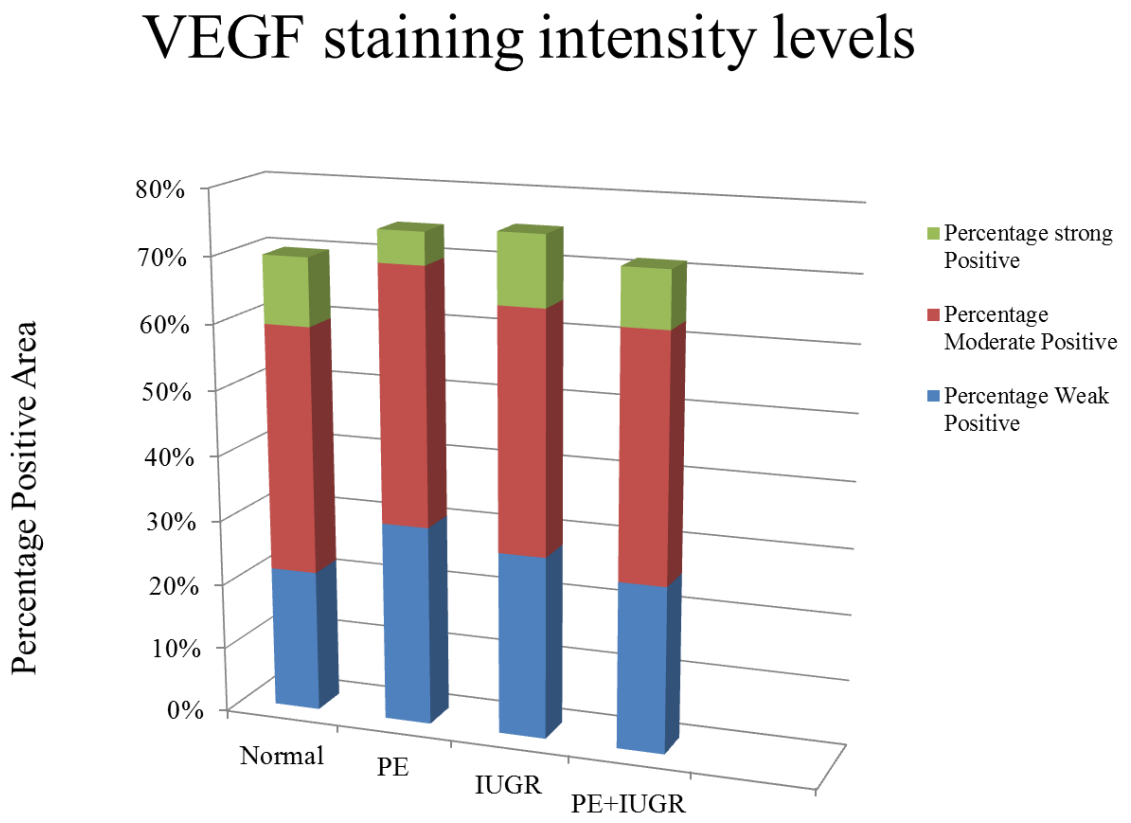

Figure 3.13 VEGF staining demonstrating percentage of the tissue staining at different levels of staining intensity (weak, moderate and strong) as generated by automated image analysis software using a positive pixel count algorithm.

The majority of the positive staining tissue for VEGF in all clinical groups was in the weak to moderate staining intensity.

3.5.7 Placental PlGF: Quantitative analysis of immunohistochemical staining.

PlGF immunostaining characteristic for positive pixel counts and staining intensity in all clinical groups are presented in Table 3.3 and Figure 3.14-3.16. The PlGF staining was lower in area and intensity in pregnancies complicated by IUGR.

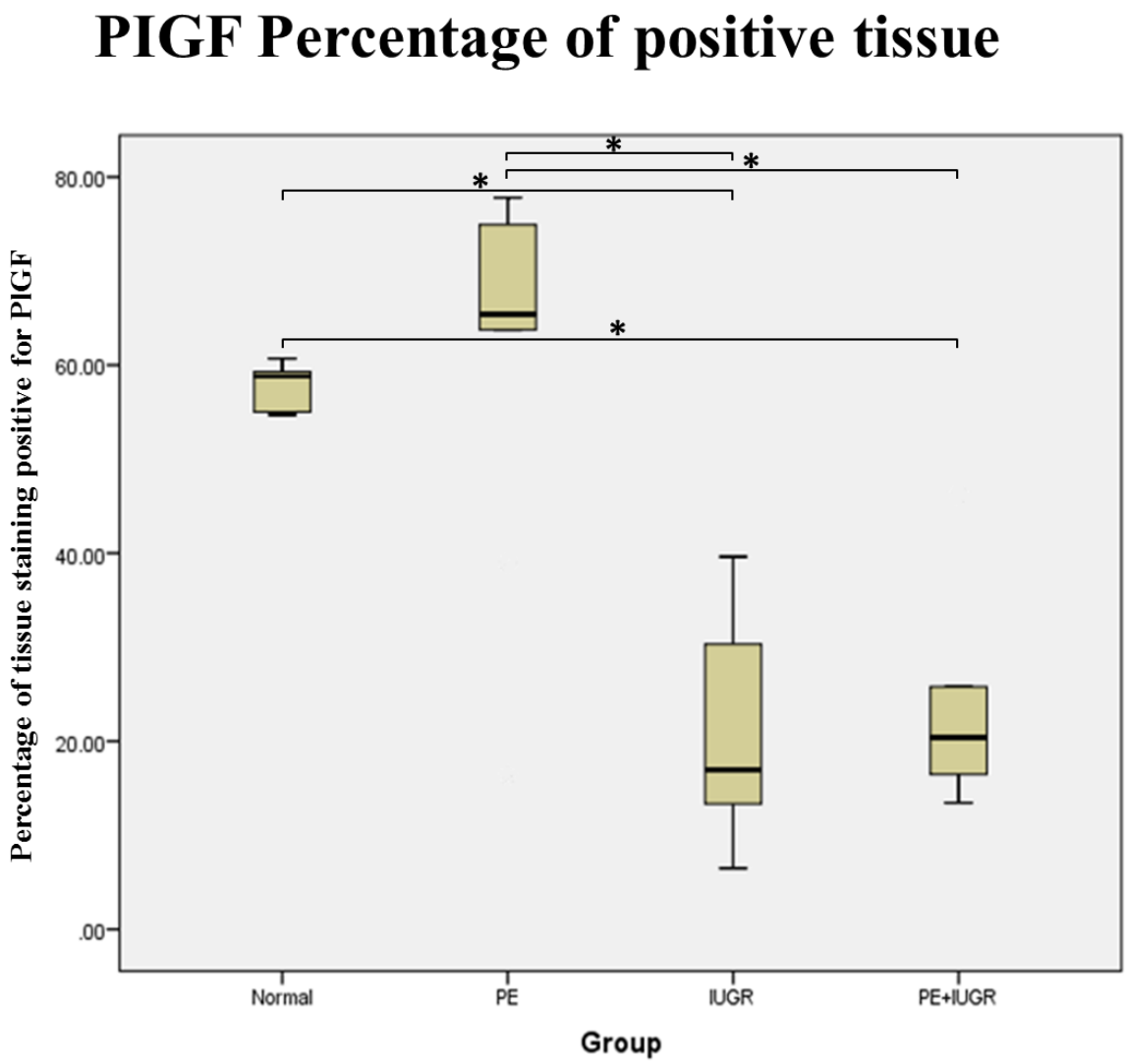

Figure 3.14 PlGF immunostaining: Percentage of positive staining pixels over total pixels. Statistically significant difference seen in the percentage of area positive for PlGF between normal pregnancy and IUGR (p = 0.001) as well as between normal pregnancy and PE+IUGR (p = 0.004) with IUGR showing a lower percentage of positive cells. Significant difference also seen between PE and IUGR (p = 0.001) as well as PE and PE+IUGR (p = 0.012). No difference was seen between Normal pregnancy and PE.

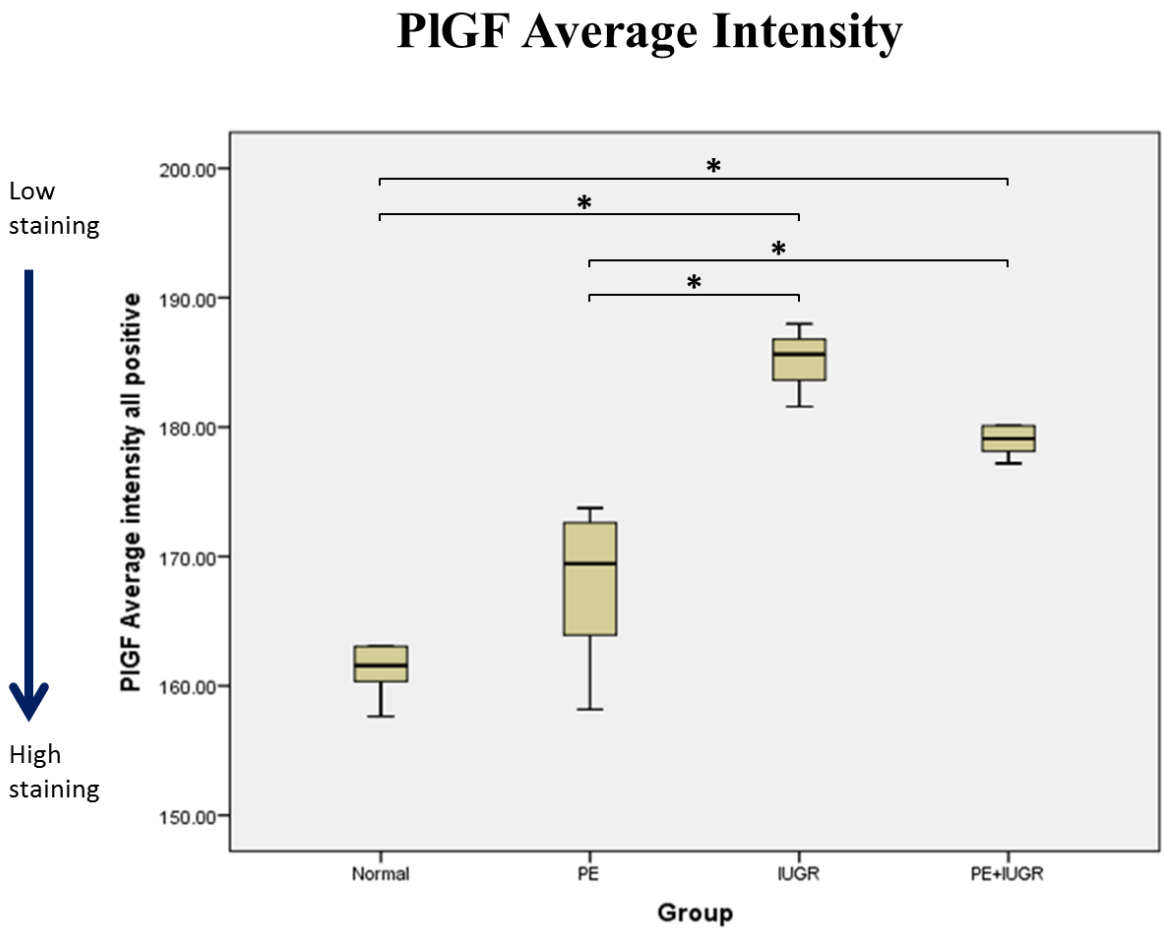

Figure 3.15 PlGF average staining intensity for all positively stained pixels.

The PlGF staining intensity was in the moderate range (intensity 100-175) for Normal pregnancy and PE, while IUGR and PE+IUGR staining intensity was weak (>175). No difference was seen between normal pregnancy and PE. Arrow indicates that higher intensity = lower staining.

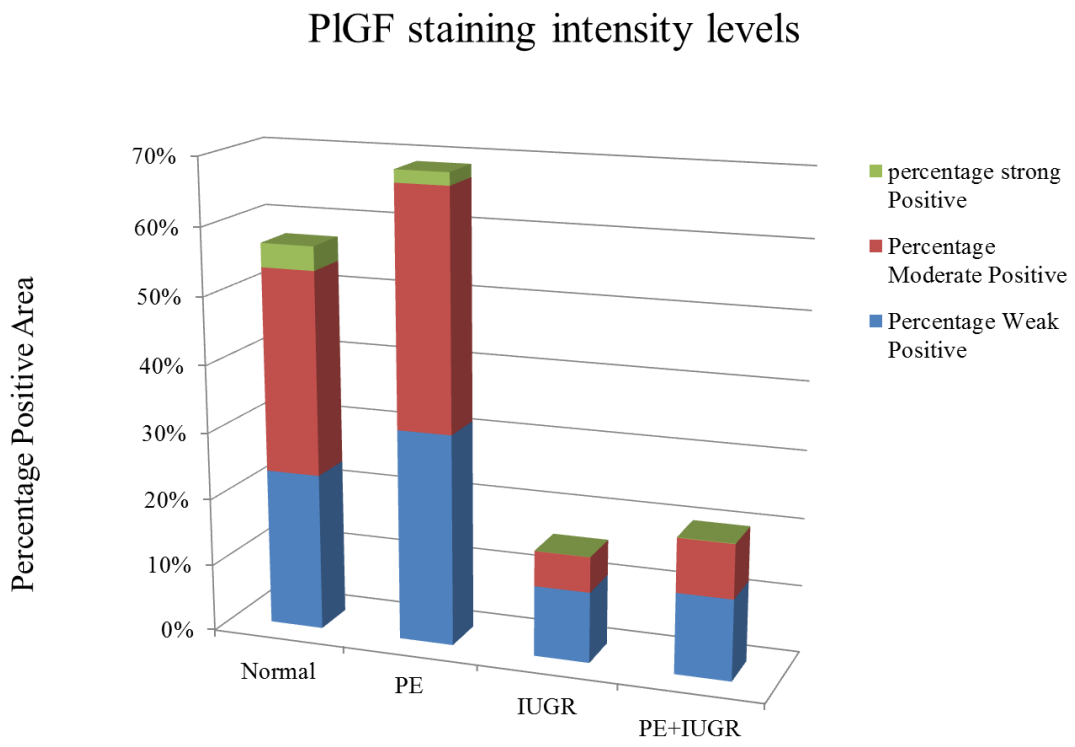

Figure 3.16 PlGF staining demonstrating percentage of the tissue staining at different levels of staining intensity (weak, moderate and strong) as generated by automated image analysis software using a positive pixel count algorithm.

The majority of the positively staining cells for PlGF in the normal and preeclamptic tissue were moderately stained while the pregnancies with IUGR demonstrated mainly weak staining.

3.5.8 Placental Flt-1: Quantitative analysis of immunohistochemical staining.

Flt-1immune-staining characteristic for positive pixel counts and staining intensity in all clinical groups are presented in Table 3.3 and Figures 3.17-3.19.

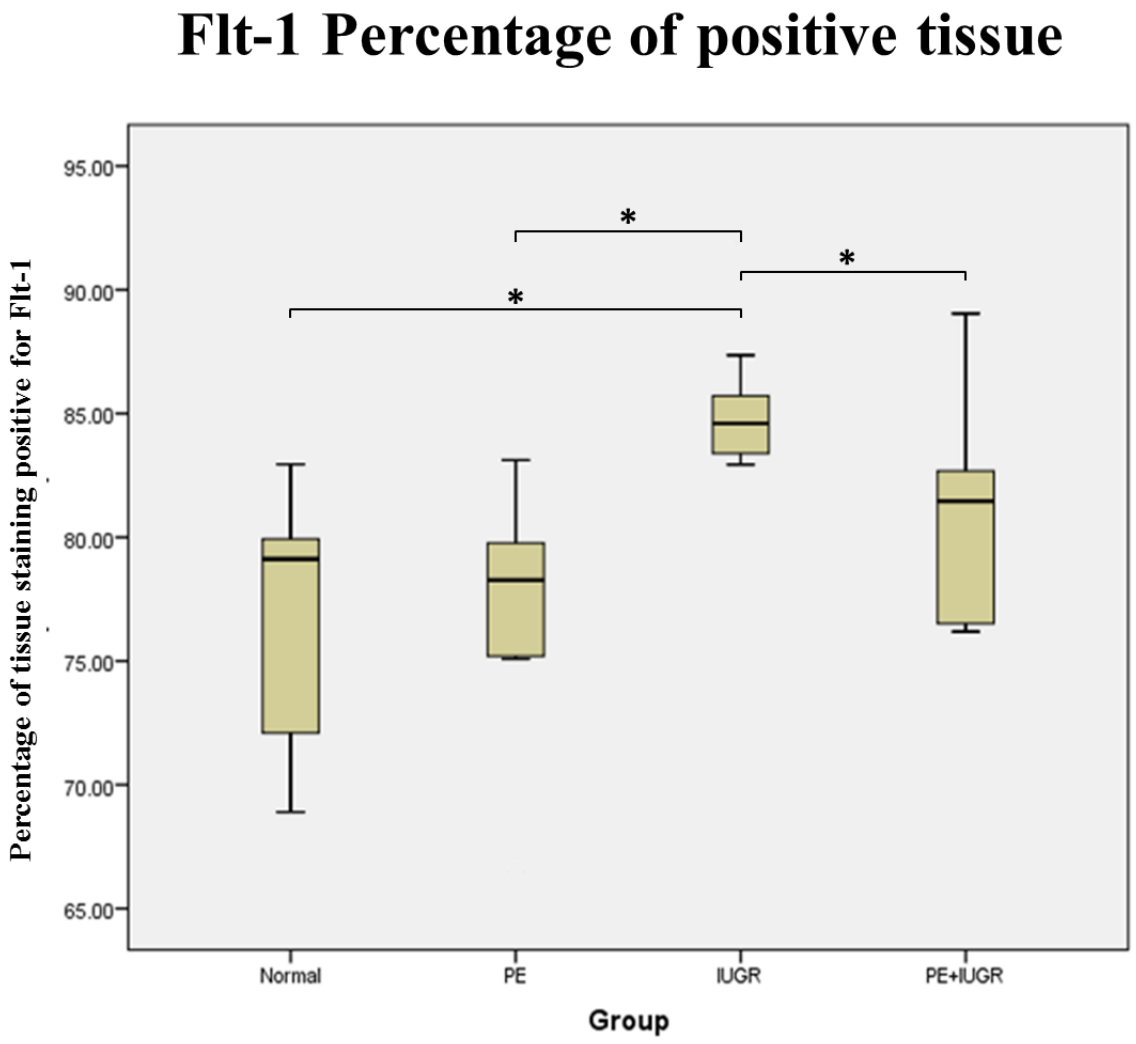

Figure 3.17 Flt-1 immunostaining: Percentage of positive staining pixels over total pixels.

All clinical groups displayed a high percentage area of positive pixels averaging over 75% of total area, median ranging from 78-85%. The area positive appeared to be marginally increased in the pathological pregnancies.

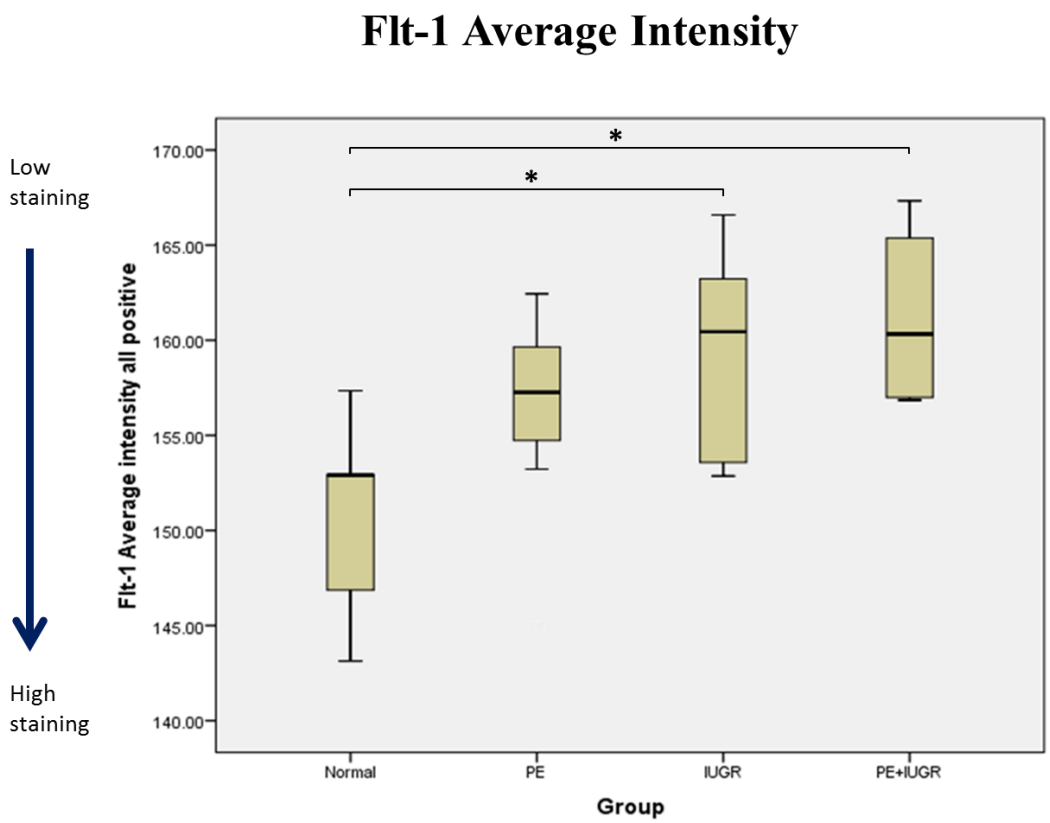

Figure 3.18 Flt-1 average staining intensity for all positively stained pixels.

The staining intensity for normal pregnancy was in the moderate range (100-175) with a small reduction in the staining in the pregnancies complicated by IUGR/PE+IUGR. Arrow indicates that higher intensity = lower staining.

In combination with Figure 3.17, Flt-1 is noted to be more widely expressed in pathological group placentas, but at a lesser intensity.

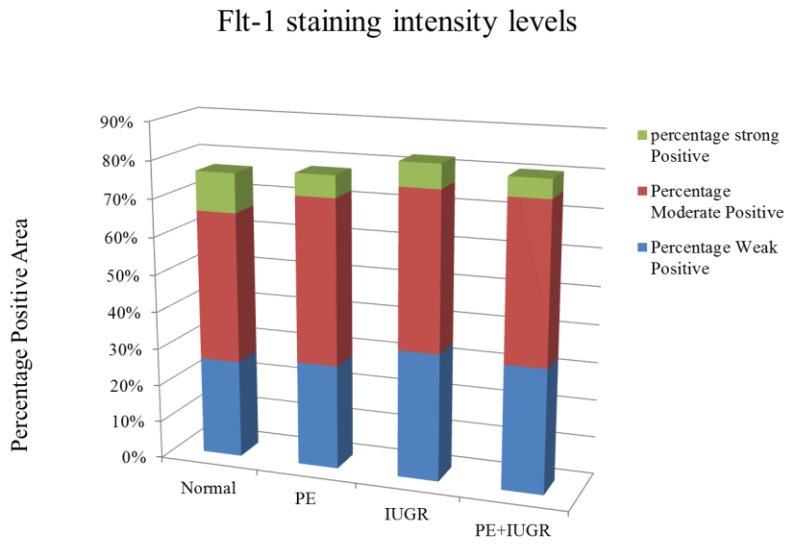

Figure 3.19 Flt-1 staining demonstrating percentage of the tissue staining at different levels of staining intensity (weak, moderate and strong) as generated by automated image analysis software using a positive pixel count algorithm.

The majority of positively stained tissue in all clinical groups demonstrated moderate intensity of staining.

3.5.9 Placental KDR: Quantitative analysis of immunohistochemical staining.

KDR immune-staining characteristic for positive pixel counts and staining intensity in all clinical groups are presented in Table 3.3 and Figure 3.20-3.22.

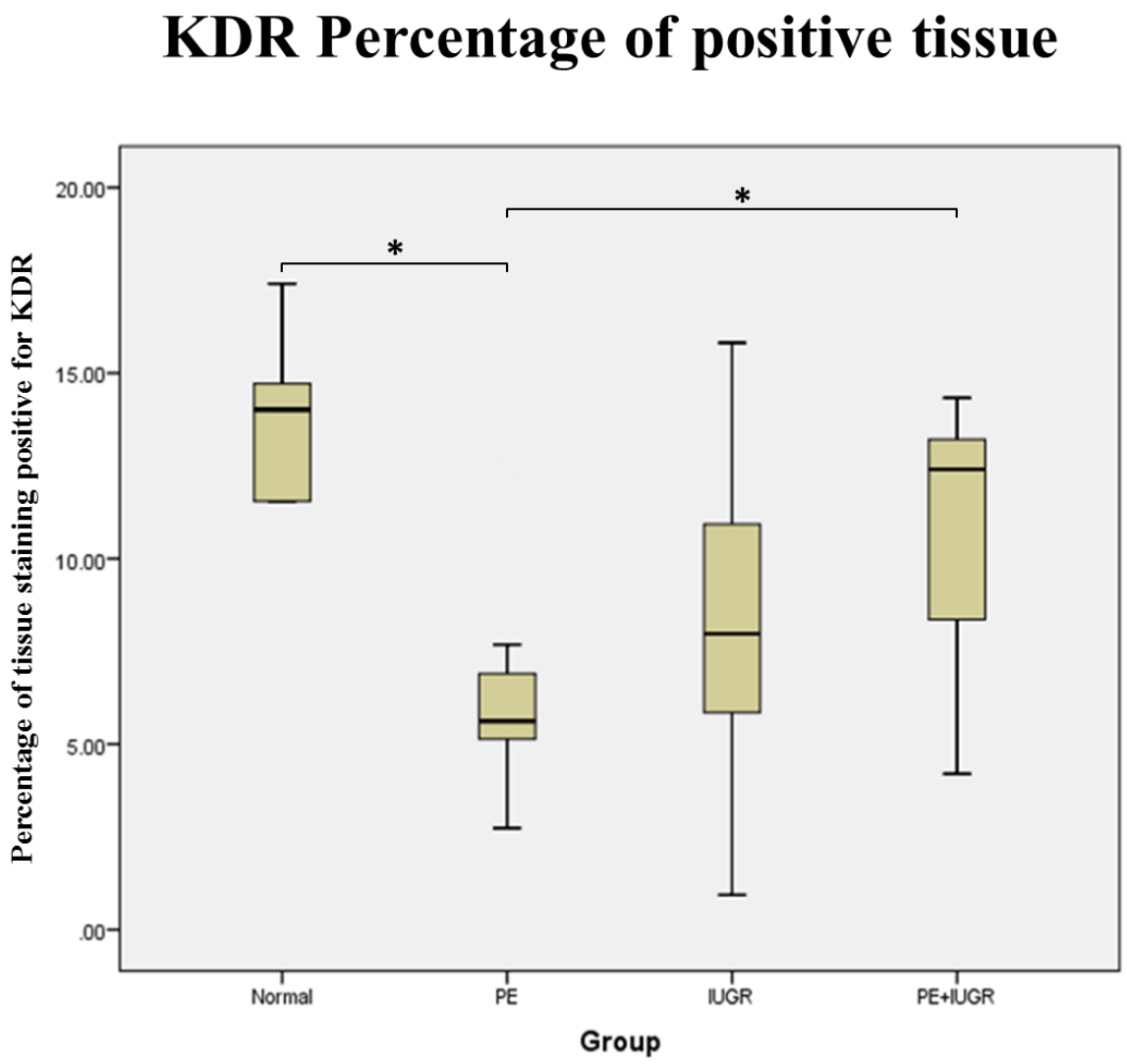

Figure 3.20 KDR immunostaining: Percentage of positive staining pixels over total pixels.

Statistically significant difference seen between normal pregnancy and PE ($p = 0.012$) and between PE and PE+IUGR ($p = 0.05$) only.

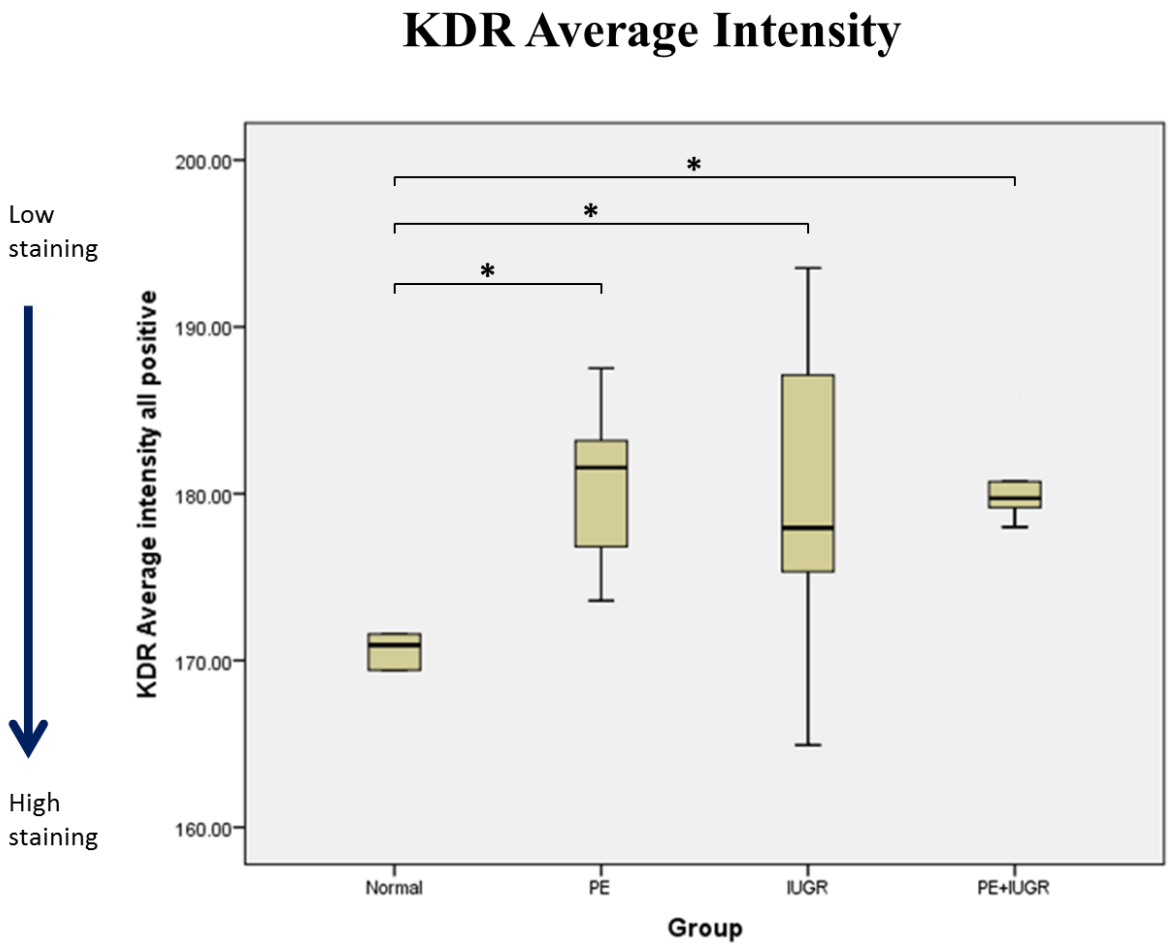

Figure 3.21 KDR average staining intensity for all positively stained pixels.

Normal pregnancy placenta had higher intensity of staining compared to the pathological pregnancies with significant difference seen between Normal and PE ($p = 0.002$), Normal and IUGR ($p = 0.013$) as well as Normal and PE+IUGR ($p = 0.004$). No difference in staining intensity was seen between the pathological groups. Arrow indicates higher intensity = lower staining.

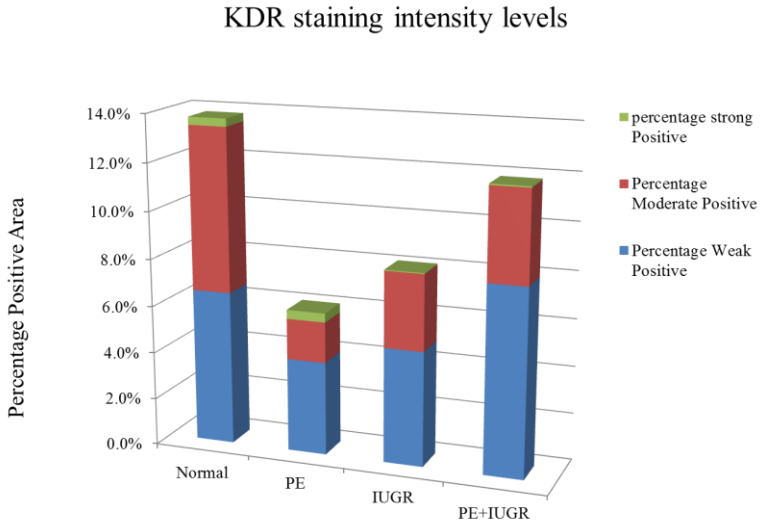

Figure 3.22 KDR staining demonstrating percentage of the tissue staining at different levels of staining intensity (weak, moderate and strong) as generated by automated image analysis software using a positive pixel count algorithm.

The majority of positively stained tissue in normal pregnancy was moderate intensity while the pathological groups PE, IUGR and PE+IUGR demonstrated mainly weak staining.

3.5.10 Inter- and intra-observer variability

To assess the inter-observer variability in semi-quantitative scoring of immunostaining, two observers manually analyzed and scored VEGF immunostaining of 120 slides from 30 placentas (Figure 3.23). Observer 2 was blinded to the clinical study groups.

The results showed a significant correlation between the scoring of the two observers (kappa correlation coefficient 0.469, $p < 0.01$). The correlation coefficient for grading of VEGF immunostaining scores between two independent observers using Bland-Altman limits of agreement was 88% ($p < 0.001$). There is a significant tendency for observer 2 to score slightly higher than observer 1, mean difference 0.12 (SD 0.03, $p<0.001$).

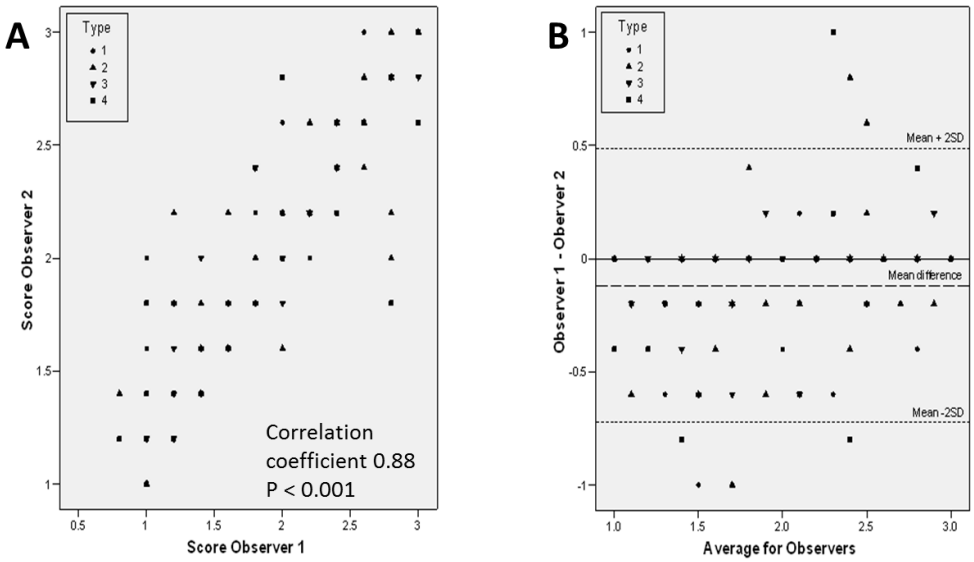

Figure 3.23 The inter-observer variability in semi-quantitative scoring of immunostaining.

A: Scatterplot of average score over 5 fields for each slide for Observer 2 versus Observer 1, using semi-quantitative analysis. B: Bland-Altman plot of inter-observer variability between Observer 1 and 2.

3.5.11 Comparison between semi-quantitative and digital image analysis of VEGF staining

The relationship between semi-quantitative and digital analysis of VEGF immunostaining in the placenta was defined (Figure 3.24) using Spearman rank correlation coefficients (SPSS1, Chicago, Illinois, USA). A moderate correlation was seen between the average score for manual reading and the score generated by the automated digital image analysis, across all study subjects in the moderate to strong VEGF intensity levels (Figure 3.24). The correlation was weaker and did not reach significance in the weak intensity levels.

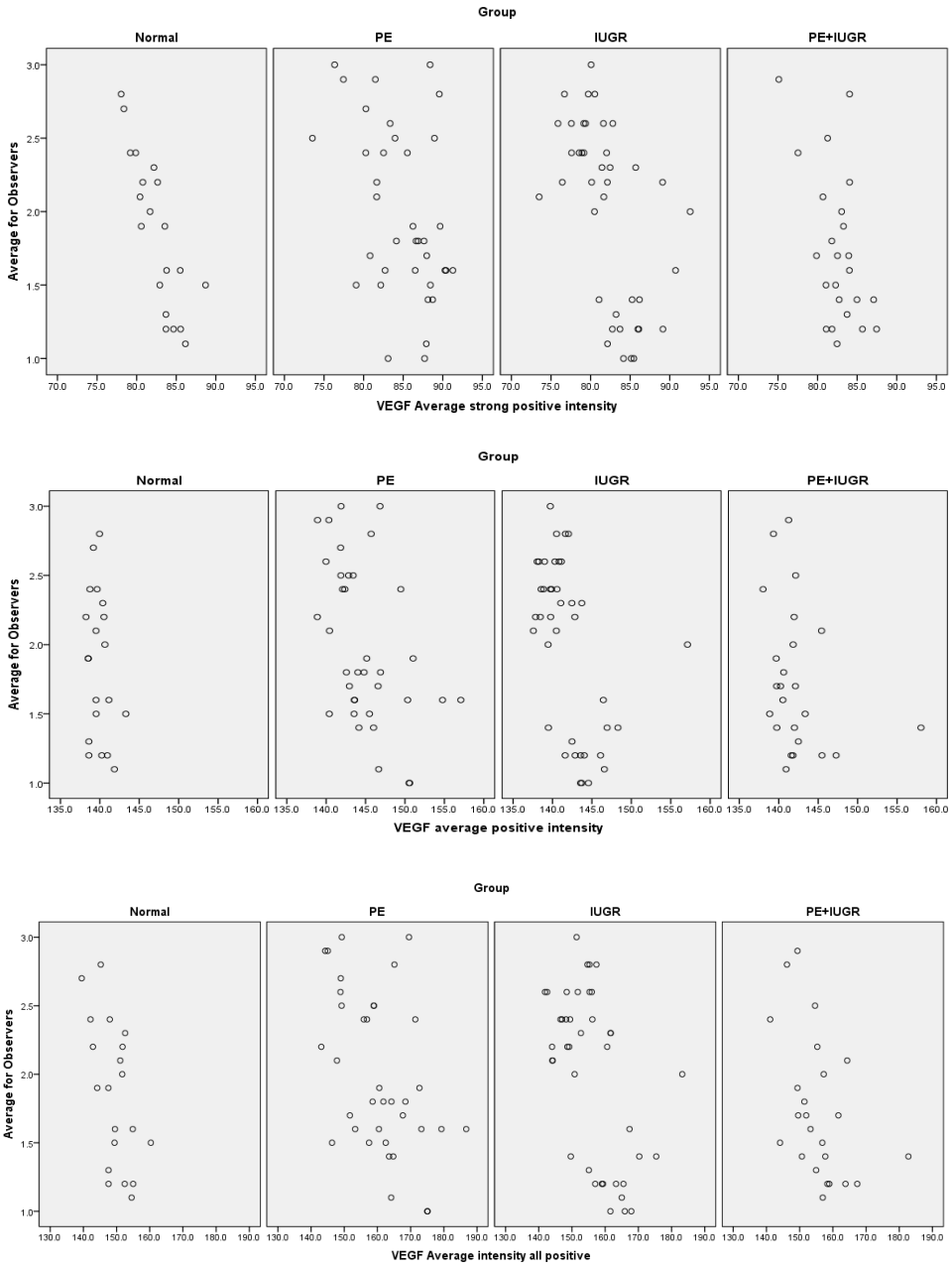

Figure 3.24 Spearman rank correlation for relationship between average scores for two manual observers and automated digital analysis of positive pixel count for VEGF intensity across all samples from four clinical groups of Normal, PE, IUGR and PE+IUGR.

Data presented for A = strong positive, B = moderate positive, C = all positive VEGF intensity. A moderate correlation was seen in the strong (correlation coefficient 0.51, p <0.01), moderate (correlation coefficient 0.37, p <0.01) and all positive staining (correlation

coefficient 0.45, $p < 0.01$). The correlation was weaker and did not reach significance in the weak intensity levels (correlation coefficient 0.06, $p = 0.34$).

3.6 Discussion

Factors that influence placental vascular development and function will have an impact on fetal growth and development, and thereby on neonatal survival and growth. A substantial body of evidence indicates angiogenic factors play a significant role in placental angiogenesis as well as restoration of the placental vasculature. In this study the expression of VEGF, PlGF and their receptors Flt-1 and KDR were determined in normal and pathological placentas to determine their role in the pathogenesis of preeclampsia and intrauterine fetal growth restriction.

3.6.1 Placental morphology

The description of placental morphology in this study is comparable to previous findings of syncytiotrophoblast knotting, villous infarcts, avascular villi and uteroplacental fibrinoid necrosis as characteristic placental morphological features of preeclampsia and intrauterine growth restriction (36, 57). Placental morphological changes were similar in PE and IUGR.

3.6.2 Villous architecture

Previous published work on stereological assessment of placental morphology in PE and IUGR have shown that PE had an effect on intervillous stroma and terminal villi volume only. IUGR, alone or in combination with PE contributed towards significant reductions in volumetric and surface area terminal villous and vascular features (388). Further studies into

early and late onset preeclampsia have shown isolated early-onset PE to be associated with abnormal placental morphology, but placentas from late-onset PE were morphologically similar to placentas from gestational age matched controls, suggesting the existence of two subsets of this condition and supporting the hypothesis that late-onset preeclampsia is a maternal disorder and not a placental disease (389). A study using digital image analysis of the morphology and composition of placental villi in pregnancies complicated by PE and IUGR (379) demonstrated significantly smaller placentas in growth restricted pregnancies. PE, with or without IUGR, had no effect on the total area occupied by villi. IUGR alone showed a real and consistent reduction in villous numbers. These measurements point to impoverished villus structure in idiopathic IUGR. The observed changes in PE with IUGR were more akin to PE without growth restriction than IUGR alone. The authors suggested that idiopathic IUGR and PE +IUGR have a separate aetiology; idiopathic IUGR arising through a reduction in villous area alone, and IUGR in PE caused by changes in syncytiotrophoblast and cytotrophoblast quantity (379).

The findings on villous architecture are consistent with the above published studies. The study has confirmed that significant morphological abnormalities exist in the placentas of PE and IUGR. The loss of villi is significant in the IUGR with abnormal umbilical artery Doppler resistance. The effect is cumulative with a combination of PE and IUGR. These results of the number of villi per high power field and villous morphology, indicate loss of villous vascularity and tissue. While both PE and IUGR have changes compared to the normal term placenta, the cumulative effect of PE and IUGR appear to lead to a more significant loss of villous architecture than either condition alone. The decreased number of villi in the IUGR and PE+IUGR is also consistent with prior histopathological studies documenting loss of terminal villi with IUGR (15, 57).

3.6.3 Pro and anti-angiogenic factor levels in the placenta

The localization of VEGF, PlGF, Flt-1 and KDR in placental tissues was similar across the four clinical groups. These results showed that positive staining for VEGF, Flt-1, and PlGF was mainly localized to the syncytiotrophoblast layer of the placenta, while KDR staining was mainly detected in the endothelial layer. There were significant differences detected in the staining intensity across the clinical groups in PlGF and KDR while VEGF and Flt-1 were in a similar immunostaining range. The interpretation of the staining intensity should take into consideration that while differences in staining intensity levels leading to different strengths of staining (weak, moderate and strong) may be clinically important, variations within a staining intensity levels are unlikely to be clinically significant or be detected by the naked eye.

3.6.3.1 VEGF

These results on the overall distribution and the intensity of VEGF in the placentas demonstrated that while a higher number of cells were positive for VEGF in the pathological placentas, the intensity of staining was in the moderate staining range in all the clinical groups. These findings are consistent with variable data from previous studies (191, 199, 201) showing increased as well as reduced VEGF levels in preeclampsia. The variation in staining may be explained by the fact that placental pathology is often patchy within the disc of the placenta and an overall assessment may not be able to demonstrate a difference. In a novel concept, the placental biopsies were analyzed according to the presence or absence of villous infarctions in the sample and have demonstrated that areas with active disease of the placenta as shown by villous infarction demonstrated significantly reduced VEGF staining and expression. A similar analysis on VEGF expression in relation to significant necrosis in tissue has been described in neuronal tumors by other researchers (80). This data seems to support

lack of VEGF as a causal factor in villous infarction or alternatively cellular death associated with infarction leading to reduced VEGF expression. The VEGF expression in placental samples without areas of infarction appears to be increased, suggesting a compensatory mechanism in the regeneration of the intact placental villi (194).

3.6.3.2 PlGF

In the current study, PlGF was significantly reduced in IUGR and PE+IUGR as compared to normal pregnancy and PE, both in the area positive as well as in intensity of staining. Consistent with previous descriptions such as, the PlGF expression was not significantly different in the preeclampsia only group (190). A recent longitudinal and cross sectional study into plasma PlGF levels (390) suggested that low maternal PlGF throughout pregnancy identifies a subset of preeclampsia patients that develop early and severe disease. That study did not include IUGR and it is not clear whether the described group of patients had significant IUGR as a result of low PlGF. The current study results on reduced PlGF correlates with published literature (58) showing that PlGF expression is reduced in the presence of placental hypoxic/ischemic morphological changes and provides further evidence that hypoxic/ischemic changes occur in IUGR placentas.

3.6.3.3 Flt-1

These results on Flt-1 parallels previously published results documenting moderate to strong immune-staining of syncytiotrophoblast and extra villous trophoblast (202) in normal pregnancy and preeclampsia. The study has shown that a high percentage of cells stained for Flt-1 in all clinical groups with an increase noted in the pathological groups. The Flt-1 immunostaining intensity was moderate in all groups with a comparatively small reduction in staining intensity in pregnancies affected by IUGR/PE+IUGR compared to normal pregnancy and preeclampsia.

3.6.3.4 KDR

Immunolocalization of KDR in the current study has demonstrated a lower membrane bound intensity and area positive for KDR expression in pathological placentas with preeclampsia and IUGR compared to normal pregnancy. The findings raise the possibility that a compromised expression of KDR may play a significant part in the pathogenesis of PE and IUGR.

3.6.4 Automatic analysis

While the conventional Hematoxylin-Eosin staining is the mainstay for pathologic diagnosis, immunohistochemical staining is increasingly used in research as well as clinical diagnosis. There is a need for standardization of analysis method for more reproducibility, less time consuming quantitative analysis methods. Newer tools using digital image analysis and algorithms are a way forward in achieving these targets. The published literature to date on angiogenic factors and their receptor expression using immunohistochemical methods has used semi-quantitative methods of interpreting and scoring the staining intensity (176, 180).

The current study used semi-quantitative analysis of immunohistochemically stained slides to describe the localization and intensity of VEGF staining. Image analysis using digitized slides and automated digital image analysis of staining characteristics were also used to objectively assess the expression of angiogenic factors VEGF and PlGF and their receptors Flt-1 and KDR in placentas of preeclamptic and IUGR pregnancies. A comparison was made between the semi-quantitative and digital analysis techniques for VEGF showing that while there is consistency between the techniques with moderate to strong staining, the correlation is not as strong with weak staining. The use of semi-quantitative techniques may explain

some of the variable results published in the last decade on immunohistochemical expression of angiogenic factors in the placenta.

This is one of the few studies presenting digital image analysis of immunohistochemical staining in human placental tissue. A published comparative analysis of manual point counting method and automated pixel-counting method of area percentage estimations has suggested that the inter-observer concordance in the point-counting technique is reasonably high and the results obtained by the point counting method approach those seen by the automated pixel-counting when at least 300 points are overlaid (391). A recently published study used a visual image analysis of formalin-fixed, wax-embedded sections stained with haematoxylin and eosin (H&E) to observe changes in the morphology and composition of placental villi in pregnancies complicated by PE and IUGR (379). A further study into placental morphometry and villous architecture in pregnancies complicated by IUGR used a computerized Video Image Analysis system for analysis (380).

Several other studies such as have published comparisons between semi-quantitative scoring systems and digital analysis techniques for immunohistochemical staining (392). The accuracy of digital techniques including Aperio computer-assisted analysis of immunohistochemical staining techniques have been assessed and validated in multiple tissue types including brain, breast and kidney (383, 392-394). In published literature, Aperio Positive Pixel Count Algorithm is comparable to the pathologist's scoring and could add benefits of automated and reproducible measurement (383).

The present study shows that angiogenic factor immunostaining in the placenta can be easily evaluated using computer assisted image analysis on completely digitized slides. Digital pathology evaluation of VEGF staining in the placenta parallels semi-quantitative analysis in strong to intense staining, bringing objectivity to the analysis with reduction of intra-observer

and inter-observer variability and is a useful tool to improve reproducibility of the scoring. The published parameters for definition of low, medium and strong staining would facilitate comparison and reproducibility of research findings from different research centers. With the adoption of digital pathology, image analysis of immunohistochemistry slides can be integrated seamlessly into the digital pathology workflow.

3.6.5 Interpretation of results

A novel feature in this study was the classification of the clinical groups according to the umbilical artery Doppler flow velocity waveforms as well as maternal hypertensive disease. A meta-analysis of twenty randomized controlled trials of umbilical artery Doppler ultrasound showed a significant reduction in the number of antenatal admissions, inductions of labour, and caesarean sections for fetal distress in the Doppler group and that the clinical action guided by Doppler ultrasonography reduces the odds of perinatal death by 38% (376). The Doppler resistance has been described as various indices using the peak systolic and end diastolic flow velocity of the umbilical artery Doppler waveform. All the described indices, Systolic / Diastolic ratio (S/D), Pulsatility index (PI) and Resistance (Resistive) Index (RI) are different forms of the same information and decrease with increasing gestation. A detailed description of the indices is provided in Figure 2.1 and 2.2 as well as Table 2.1 (376). A birth weight of <10th percentile may be a result of multiple aetiological factors, including constitutionally small babies with normal placental function as well as in-utero growth restricted fetuses with placental vascular disease. Study of these placentas as one group is unlikely to provide us with clarification of the pathophysiology of placental vascular disease. Patient selection based on maternal symptoms, especially if mild may also not be very reflective of significant placental pathology. The use of umbilical artery Doppler waveform

analysis in the selection of cases ensured that the samples were collected from pregnancies affected by placental vascular disease.

The strength of this study is the strict criteria used for patient selection. The use of umbilical artery resistance in the identification of patients with a placental cause of IUGR has removed the ambiguity generated by previous studies and has added to the reliability of these findings. This study was limited by small patient numbers, limited placental samples and the cross sectional design, although comparable to previously published studies using similar techniques (379). The use of the digital image analysis of the whole placental biopsy has increased the area of placenta analysed for staining as compared to a limited number of high power fields usually analysed by the manual assessment of staining.

To our knowledge, this is the first study to look at the expression and immunolocalisation of VEGF and receptors in preeclampsia and intrauterine growth retardation characterized by abnormal umbilical artery Doppler studies. The study has correlated the angiogenic effects of VEGF, PlGF as well as anti-angiogenic response of Flt-1 and KDR in the placenta to histopathological changes as well as clinical features of umbilical artery Doppler waveforms and preeclampsia. The current study adds to the existing literature as the first description of digital image analysis techniques in the assessment of angiogenic factor expression in the placenta.

The findings of this study show that the level of overall VEGF and Flt-1 are in the same range for normal pregnancy and pregnancies complicated by PE and IUGR. The intensity of VEGF staining has been shown to be reduced in the areas of significant pathology such as villous infarction compared to the non-infarcted areas suggesting compensatory villous regeneration in these areas. One of the most important findings in the present study is that PlGF and KDR are significantly reduced in expression in the placentas from pregnancies complicated by

IUGR compared to normal and preeclamptic pregnancies. The intensity of Flt-1 was noted to be moderate and within similar range across all the clinical groups. The area positive was however higher in PE and IUGR. It is possible that the overall effect of the combination of moderate intensity and increased area is seen as higher Flt-1 staining on manual reading with photo-microscopy.

The results of this study raise the possibility that changes in VEGF and Flt-1 expression may be a consequence rather than the cause of placental vascular disease and preeclampsia and that lack of PlGF and KDR may be a main cause for the development of intrauterine fetal growth restriction.

We have also shown that automated digital image analysis using software such as Aperio positive pixel algorithm could be successfully used as an alternative method to the manual reading of placental immunohistochemical staining.

Chapter 4 - Expression of angiogenic factors and their receptors in the maternal peripheral circulation and fetal cord blood in normal and complicated pregnancies.

4.1 Summary

Introduction: Literature to date has described aberrant angiogenic factors and their receptor levels in placental disease. Whether there is a difference between preeclampsia and IUGR is not clear. It is still not established whether these changes are part of the pathogenesis of placental disease or a reactionary change to the disease process in the placenta. Circulating angiogenic factors are being evaluated as potential biomarkers for pregnancy complications such as PE and IUGR.

Aim: To evaluate the plasma levels of angiogenic factors in preeclampsia (PE) and intrauterine fetal growth restriction (IUGR) and their potential as biomarkers to distinguish normal from pathologic pregnancies.

Methods: Case control study included singleton pregnancies in four clinical groups of normal pregnancy, preeclampsia (PE), preeclampsia with intrauterine fetal growth restriction (PE+IUGR) and IUGR. The classification of IUGR included umbilical artery Doppler resistance. Maternal plasma placental growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1), soluble kinase domain receptor (sKDR) and soluble Endoglin (sEng) as well as fetal umbilical artery sFlt-1 levels were determined. Each individual marker and their ratios were assessed for their potential to distinguish normal pregnancy from pregnancies affected by PE and/or IUGR.

Results: The study found (i) elevated plasma sFlt-1, sEng and reduced PlGF, sKDR in PE and IUGR; (ii) similar angiogenic profiles in PE and IUGR and (iii) sEng and sFlt-1*sEng/PlGF performed best as biomarkers in identifying pathologic pregnancies. (iv) Fetal sFlt-1 levels were well below the maternal levels and no difference between clinical groups.

Conclusions: PE and IUGR have similar angiogenic profiles, suggesting that angiogenic marker profiles lack specificity in identifying PE and that other factors are required for the identification of PE from IUGR. sEng is a predictor of established PE and IUGR and could be part of a biomarker profile for predicting PE or IUGR.

This chapter is published in part in the journal article: Alahakoon TI, Zhang W, Trudinger BJ, Lee VW. Discordant clinical presentations of preeclampsia and intrauterine fetal growth restriction with similar pro- and anti-angiogenic profiles. J Matern Fetal Neonatal Med. 2014 2014; 27(18): 1854-1859.

4.2 Introduction

The balance between angiogenic factors and their pro and anti-angiogenic receptors are thought to play an important role in the ongoing control of placental angiogenesis in normal and complicated pregnancies (215). The last decade has seen a plethora of publications investigating the role of angiogenic factors in placental disease, in particular the VEGF family, and their receptor levels in the maternal circulation. Current knowledge of the changes in the angiogenic factors in preeclampsia and intrauterine growth restriction has been explored in detail in Chapter 1 of this thesis. While it is evident that the angiogenic factors and their receptor levels are aberrant in placental disease, whether there is a difference in preeclampsia and IUGR is not clear. It is still not established whether these changes are part of the pathogenesis of placental disease or a reactionary change to the disease process in the placenta. Circulating angiogenic factors are being evaluated as potential biomarkers for pregnancy complications such as PE and IUGR (215, 219).

4.3 Aims

- Measure VEGF family angiogenic factors and their receptors in maternal and fetal plasma.

- Identify any differences in angiogenic factors and their receptor levels between normal pregnancy and pregnancies complicated by preeclampsia and/or fetal growth restriction.

- Evaluate the predictive value of maternal and fetal plasma angiogenic factors and their receptors in identifying pregnancy complications such as preeclampsia and intrauterine fetal growth restriction

4.4 Methods

A case control study was conducted including singleton pregnancies from four clinical groups of normal pregnancy, PE, PE+IUGR and IUGR. Maternal plasma PlGF, sFlt-1, sKDR and sEng as well as fetal umbilical artery sFlt-1 levels were determined. Each individual marker and their ratios were assessed for their potential to distinguish normal pregnancy from pregnancies affected by PE and/or IUGR.

The sample collection and measurement of maternal plasma biomarkers using ELISA techniques were as described in Chapter 2.5. Statistical analysis detailed in Chapter 2.8.9.

4.5 Results

4.5.1 Clinical characteristics of the study population

Results are presented for 84 maternal peripheral venous samples and 84 fetal umbilical arterial cord blood samples. The demographic data for the four groups are presented in Table 4.1. There were no significant differences in maternal age, parity and gestational age at blood sampling between the four groups. A higher proportion of the pathological pregnancies were to primigravid mothers. The gestational age at delivery and birth weight were significantly different in the pregnancies complicated by preeclampsia and fetal growth restriction as compared to normal pregnancies. No significant differences in demographic data were noted between pregnancies complicated by PE, PE+IUGR and IUGR.

	Normal Pregnancy	**Preeclampsia (PE)**	**Preeclampsia + IUGR**	**IUGR**
Patient number	29	15	16	24
Maternal age (years)	30.9 (27.5-34.30)	27.9 (25.0-30.0)	27.8 (22.3-35.5)	31.0 (26.0-37.0)
Primigravida %	21%	53%*	63%*	66%*
Gestational age At venesection	34.9 (32.2-39.1)	31.9* (30.3-34.1)	31.9* (27.7-34.8)	33.9* (31.2-37.0)
Gestational age At delivery	39.1 (38.1-40.1)	31.9* (30.3-34.1)	31.9* (27.7-34.8)	33.9* (31.2-37.0)
Birth weight (g)	3452 (3052-3850)	1727 * (1340-2275)	1392* (745-1918)	1627* (1070-2206)
Aspirin treatment	0/29	1/15	2/16	1/24
Smoking	1/29	2/15	1/16	2/24
Antenatal steroids	0/29	9/15	13/16	10/24
Antihypertensive	0/29	11/15	9/16	0/24

Table 4.1 Clinical characteristics of the study population.

Results are presented as mean ± standard deviation unless otherwise specified.

* Significantly different to normal pregnancies $p < 0.05$.

4.5.2 Circulating plasma pro-angiogenic and anti-angiogenic factors: individual factor levels

The plasma pro-angiogenic factor levels (PlGF) and anti-angiogenic soluble receptor levels sFlt-1, sKDR and sEng are listed in Table 4.2.

4.5.2.1 Maternal VEGF levels

The free or unbound plasma VEGF levels were below the sensitivity of the assay in preliminary ELISA experiments. Further testing of samples was not performed for VEGF.

	Group			
	Normal Median (Interquartile range) N = 29	**PE only** Median (Interquartile range) N = 15	**PE & IUGR** Median (Interquartile range) N = 16	**IUGR only** Median (Interquartile range) N = 24
Maternal plasma sFlt-1 level (pg/ml)	2798.0 (1800,5732)	8672* (8453,8904)	8934.0* (8706,9175)	7023* (5848,10093)
Fetal/Cord blood sFlt-1 level (pg/ml)	161.0 (115.0,220.0)	322.0 (198,719)	415.5 (277,562)	288 (159,460)
Maternal sKDR level (pg/ml)	9744 (7337.0,11691.0)	6229* (4856,7986)	5466* (4519,6867)	6441* (4165,7269)
Maternal plasma sEng level (ng/ml)	4.5 (3.7,5.9)	29.4* (16,46)	97*# (50,134)	34* (11,55)
Maternal plasma PlGF level (pg/ml)	226.0 (128.0,458.0)	46.0* (19,57)	31* (8,47)	30* (7,40)
sFlt-1 / PlGF	11.76 (4.1,39.8)	187.70* (139,468)	289* (192,1152)	281* (156,1088)
sKDR / sFlt-1	3.88 (1.4,5.8)	0.72* (0.56,0.90)	0.61* (0.53,0.79)	0.88* (0.54,1.16)
sKDR/PlGF	42.09 (23.70,63.17)	169.09* (91,247)	190* (107,793)	241* (104,671)
PIGF / sEng	42.80 (17.9,158.9)	2.20* (0.53,2.43)	0.27*# (0.09,0.58)	0.58* (0.37,2.41)
sKDR / sEng	2132.20 (1276,3183)	211.65 (76,449)	60*# (47,93)	164* (87,531)
sFlt-1 / sEng	513.84 (349.5,771.4)	292.69 (189,554)	87*#† (65,179)	197* (136,560)

Table 4.2 Maternal plasma angiogenic factors and their receptor levels.

Results are presented as median ± interquartile range for each continuous variable unless otherwise specified. Ratios are presented as log transformed values.* Significantly different to normal pregnancies, $p < 05$. # Significantly different from PE. † Significantly different between pathologic groups PE, PE+IUGR and IUGR. $p < 0.05$.

4.5.2.2 Maternal plasma PlGF levels

Patients with preeclampsia (PE), intrauterine growth restriction (IUGR) and co-existing PE and IUGR had significantly reduced median plasma PlGF levels as compared to the normal pregnancies. There were no significant differences in median plasma PlGF concentrations between the complicated pregnancies PE, PE+IUGR and IUGR (Figure 4.1).

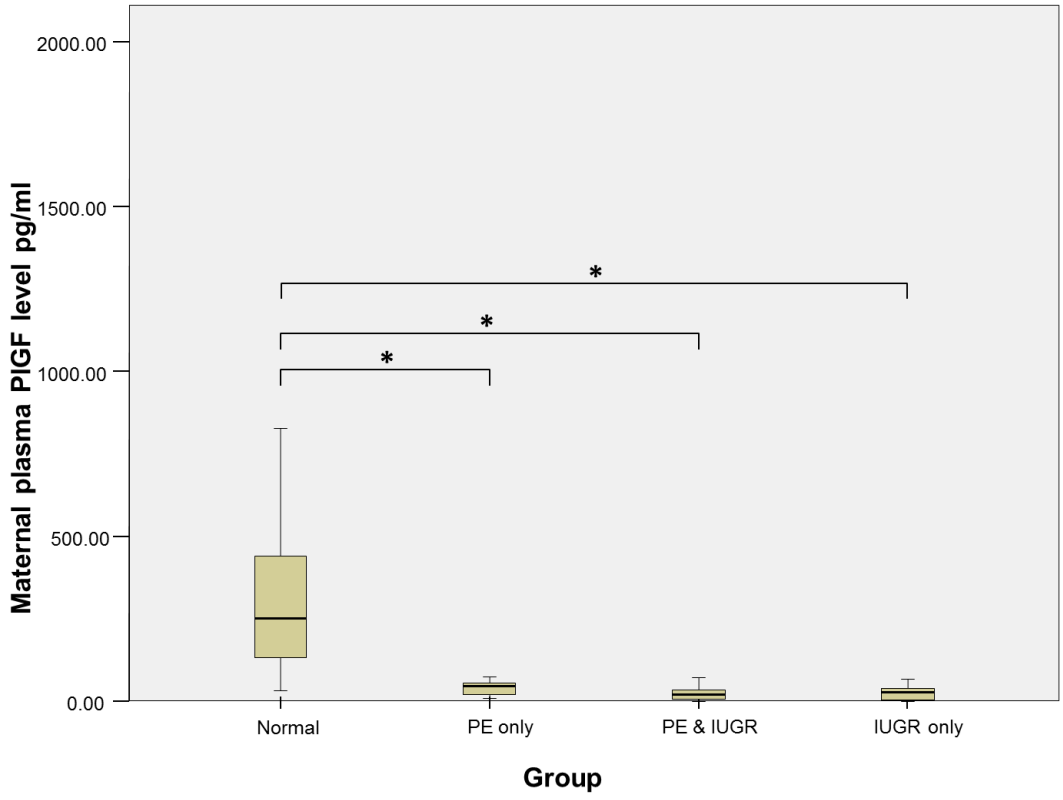

Figure 4.1 Maternal plasma PlGF levels by group.

Statistically significant difference between groups * $p < 0.001$.

4.5.2.3 Maternal plasma sFlt-1 levels

Patients with preeclampsia (PE), intrauterine growth restriction (IUGR) and co-existing PE and IUGR had elevated median plasma sFlt-1 levels as compared to the normal pregnancies. There were no significant differences in median plasma sFlt-1 concentrations between the complicated pregnancies PE, PE+IUGR and IUGR. * $p < 0.001$ (Figure 4.2).

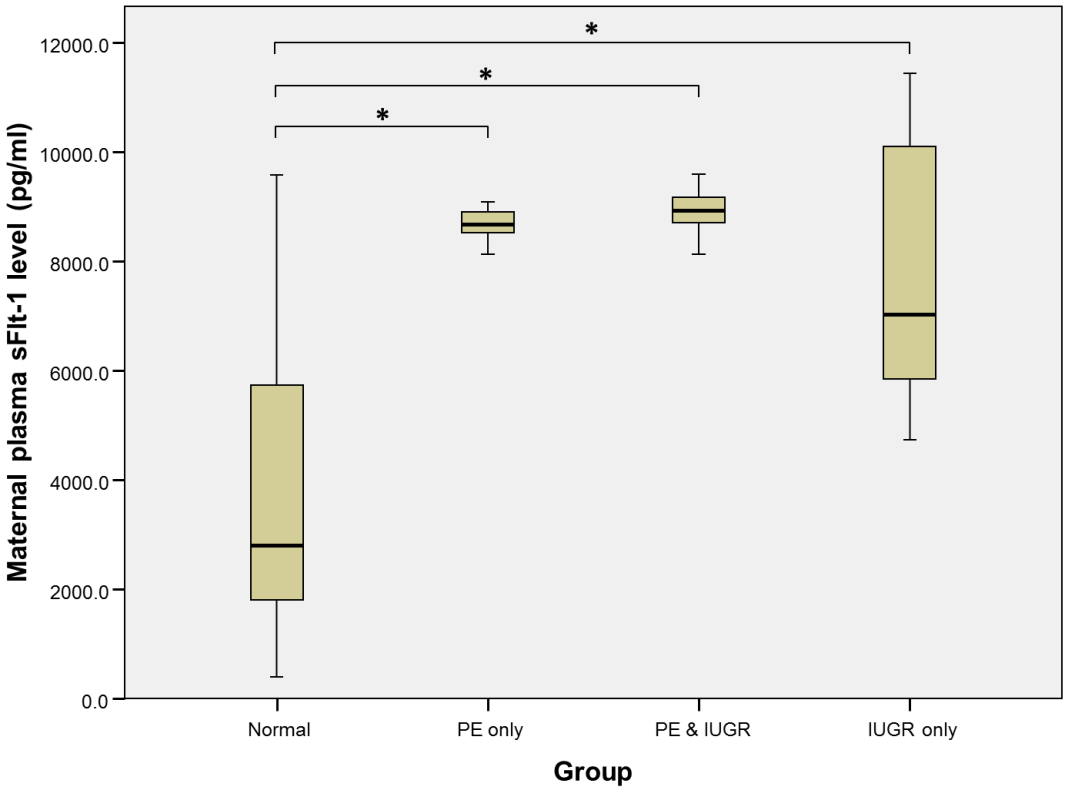

Figure 4.2. Maternal plasma sFlt-1 by group.

Statistically significant difference between groups * $p < 0.001$.

4.5.2.4 Maternal plasma sKDR levels

Patients with preeclampsia (PE), intrauterine growth restriction (IUGR) and co-existing PE and IUGR had reduced median plasma sKDR levels as compared to the normal pregnancies. There were no significant differences in median plasma KDR concentration between the complicated pregnancies PE, PE+IUGR and IUGR. * $p < 0.001$ (Figure 4.3).

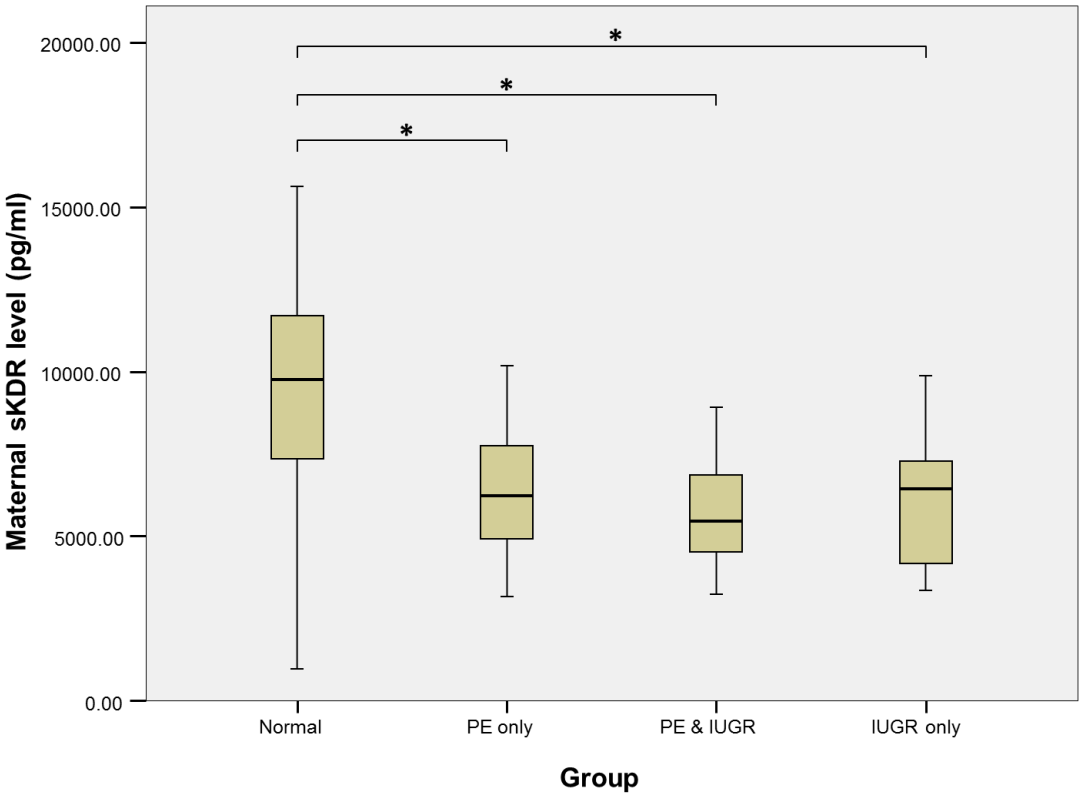

Figure 4.3 Maternal plasma sKDR levels by clinical group.

Statistically significant difference between groups * $p < 0.001$.

4.5.2.5 Maternal plasma sEndoglin

The sEndoglin levels were significantly higher in preeclampsia and intrauterine fetal growth restriction compared to normal pregnancy. Significant differences were seen between the complicated pregnancies with sEng levels higher in PE+IUGR as compared to PE or IUGR alone * $p < 0.05$, ** $p = 0.001$ (Figure 4.4).

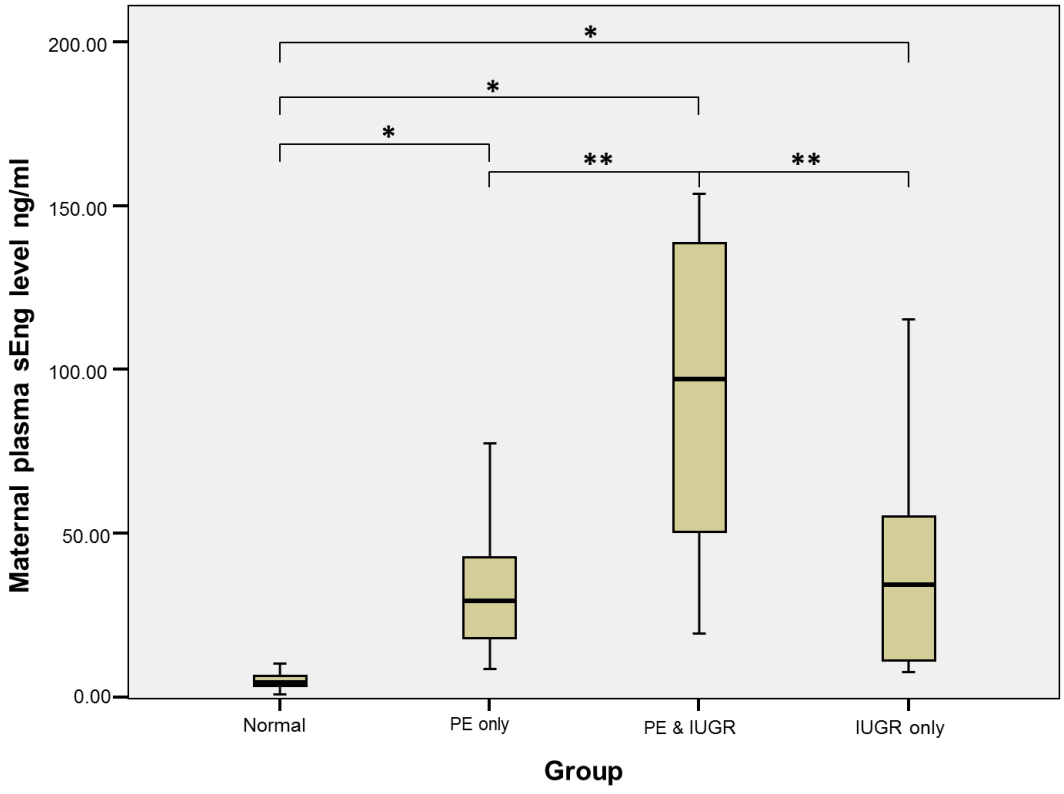

Figure 4.4 Maternal plasma sEndoglin levels by clinical group. Statistically significant difference between groups * $p < 0.001$.

4.5.3 Correlation between the levels of circulating receptors sFlt-1 and sKDR by clinical group

The data were tested for a correlation between plasma sFlt-1 and sKDR levels using Spearman's correlation coefficient (Figure 4.5). As both of these are anti-angiogenic factors, a correlation was sought between these two factors.

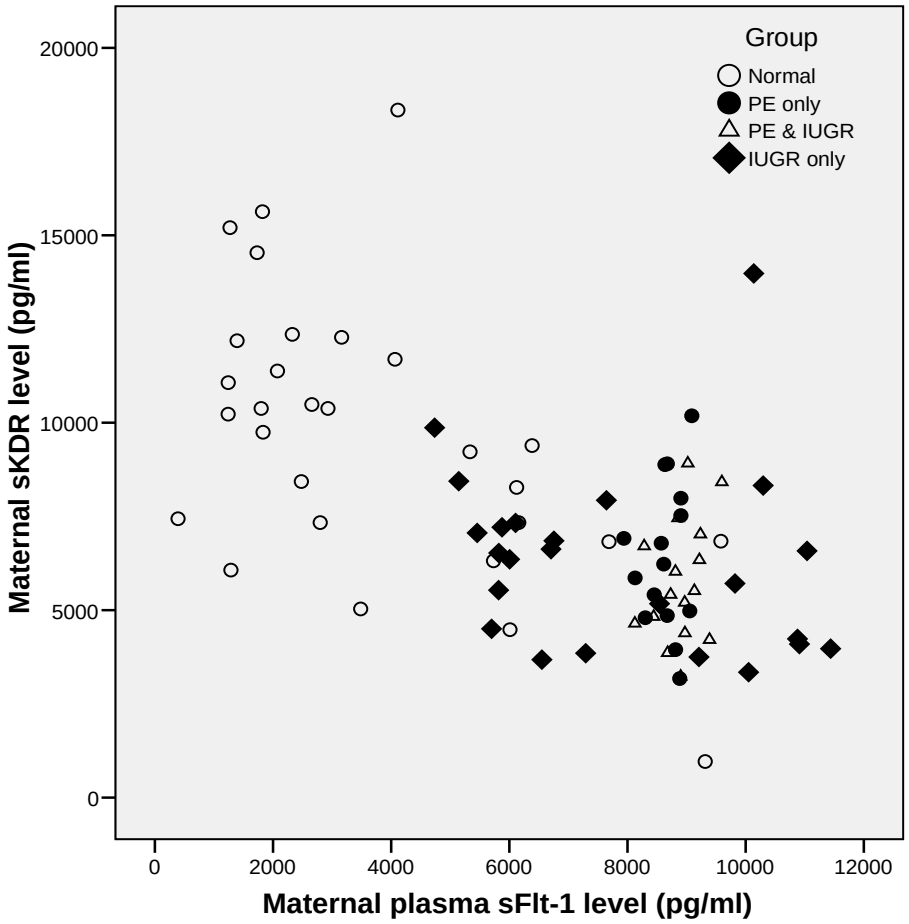

Figure 4.5 Correlation of plasma sFlt-1 and sKDR by clinical group.

A significant negative correlation was demonstrated ($r = -0.54$) at a significance level $p < 0.01$ level (2-tailed).

4.5.4 Variation in angiogenic factors and their receptor levels with gestational age according to clinical group

The level of association between gestational age and each angiogenic marker of interest was further evaluated using Spearman rank correlation. The results are displayed below in Figures 4.6 - 4.9.

4.5.4.1 sFlt-1 levels by gestational age and clinical group

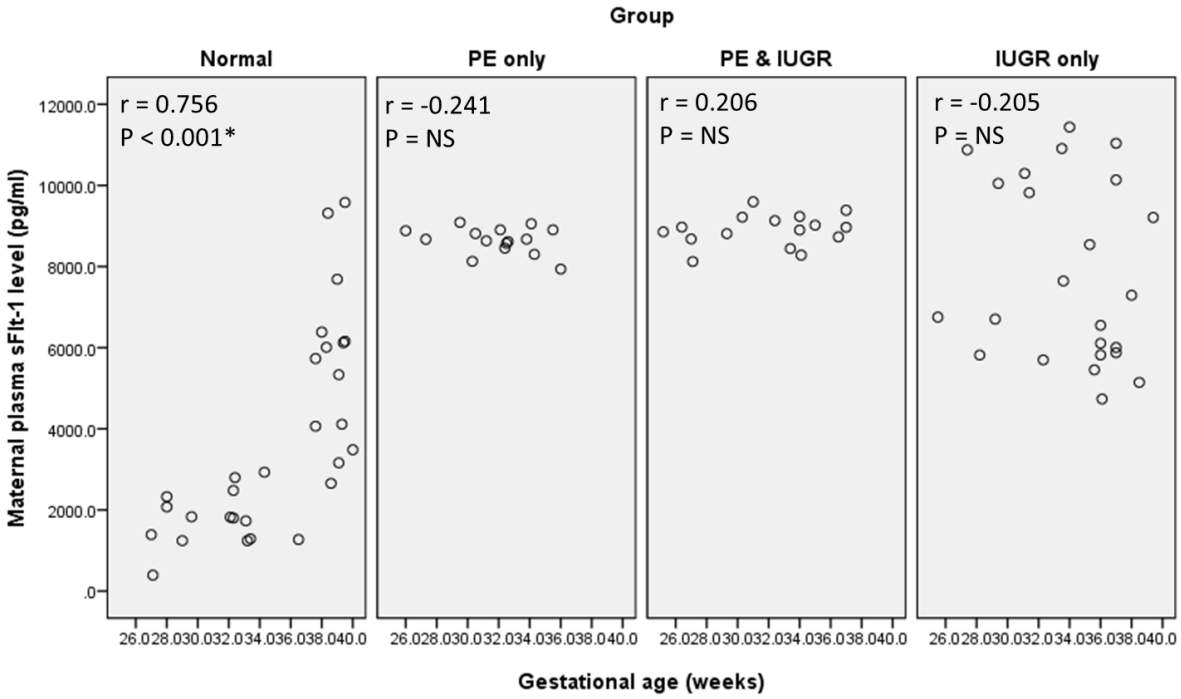

Figure 4.6 Spearman rank correlation for the level of association between gestational age and sFlt-1 levels for each clinical group.

The plasma concentrations of sFlt-1 in normal pregnancies were higher in later gestations. No significant association demonstrated between gestational age and sFlt-1 levels in preeclampsia PE, PE+IUGR or IUGR. This could be explained by the fact that the PE and IUGR cases in the study had established pathology, even at an earlier gestation, with already

elevated levels of sFlt-1 and sEng. * = statistically significant $p < 0.05$. NS = Not statistically significant.

4.5.4.2 sKDR levels by gestational age and clinical group

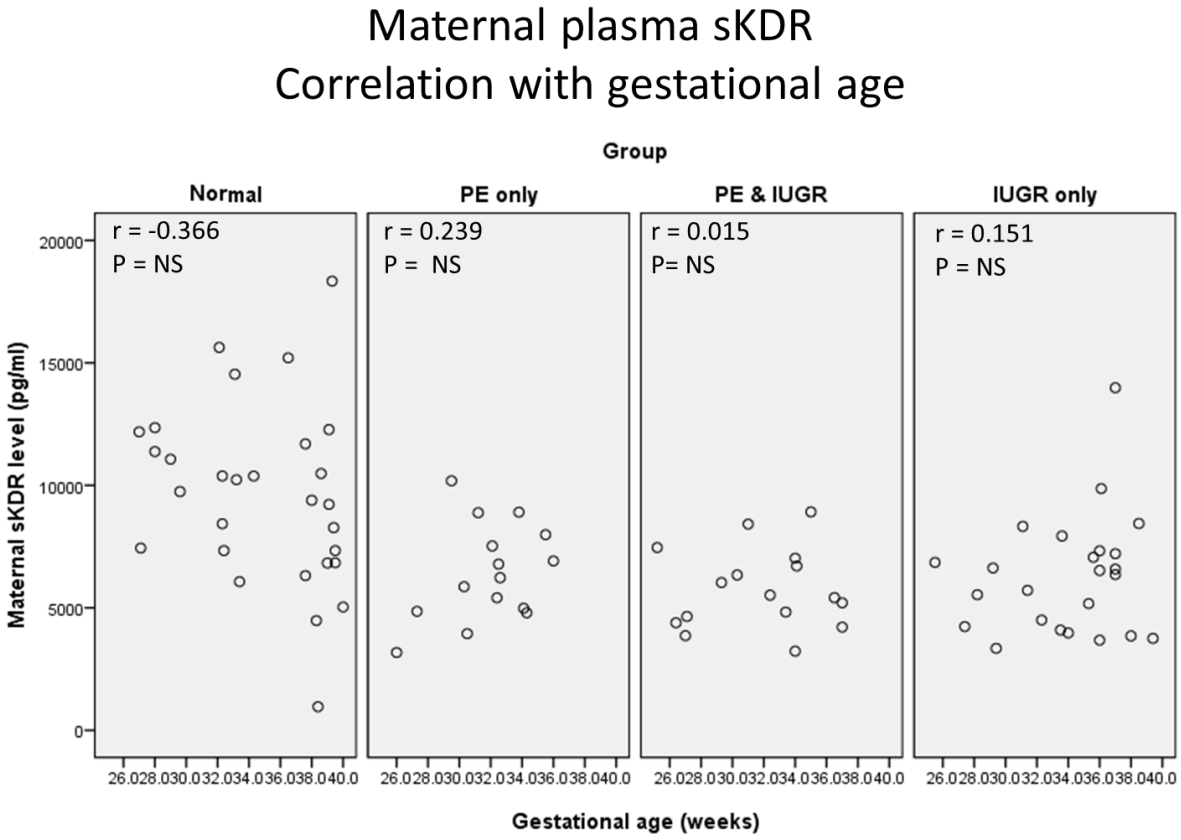

Figure 4.7 Spearman rank correlation for the level of association between gestational age and sKDR levels for each clinical group.

* = statistically significant $p < 0.05$. NS = Not statistically significant. There was no significant association demonstrated between gestational age and sKDR levels in any of the clinical groups.

4.5.4.3 sEng levels by gestational age and clinical group

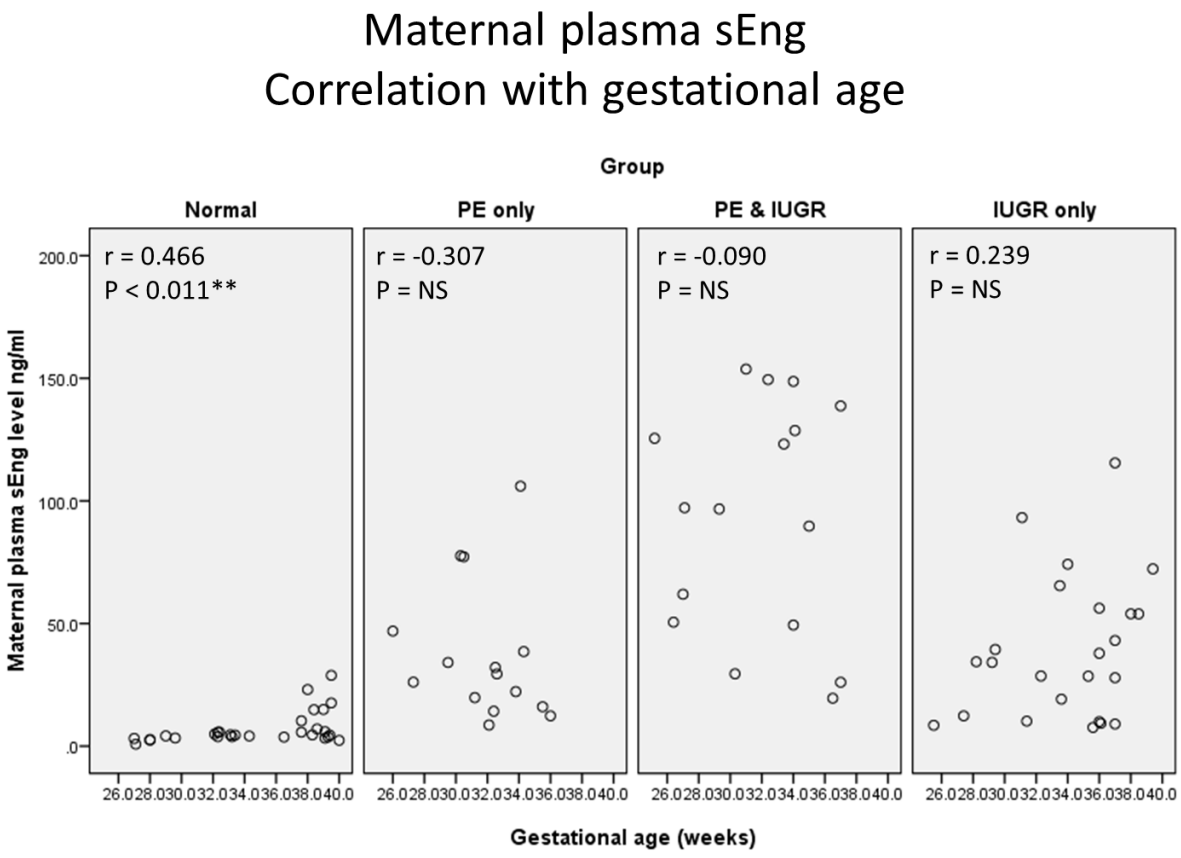

Figure 4.8 Spearman rank correlation for the level of association between gestational age and sEng levels for each clinical group.

A significant association noted between gestational age and sEng for normal pregnancy (r = 0.466, ** $p < 0.05$). There was no significant association (NS) demonstrated between gestational age and sEng levels in PE, IUGR or PE+IUGR.

4.5.4.4 PlGF levels by gestational age and clinical group

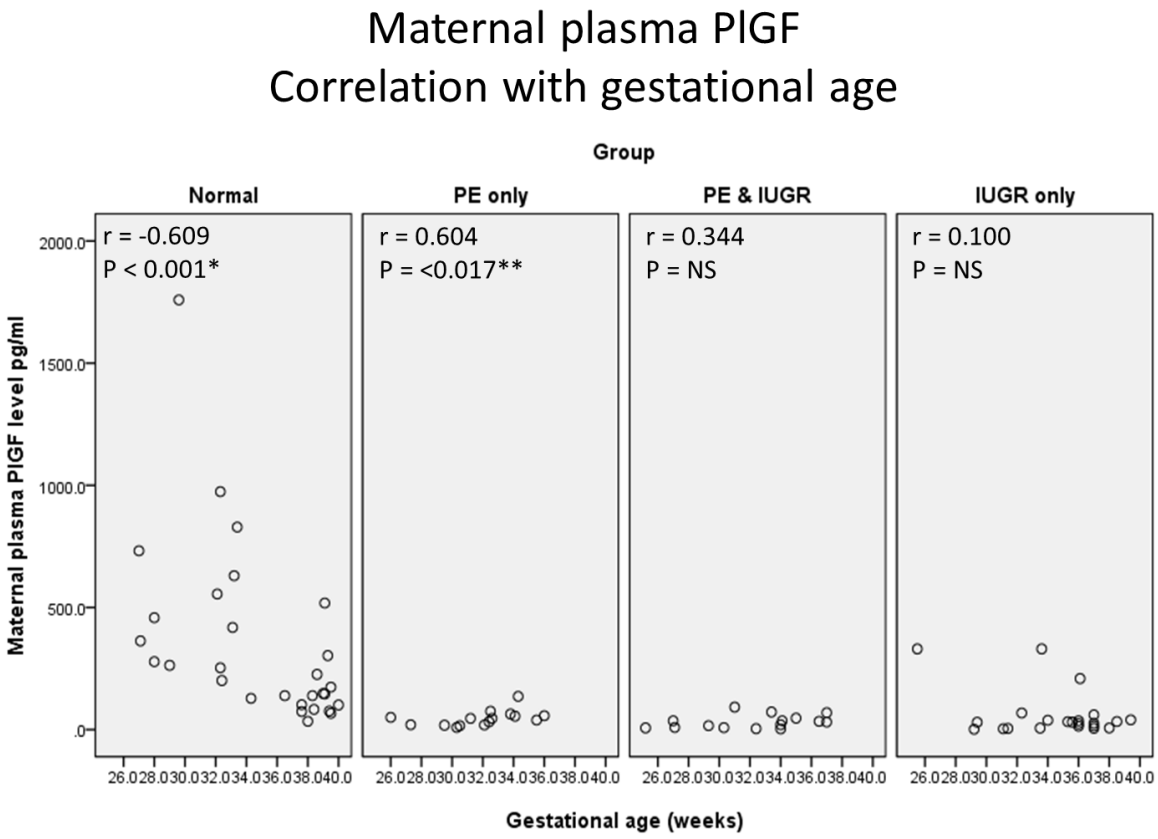

Figure 4.9 Spearman rank correlation for the level of association between gestational age and PlGF levels for each clinical group.

A significant negative association noted between gestational age and plasma PlGF levels for normal pregnancy ($r = -0.609$, * $p < 0.001$). A positive association was noted between gestational age and PE ($r = 0.604$, * $p < 0.05$). No significant association was demonstrated between gestational age and PlGF levels in PE+IUGR or IUGR.

4.3.5 Angiogenic factors and their receptors: Ratios between pro and anti-angiogenic factors

The ratios between various angiogenic and anti-angiogenic factors in the four clinical groups are described in Table 4.2 and Figures 4.10- 4.13. The results are displayed as median ± interquartiles. For all figures, the significant differences from normal pregnancy are identified as *p < 0.05.

For sFlt-1/PlGF (Figure 4.10), sKDR/sFlt-1, sKDR/PlGF and sFlt-1*sEng//PlGF (Figure 4.13), significant differences were seen between normal pregnancy and the pathological pregnancies. No significant differences seen between the pathological pregnancies PE, PE+IUGR and IUGR * p < 0.001.

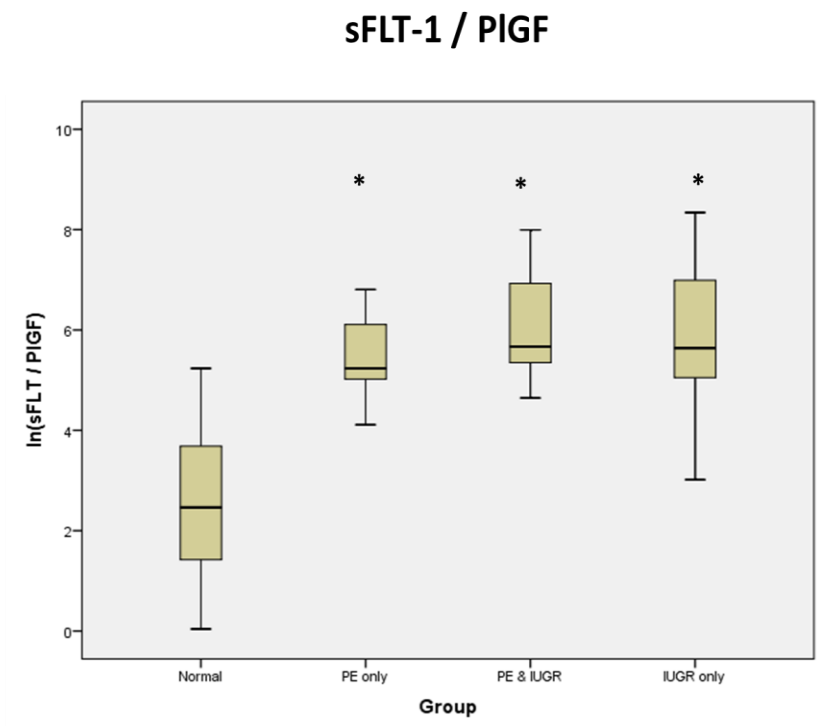

Figure 4.10 Maternal plasma sFlt-1/PlGF ratio by clinical group.

For the ratios PlGF/sEng (Figure 4.11) and sKDR/sEng, there were significant differences seen between normal pregnancy and pathological pregnancies (* $p < 0.001$) as well as between PE+IUGR and IUGR only (** $p < 0.01$). No difference was seen between preeclampsia and IUGR.

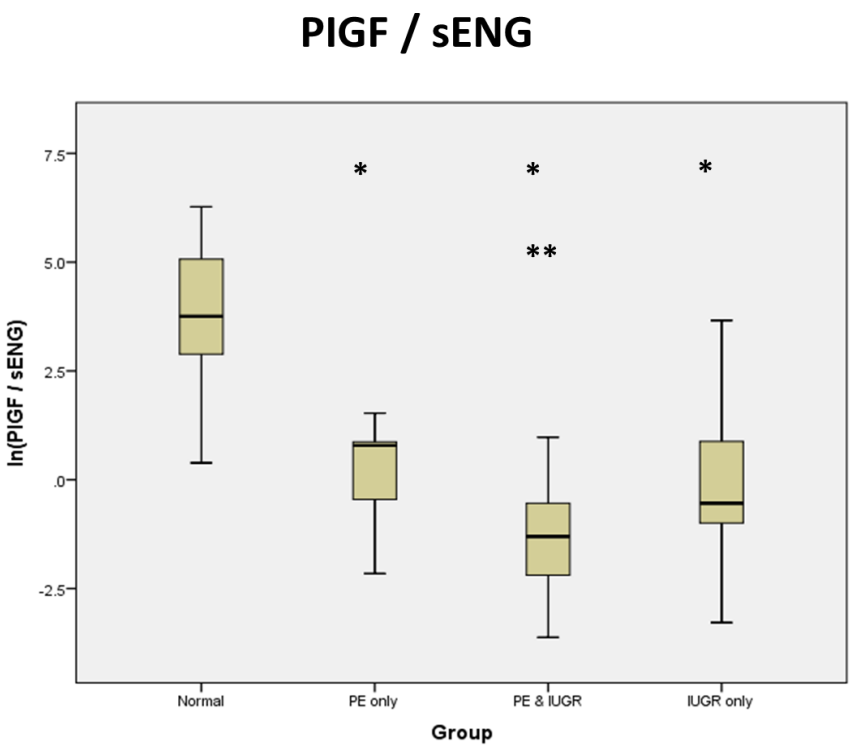

Figure 4.11 Maternal plasma PlGF/sEng ratio by clinical group

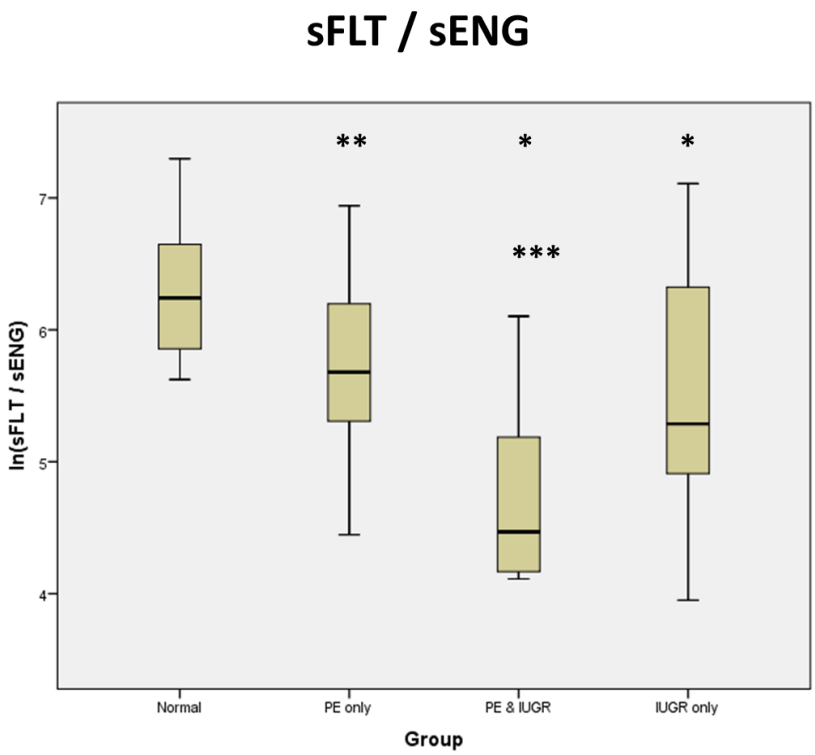

Figure 4.12 Maternal plasma sFlt-1/ sEng ratio by clinical group.

For the ratio sFlt-1/sEng (Figure 4.12) significant differences were seen between normal pregnancy and PE (**p <0.01), PE+IUGR and IUGR (* p < 0.001). No difference was seen between preeclampsia or intrauterine fetal growth restriction groups while the biomarker ratio of the pregnancies complicated by preeclampsia and intrauterine growth restriction (PE+IUGR) is different to preeclampsia or IUGR only (***p <0.001)

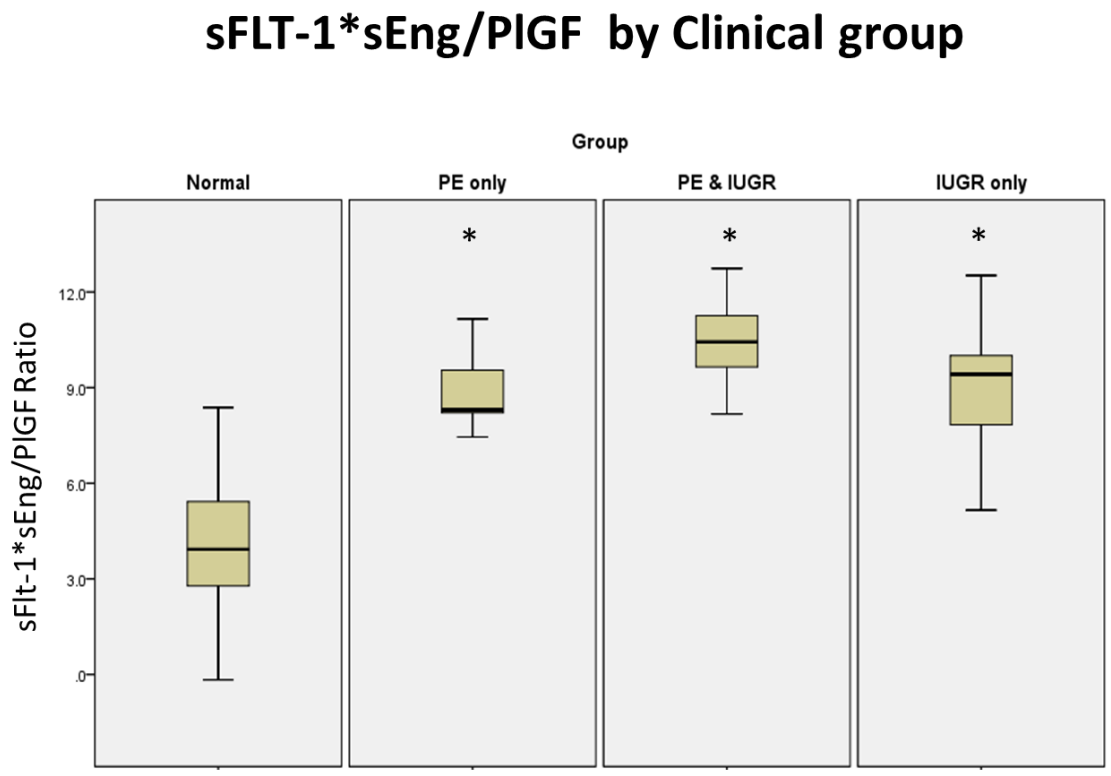

Figure 4.13 Maternal plasma sFlt-1*sEng/PlGF ratio by clinical group.

Significant differences were seen between normal pregnancy and the pathological pregnancies. No significant difference was seen between the pathological pregnancies PE, PE+IUGR and IUGR.

* $p < 0.001$.

4.5.6 Circulating angiogenic and anti-angiogenic factor levels in identifying pregnancies complicated by preeclampsia and/or intrauterine fetal growth restriction.

The predictive value of the circulating angiogenic factors, anti-angiogenic factors and their ratios to distinguish between normal vs pathological pregnancies complicated by preeclampsia, preeclampsia with fetal growth restriction or fetal growth restriction was assessed using ROC graphs. The relevant ROC graphs are displayed in Figures 4.14-4.16.

Table 4.3 summarizes the sensitivity and specificity of each individual or combination of factors as assessed by the area under the ROC graph.

4.5.6.1 Individual biomarkers

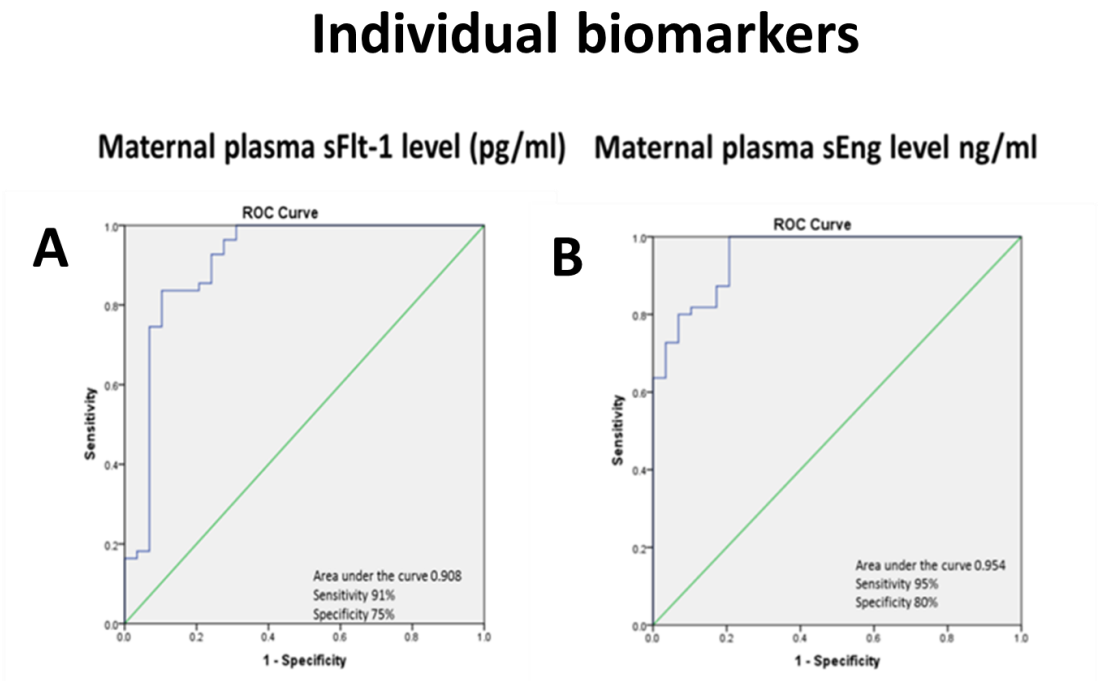

Figure 4.14 ROC curves for anti-angiogenic factors as individual biomarkers.

A = Maternal plasma sFlt-1 level (pg/ml). B = sEng. AUC represent sensitivity and specificity in detecting normal vs pathological pregnancies.

4.5.6.2 Composite markers in identifying pathological pregnancies

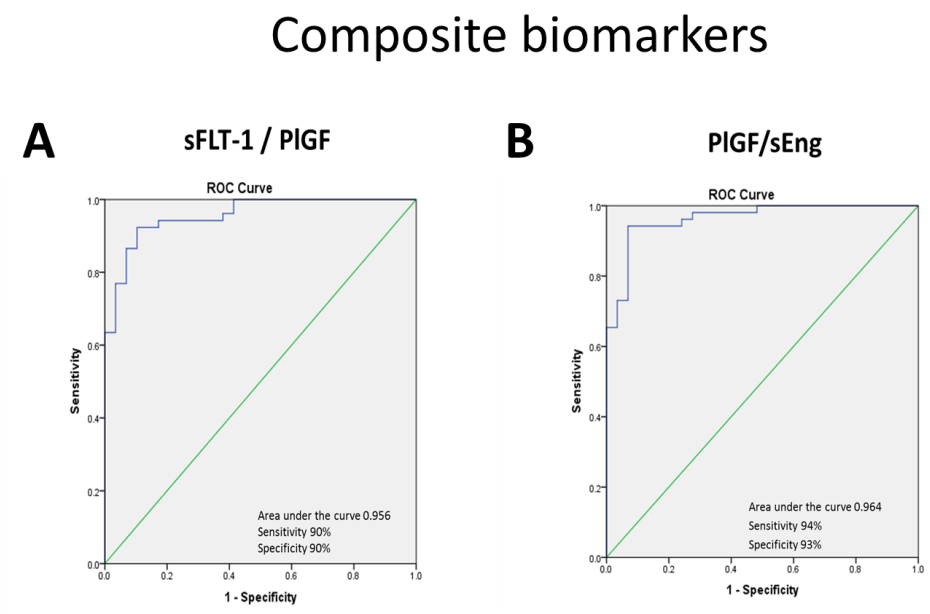

Figure 4.15 ROC curves for combination of pro and anti-angiogenic factors as biomarkers.

A = sFlt-1/PlGF, B = PlGF/sEng, AUC displayed for sensitivity and specificity in detecting normal vs pathological pregnancies.

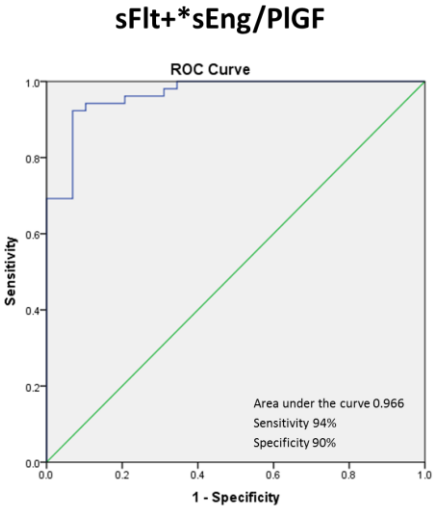

Figure 4.16 Maternal plasma sFlt-1*sEng/PlGF Ratio. Sensitivity and specificity in detecting normal vs pathological pregnancies.

Summary of screening performance using ROC curves for Normal vs Pathological pregnancies

Maternal marker	Area under curve	Sensitivity	Specificity
SFlt-1	0.908	91%	75%
sKDR	0.813	90%	59%
sEng	0.954	95%	80%
PlGF	**0.941**	**90%**	**90%**
sFlt-1/PlGF	**0.956**	**90%**	**90%**
sKDR/PlGF	0.901	90%	76%
sEng/PlGF	**0.964**	**94%**	**93%**
sKDR/sFlt-1	0.907	92%	79%
sKDR/sEng	0.940	91%	79%
sFlt-1/sEng	0.814	90%	25%
sFLT-1*sEng/PlGF	**0.966**	**94%**	**90%**
sFLT-1*sEng/PlGF ^{2}	**0.966**	**94%**	**90%**

Table 4.3 Summary of screening performance using ROC curves of individual biomarkers and their ratios for identification of pregnancies complicated by preeclampsia and/or fetal growth restriction as different to normal pregnancies.

The ratios with the best performance in sensitivity and specificity are highlighted in red.

^{2} = number squared of the given value

4.5.7 Angiogenic factors in the fetal circulation

4.5.7.1 Umbilical artery sFlt-1 levels

The levels of fetal plasma Flt-1 levels from umbilical artery cord blood are presented in Figure 4.17, comparison with maternal plasma levels have been presented in Figures 4.18.

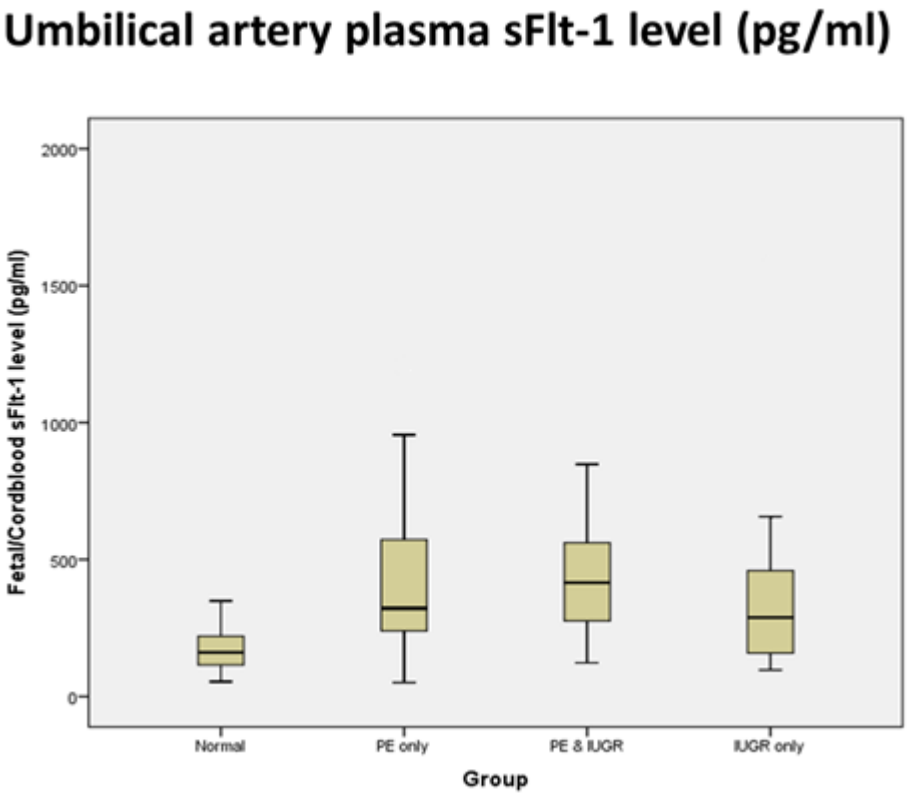

Figure 4.17 Fetal umbilical artery plasma sFlt-1 levels.

No significant differences were seen between the clinical groups normal pregnancy, PE, PE+IUGR and IUGR.

4.5.7.2 Comparison between maternal and fetal levels of sFlt-1 levels

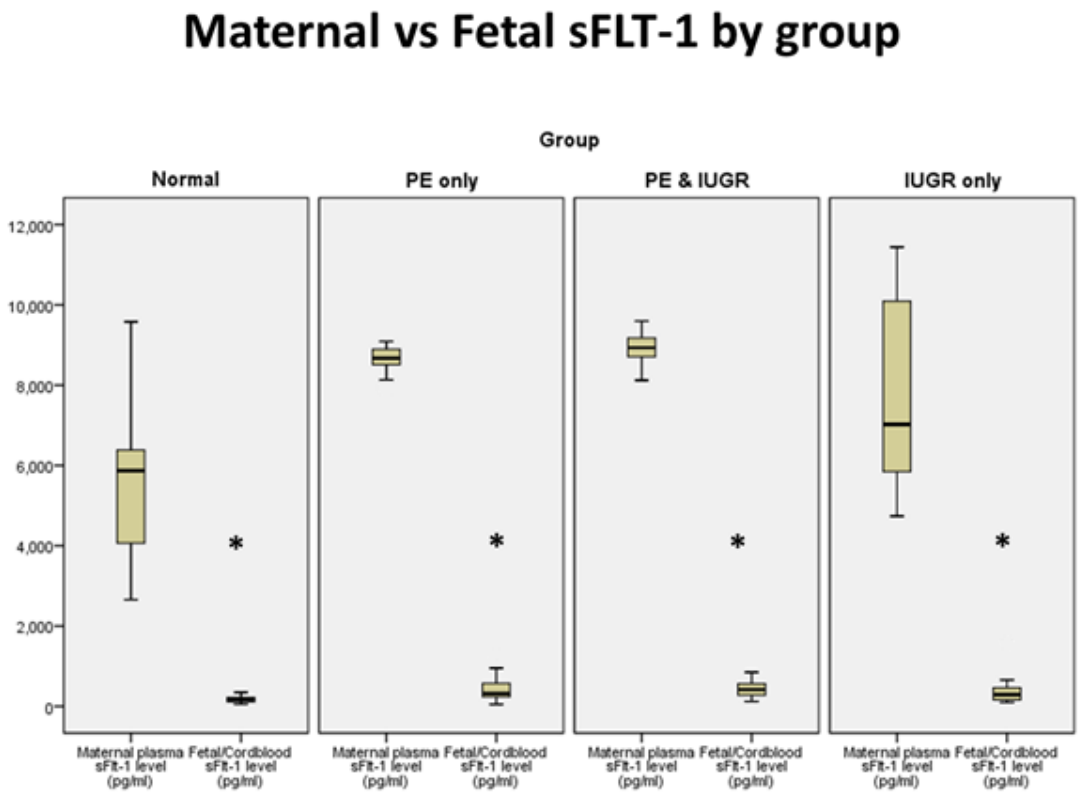

Figure 4.18 Comparison between maternal vs fetal plasma sFlt-1 levels using paired t tests. The fetal levels were significantly lower than the maternal levels for each clinical group including normal pregnancies. * $p < 0.001$.

4.6 Discussion

4.6.1 Individual angiogenic factors

In the current research, circulating levels of pro and anti-angiogenic factors were measured by ELISA in maternal and fetal (umbilical artery) plasma, and the levels were compared between four clinical groups of normal pregnancy, preeclampsia, preeclampsia with fetal growth restriction and isolated fetal growth restriction. The results indicate that while anti-angiogenic receptor levels of sFlt-1and sEndoglin appear to be significantly elevated, the anti-angiogenic sKDR levels were reduced in the pathological pregnancies as compared to normal pregnancy. The pro-angiogenic factor PlGF was significantly lower in the pathological groups. No significant differences in pro or anti-angiogenic factor level were observed between the pathological pregnancies except for with sEndoglin.

It is now well established that serum/plasma levels of sFlt-1, free PlGF, and free VEGF are altered in women with clinical preeclampsia (155, 209, 395). Higher levels of sFlt-1 (155, 178, 215, 395, 396), elevated sEng (170, 188) and lower levels of PlGF (155) and VEGF have been documented in "end-point specimens" after the onset of preeclampsia as compared to normal pregnancy controls (215). In a landmark study, Maynard *et al* (155) showed that preeclampsia is associated with elevated circulating sFlt-1 protein. This work also suggested that sFlt-1 acts through its antagonism of both VEGF and PlGF.

In a similar study, Venkatesha et al (170) used adenoviral expression of sEng and sFlt-1, alone or in combination in pregnant rats to induce hypertension and proteinuria in pregnant rats. Proteinuria was modest in sEng treated rats, but severe in the sFlt1-treated group. The sFlt1+sEng group showed nephrotic range proteinuria, severe hypertension and biochemical evidence of HELLP syndrome (elevated lactate dehydrogenase and aspartate aminotransferase

and decreased platelet counts). Fetal growth restriction was observed in the litters born to the sFlt1+sEng group, probably related to the placental vascular ischemia and damage. These findings are consistent with these studies, with PE+IUGR group associated with elevated sFlt-1 and higher levels of sEng than the other pathological groups.

Accumulated evidence to date on angiogenic factors suggests that the balance between VEGF and PlGF as well as their receptors Flt-1 and KDR is important for effective vasculogenesis, angiogenesis, and placental development during pregnancy (178). The anti-angiogenic effect of sFlt-1 and sEndoglin has been suggested as the basis for the endothelial dysfunction and the clinical presentation of preeclampsia (178). The mechanism for initiating increased expression of membrane bound Flt-1 or sFlt-1 has not yet been elucidated but has been postulated to be in response to placental hypoxia (397).

The findings on the angiogenic factor profile of this study parallels published data to date showing elevated sFlt-1 and sEng and low PlGF in pregnancies complicated by preeclampsia in comparison to normal pregnancies. This data clearly demonstrates that the deranged angiogenic profile in isolated/idiopathic fetal growth restriction, as documented with elevated sFlt-1, sEng and low PlGF, is similar to pregnancies complicated by preeclampsia and preeclampsia with fetal growth restriction. The presented data are concordant with recently published results showing that patients destined to deliver a small for gestational age (SGA) neonate had higher plasma concentrations of sEng throughout gestation than those with normal pregnancies (230) but discordant with the published angiogenic factor profiles describing no significant differences in the plasma concentrations of sFlt-1 between patients destined to deliver an SGA neonate and those with normal pregnancies (230). A previous study on maternal plasma concentrations of soluble Endoglin in pregnancies with intrauterine growth restriction have shown similar results to the current data showing that the sEng levels

are elevated in SGA/IUGR patients (239). The levels of sEng in IUGR have been shown to be less than in preeclampsia in a published study (230). These discrepancies in results may be explained by the patient selection criteria where fetal growth restriction have been variably defined as SGA (birth weight $<10^{th}$ centile) only or SGA with elevated umbilical artery Doppler resistance in the current study. Population and methodological differences may have also contributed. The inclusion of elevated umbilical artery Doppler resistance in the selection criteria of this ensured that IUGR of placental origin was included and constitutionally small fetuses and SGA due to other etiologies such as infection were excluded.

PlGF is pro-angiogenic member of the VEGF family, with substantially higher plasma concentrations in pregnant women (248). Abundant PlGF is shown to be released by activated endothelial cells (141) while hypoxia is thought to reduce PlGF production (182). Chronic uteroplacental ischemia may account for the low maternal plasma concentration of PlGF in pregnancies complicated by preeclampsia or a SGA neonate (230). The measured PlGF levels in normal pregnancies show a trend towards higher values earlier in gestation and with a decline in late gestation. The pregnancies complicated by preeclampsia, preeclampsia with fetal growth restriction and idiopathic fetal growth restriction all show extremely reduced levels of PlGF.

The key observation of this study is that changes in the concentrations of pro-angiogenic (PlGF) and anti-angiogenic factors (sEng and sFlt-1) are similar in pregnancies complicated by preeclampsia, preeclampsia with intrauterine fetal growth restriction and idiopathic fetal growth restriction. The angiogenic profiles in the normal uncomplicated pregnancies were significantly different to the pathological pregnancies, but each angiogenic factor profile showed a trend towards the pathological pattern with advancing gestation. The findings

suggest that the placental disease present in preeclampsia and intrauterine growth restriction represents an accelerated process of physiological changes that may happen in normal pregnancy with advanced gestation. The only demonstrable difference between pathological pregnancy groups was an elevated sEng and elevated ratios incorporating sEng in the pregnancies complicated by preeclampsia and fetal growth restriction. This is in contrast to previously published data showing elevated sEng and decreased PlGF, but no demonstrable increased in sFlt-1 in small for gestational age fetuses (209, 230).

Transforming Growth Factor-β1 (TGF-β1) induces vasorelaxation through activation of eNOS by triggering de-phosphorylation of Thr495, providing a novel mechanism for endothelium-dependent vasoregulation (209). sEng interferes with TGF-β1 receptor binding and downstream signaling in endothelial cells, and attenuates eNOS activation. It has been proposed that the contributions of sEng and sFlt1 to the pathogenesis of maternal preeclampsia are, at least in part, related to their inhibition of VEGF and TGF-β1 stimulation of endothelial dependent NO activation and vasomotor effects (209). It has been speculated that sEng is produced by the placenta as a compensatory mechanism to limit the effects of cell surface Endoglin. In preeclampsia, excessive production of cell surface Endoglin would lead to increased sEng in the maternal circulation, which in turn may be responsible for the clinical manifestations of preeclampsia (209).

Membrane bound KDR is known to be the pro-angiogenic receptor for VEGF, mediating most of its actions and upregulated by VEGF (398). Its soluble receptor sKDR however has been shown to have anti-angiogenic properties. Few studies to date such as have explored the role of sKDR in pregnancy complications, showing a reduced level in both preeclampsia and fetal growth restriction (222, 399). This data confirms the previously documented pattern of low KDR in preeclampsia, preeclampsia with fetal growth restriction and idiopathic fetal

growth restriction of placental origin. The mechanism resulting in reduced sKDR is unclear with a possible explanation being the reduced bioavailability of VEGF to up-regulate KDR due to the blocking effect of the elevated sFlt-1 in these conditions (24). The binding of VEGF by sFlt-1 can lead to low free VEGF in conditions of elevated sFlt-1.

The levels of free VEGF in the preliminary optimization steps were below the threshold values for the test (ELISA kit, R&D Systems). Published data from several authors reported increased systemic VEGF levels in women with preeclampsia in some studies (210, 400, 401) with decreased levels in others (155, 195). This discrepancy can be explained by the fact that the studies showing increased VEGF levels tested the total circulating VEGF using radioimmunoassay or non-R&D ELISA systems while a reduced VEGF was documented by studies using commercially available ELISA kits testing free VEGF only.

4.6.2 Ratio of angiogenic factors

In the current study maternal plasma angiogenic factors ratios were evaluated by clinical group. Significant differences were seen between normal pregnancy and the complicated pregnancies, similar to the individual markers in the pro/anti angiogenic marker ratios of sFlt-1/PlGF, sKDR/PlGF, sKDR/sFlt-1 and sFlt-1+sEng/PlGF. No significant differences were seen between the complicated pregnancies. Only ratios including sEng showed a statistically significant difference between the pathological groups with ratios sEng/PlGF, sKDR/sEng and sFlt-1/sEng higher in the preeclampsia with fetal growth restriction as compared to the preeclampsia or fetal growth restriction alone. These data suggests sEng to be a valuable screening or diagnostic marker of placental disease.

In a study by Levine et al (188), angiogenic biomarkers and ratios including sEng were assessed for the predictive value of developing preeclampsia. Circulating soluble Endoglin

levels increased markedly beginning 2 to 3 months before the onset of preeclampsia. An increased level of soluble Endoglin was usually accompanied by an increased ratio of sFlt-1:PlGF. The risk of preeclampsia was greatest among women in the highest quartile of the control distributions for both biomarkers but not for either biomarker alone. They concluded that rising circulating levels of soluble Endoglin and ratios of sFlt-1:PlGF herald the onset of preeclampsia. While the data presented here are concordant with the findings of Levine et al, that the circulating levels of sFlt-1 increases with gestation in normal pregnancy and earlier with preeclampsia, they also show that significantly elevated levels of sFlt-1 and low PlGF are also seen in pregnant women with isolated IUGR with no clinical evidence of preeclampsia.

4.6.3 Plasma levels with gestational age

The relationship of the biomarkers to gestational age was investigated in the four clinical groups. It is important to note that this analysis has been done on cross-sectional rather than longitudinal data. The sFlt-1 and sEndoglin levels in normal pregnancy increased with gestational age while the PlGF levels decreased. Among normal pregnant women, there was a positive correlation between plasma concentrations of sFlt-1 and advancing gestational age. In comparison, there was no association detected between plasma sFlt-1 levels and gestational age in preeclampsia, intrauterine fetal growth restriction or a combination of these pregnancy complications. This could be explained by the fact that the PE and IUGR cases in the study had established pathology, even at an earlier gestation, with already elevated levels of sFlt-1 and sEng as compared to the studies published that show increasing levels of sFlt-1 approximately 5 weeks prior to established disease (215).

A similar correlation was seen in the plasma sEndoglin levels with gestational age, in the four studied clinical groups. In contrast, the plasma PlGF levels showed a significantly negative

correlation between plasma concentrations and gestational age in normal pregnancy while only a mildly positive correlation was detected in preeclampsia, and no correlation in IUGR or PE+IUGR. sKDR did not reveal a gestation related change in any clinical group. Previous studies have suggested that circulating sFlt-1 and sEndoglin rise with normal pregnancy in later gestation to counteract increased VEGF production in the increasing placental mass. The low levels and the anti-angiogenic properties of KDR are less likely to play such a role.

4.6.4 Pro and anti-angiogenic factors as biomarkers in identifying normal vs pathological pregnancies complicated with preeclampsia and/or intrauterine fetal growth restriction.

Many studies to date have identified sFlt-1, PlGF and sEng as biomarkers for pregnancy complications of preeclampsia, intrauterine fetal growth restriction, placental abruption, mirror syndrome and fetal demise (230, 402-404).

The angiogenic factor levels from the current study were evaluated for their effectiveness in discriminating normal from complicated pregnancies. As the angiogenic profiles of preeclampsia, preeclampsia with fetal growth restriction and idiopathic fetal growth restriction were similar, they were grouped together as pathological pregnancies as opposed to uncomplicated normal pregnancies. Individual factor levels and ratios of PlGF, sFlt-1 and sEng were assessed using receiver operating characteristics curves and calculation of sensitivity and specificity to identify the biomarkers. As illustrated in Figures 4.17-4.19 and Table 4.3, while individual markers sFlt-1 (91% sensitivity, 75% specificity, AUC 0.908) and sEng (95% sensitivity, 80% specificity, AUC 0.95) performed well, the composite ratios sEng/PlGF (94% sensitivity, 93% specificity, AUC 0.966) and sFlt-1*sEng/PlGF (94% sensitivity, 90% specificity, AUC 0.966) performed best as a diagnostic marker in distinguishing pathological pregnancies from normal pregnancies. These data are concordant

with previously published predictors of preeclampsia and fetal growth restriction (24, 178, 188, 215, 252, 256, 257) that supports the use of a composite marker at mid gestation using sFlt-1, sEng and PlGF in screening for pregnancy complications associated with placental disease. The ROC graph followed a similar pattern for normal vs preeclampsia and normal vs intrauterine fetal growth restriction, but the number of cases was inadequate to reach any conclusions. It is important to note that these data do not suggest that preeclampsia and fetal growth restriction can be identified separately to each other by using the given individual biomarkers or their ratios.

The circulating soluble Endoglin and sFlt-1 may both contribute to the clinical phenotypes of preeclampsia and fetal growth restriction. There is evidence suggesting that sFlt, PlGF as well as sEng may show elevated levels several weeks prior to the clinical onset of the disease (188, 215). Prospective longitudinal studies are needed to assess whether these biomarkers can be of value as a screening test to predict the imminent onset of clinical disease.

4.6.5 Fetal levels

This study also measured the levels of sFlt-1 in fetal umbilical artery. Plasma levels were significantly lower than maternal circulating levels and there was no demonstrable difference between normal and complicated pregnancies. This finding suggests that the fetal contribution to the overall levels of circulating sFlt-1 in the maternal circulation is negligible and unlikely to play a part in the pathogenesis of preeclampsia. Since the fetal sFlt-1 levels were not significantly different between clinical groups, it is unlikely that fetal sFlt-1 contributes to complications such as fetal growth restriction.

4.6.6 Preeclampsia and Intrauterine fetal growth restriction: Disparity in clinical presentation with similar angiogenic profiles

Shibata et al (209) looked at whether, as with preeclampsia, sFlt-1 is increased and free PlGF is decreased in villous placenta and maternal serum of normotensive women with small-for-gestational-age (SGA) neonates. In a case-control study using banked samples, three groups of patients classified into normal pregnancy, preeclampsia and small for gestational age were studied for their circulating levels of serum sFlt and PlGF. sFlt-1 levels were higher in preeclamptics than controls but not increased in SGA pregnancies. PlGF was lower in both preeclampsia and SGA as defined by birth weight $<10^{th}$ centile as compared to normal pregnancy. PlGF did not differ between preeclampsia and SGA. They concluded that while there is a role for sFlt-1 in the maternal manifestations of preeclampsia, sFlt-1 does not appear to contribute substantially to decreased circulating free PlGF in SGA pregnancies in the absence of a maternal syndrome.

In a longitudinal study of angiogenic (PlGF) and anti-angiogenic (sEng and sFlt-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate, Romero et al concluded changes in the maternal plasma concentration of sEng, sFlt-1, and PlGF precede the clinical presentation of preeclampsia, but only changes in sEng and PlGF precede the delivery of an SGA neonate (230). In comparison, the current study results are concordant with an elevation in sEng and a decrease in PlGF in both preeclampsia and IUGR as compared to normal controls but disparate in the results showing elevated sFlt-1 levels in both preeclampsia and IUGR. The results of this study raise the question why some women with placental disease and anti-angiogenic profile develop the maternal syndrome of preeclampsia while other develops features of fetal growth restriction.

In a review on shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia, Ness et. al. (65) suggested a difference in maternal metabolic syndrome as an explanation for the varied phenotypical expression of an anti-angiogenic profile in the mother. They hypothesize that both women experiencing preeclampsia and IUGR enter pregnancy with some degree of endothelial dysfunction, a lesion that predisposes to shallow placentation. Preeclampsia develops when abnormal placentation, through the mediator of elevated circulating cytokines, interacts with maternal metabolic syndrome, comprised of adiposity, insulin resistance / hyperglycaemia, hyperlipidemia, and coagulopathy. IUGR develops in the absence of antenatal metabolic syndrome. Among these women, the baby is affected by shallow placentation but the mother does not develop clinically apparent disease.

4.6.7 Strength and limitations

The strength of this study is in patient selection. The use of umbilical artery resistance in identification of patients with a placental cause of intrauterine fetal growth restriction has removed the ambiguity generated by previous studies showing variable angiogenic profiles in fetal growth restriction. In the literature to date, small for gestational age with birth weight less than 10th centile has been the most used definition for fetal syndrome of placental disease. The use of umbilical artery Doppler resistance to select true placental fetal growth restriction in this study has added to the reliability of these findings. With multiple varying causes of small for gestational age, including constitutionally normal fetuses, this raises the question whether most of the published literature on SGA and angiogenic factors had studied fetal growth restriction due to placental dysfunction. The current study has attempted to rectify this by selecting pregnancies with not only SGA but also abnormal umbilical artery Doppler studies to identify fetal growth restriction of placental origin. This is the only study

to date to present angiogenic factors in clearly defined groups of preeclampsia, fetal growth restriction and a combination of both conditions. The results presented in this study are limited by relatively small case numbers. The gestational age related changes are limited by the cross sectional nature of the data. The potential confounding effect of steroids have not been quantified.

4.6.8 Conclusion

We conclude that PE and IUGR have similar angiogenic profiles, suggesting that angiogenic marker profiles lack specificity in identifying PE and that other factors are required for the development of PE instead of IUGR. sEng is a predictor of established PE and IUGR and could be a biomarker profiles for predicting PE or IUGR. Fetal sFlt-1 does not appear to make a significant contribution to the pathogenesis of preeclampsia or intrauterine fetal growth restriction.

Chapter 5 - Characterization of monocyte phenotype and polarization in preeclampsia and intrauterine fetal growth restriction

5.1 Summary

Background: Mononuclear cells arising from bone marrow precursors are known to circulate in blood and eventually migrate into body tissues where they further mature into variable forms of macrophages and perform multiple functions in the body including roles in homeostasis, immune defense, and tissue repair. Monocytes, the peripheral precursor of macrophages have been less studied and may play a significant role in the pathogenesis of pregnancy complications including preeclampsia and IUGR.

Aim: To determine whether monocyte subset phenotype and polarization is altered in pregnancy complications such as preeclampsia and fetal growth restriction as compared to normal pregnancy.

Methods: A prospective cross-sectional case control study was conducted. A total of 54 pregnant women between 24-40 weeks of gestation, delivering at Westmead Hospital during the period 2013-2014 were recruited and classified into four clinical groups of Normal pregnancy, PE, IUGR and PE+IUGR. The distribution of maternal monocyte subtypes were characterized and compared for each clinical group using a classification of classical, intermediate and non-classical subtypes. Monocyte polarization towards M1 (inflammatory) and M2 (healing) phenotypes was assessed by surface expression of CD86 and CD163 respectively using flow cytometry. Gestational age related changes were explored in normal pregnancy.

Results: There was no demonstrable change in the total monocyte count with gestational age. In normal pregnancy, a positive correlation was seen between gestational age and the following variables: percentage of non-classical monocytes, CD86 MFI, CD163 MFI and the percentage of monocytes expressing CD163. The percentage of intermediate monocytes was elevated and classical monocyte percentage decreased in PE, IUGR and PE+IUGR compared to normal pregnancy. There was no difference in the non-classical monocytes. This change is more prominently seen in earlier gestation <38 weeks. The percentage of monocytes expressing CD163 and the MFI of CD163 was increased in pregnancies complicated by IUGR.

Conclusion: This study is consistent with previously published findings that intermediate monocytes are increased in pregnancies complicated by preeclampsia. The study has shown for the first time that there is also a shift towards increased intermediate maternal monocyte subtype in IUGR and PE+IUGR. The study has also demonstrated polarization of maternal peripheral monocytes towards M2 in pregnancies complicated by IUGR. This change appears to be across all the monocyte subtypes classical, intermediate and non-classical. This M2 polarization may represent the body's response to and repair of significant placental injury.

5.2 Introduction

There is evidence that decidual macrophages play a major role in vascular remodeling in early placentation as well as a role in the immune mechanism responsible for the acceptance of an allogenic fetus by the mother (260). Suboptimal vascular remodeling of spiral arteries is thought to play a key role in the pathogenesis of preeclampsia (30). Monocytes, the peripheral precursor of macrophages, have been less studied in the pathogenesis of pregnancy complications and may play a significant role in pathogenesis of pregnancy complications including preeclampsia and IUGR.

5.3 Aims:

1. Classification of maternal peripheral monocytes into classical, intermediate and non-classical subtypes.

2. Identify any variation of monocyte subtypes across different gestational periods in third trimester normal pregnancy.

3. To determine whether monocyte subset phenotype is altered in pregnancy complications such as preeclampsia and fetal growth restriction as compared to normal pregnancy.

4. Determine differences in the ratio of CD86/CD163 as an indicator or biomarker of monocyte activation between normal, preeclamptic and IUGR pregnancies.

5.4 Methods

A prospective cross-sectional case control study was conducted. Maternal venous samples were collected within 7 days prior to delivery. Cord blood venous samples were collected at delivery.

Sample preparation, staining protocols, experiment design, flow cytometry optimization and technique as well as the gating strategy have been described in detail in Chapter 2. Briefly, monocyte phenotype assessment and cell marker profile of whole blood monocytes was assessed by flow cytometry using a BD Canto II flow cytometer and Flow Jo software version 10.6. Cell processing involved incubation of whole blood aliquots with cell surface antibodies followed by incubation with Optilyse C for lysing red blood cells and fixation. Two flow cytometry protocols using a three colour and a fluorescence minus one principle multicolour protocol were used for data acquisition. Data analysis involved the identification of the 3 main monocyte populations classical CD14++CD16-, intermediate CD14++CD16+ and non-classical CD14+CD16++ across the different clinical groups. Surface expression of inflammatory markers CD86 and CD163 and their Mean Fluorescent Intensity (MFI) over that of the isotype control was calculated for the different monocyte populations. The gating technique used in this study for monocyte subtypes parallel those previous published in pregnancy (274) as well as in other fields (270, 405).

5.5 Results

5.5.1 Maternal and neonatal demographic data and clinical characteristics of the study population

Results are presented for 54 maternal peripheral venous samples from four clinical groups as listed below, ranging from 24 – 40 weeks of gestation. Maternal and fetal demographic data and clinical characteristics of the study population are described in Table 5.1.

Thirty six samples were collected from umbilical vein at delivery of the pregnancy. A significant number of samples (9) were clotted and adequate monocyte numbers could not be harvested for flow cytometry. Twenty seven cord blood samples were analyzed and included in the study for monocyte phenotype. The study was conducted as an exploratory study to identify aspects for future research.

The maternal age was higher in the PE+IUGR group compared to PE ($p = 0.035$) and IUGR ($p = 0.04$) and non-significant compared to normal pregnancy. There was no significant difference in BMI between groups except the pregnancies complicated by IUGR, where the BMI was noted to be lower as compared to the PE group ($p = 0.044$). There were no statistically significant differences noted between clinical groups in gestational age at sample collection. The gestational age at delivery and birth weight showed significant differences between clinical groups (Table 5.1 and Figure 5.1). No differences in birth weight were noted between IUGR and PE+IUGR.

	Clinical Group			
	Normal (SD)	**PE (SD)**	**IUGR (SD)**	**PE+IUGR (SD)**
Number of maternal samples	24	9	12	9
Number of fetal samples	8	4	9	6
Maternal age (years)	29.4(3.6)	27.9(6.1)	28.3(4.9)	33.0(7.1)*, #
BMI	27.3(5.3)	29.6(8.1)	24.18(5.3) #	28.2(6.3)
Gestation at sample collection (weeks)	34.8(4.2)	35.7(3.3)	34.8(4.2)	32.3(2.3)
Gestation at delivery (weeks)	39.2(0.8)	36.18(3.5)*	35.48(3.6)*	32.68(2.5)*# †
Birth weight (g)	3316(505)	2749(821)*	1879(670)*#	1509(540)*#
Primiparous (%)	37.5	77.8*	58.3	88.9*
Antihypertensive treatment (%)	0%	88%	0%	44%
Smoking	8.3%	0.0%	8.3%	7.4%
Mode of delivery (Rate of LSCS)	71%	78%	100%	100%
Antenatal steroid within 7 days of delivery	0%	11%	50%	33%

Table 5.1 Maternal and fetal demographic data and clinical characteristics of the study population.

Results are presented as mean ± SD for each continuous variable unless otherwise specified.

* Significantly different from normal pregnancy. # Significantly different to PE.

† Significantly different to IUGR. $p < 0.05$.

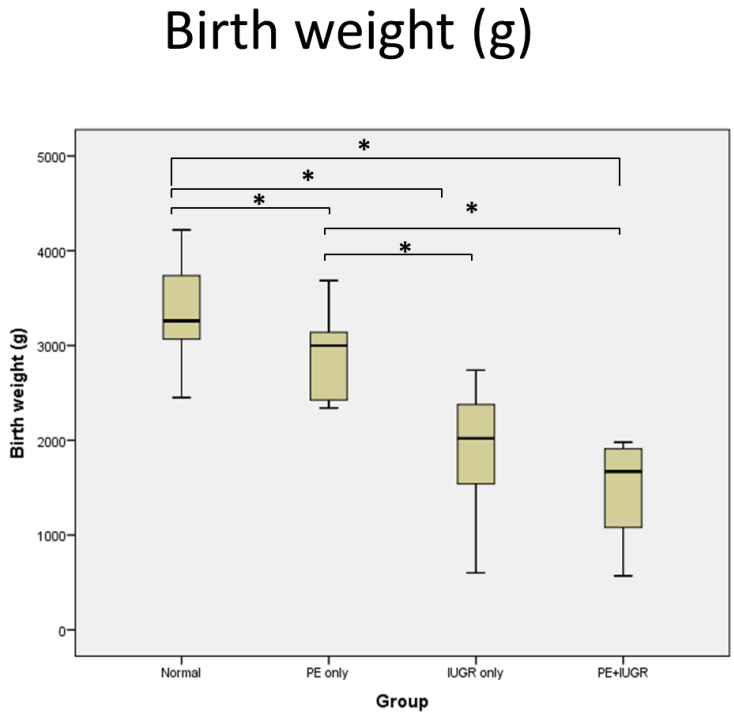

Figure 5.1 Birth weight by clinical group.

* Significant difference between clinical groups $p < 0.05$.

Analysis of the effect of confounding variables using Fishers exact test showed that preeclampsia and PE+IUGR groups included a higher percentage of primiparous patients as compared to normal pregnancy and IUGR only ($p = 0.033$). The majority of preeclamptic patients received anti-hypertensive treatment whereas only half of the PE+IUGR patients were medicated ($p < 0.001$). No difference was noted in the incidence of smoking and mode of delivery between the different clinical groups.

5.5.2 Maternal leukocyte and monocyte counts in normal and pathological pregnancies

Maternal circulating white cell count and the monocyte count as calculated by Sysmex cytometer is presented below (Table 5.2). Forward and side scatter as well as surface expression of CD14 was used to identify the monocyte populations (Figure 5.2).

	Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (percentile 25, 75)	Median (percentile 25, 75)	Median (percentile 25, 75)	Median (percentile 25, 75)
WBC (x10^9 cells/L)	9.71 (8.47, 11.83)	7.92 (7.60, 10.07)	10.38 (9.03, 10.86)	10.52 (9.51, 12.22**)**
Monocyte %	6.0 (5.5, 6.8)	6.4 (5.8, 7.7)	7.0 (6.2, 8.1)	6.3 (2.2, 6.7)
Monocyte Classical %	72.4 (47.2, 76.9)	52.1 (45.2, 71.9)	54.7 (32.5, 62.8)*	52.4 (40.8, 72.6)
Monocyte Intermediate %	22.5 (17.6, 48.3)	41.5 (16.6, 49.3)	38.7 (30.9, 58.6)*	41.7 (25.4, 52.8)
Monocyte Non-classical %	4.2 (3.2, 5.6)	4.5 (4.3, 5.4)	4.6 (3.5, 6.9)	5.7 (2.7, 7.1)

Table 5.2 Maternal leukocyte count, monocyte count and the proportion of monocyte subtypes as a percentage of total count.

Results are presented as median ± interquartile range for each continuous variable unless otherwise specified. * Significantly different to normal pregnancy $p < 0.05$.

No differences were observed in the maternal peripheral leukocyte count and the total monocyte percentage between clinical groups normal pregnancy, preeclampsia, IUGR and PE+IUGR (Table 5.2).

5.5.3. Maternal peripheral blood monocyte subsets

To determine the changes in monocyte subsets during preeclampsia and intrauterine fetal growth restriction, surface expression of CD14 and CD16 were used to gate the total monocytes into three subsets classical (CD14++CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16++) using flow cytometry. The results for monocyte subsets as a percentage of total monocytes are presented in Table 5.2 and Figure 5.3. The percentage of classical monocytes was lower in the IUGR group compared to normal pregnancy (54.7% vs 72.4%, $p = 0.016$). The percentage of intermediate monocytes is increased in the IUGR group as compared to Normal pregnancy (38.7% vs 22.5%, $p = 0.033$). There was no significant difference between the groups in the percentage of non classical monocytes.

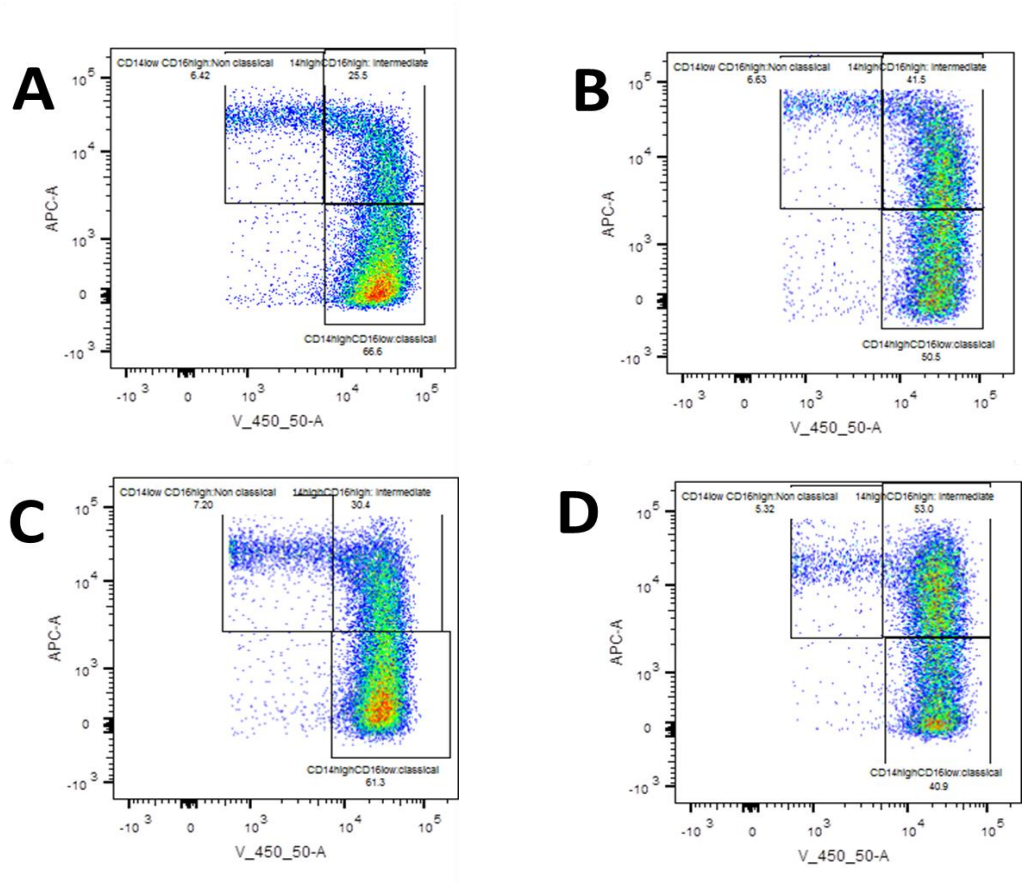

Figure 5.2 Gating strategy and distribution of monocyte subgroups in a representative sample of each clinical group.

Classical monocytes CD14++CD16-, Intermediate monocytes CD14++CD16+, Non-classical monocytes CD14+CD16++. A = Normal pregnancy, B = Preeclampsia, C= Intrauterine fetal growth restriction, D= Preeclampsia and intrauterine fetal growth restriction.

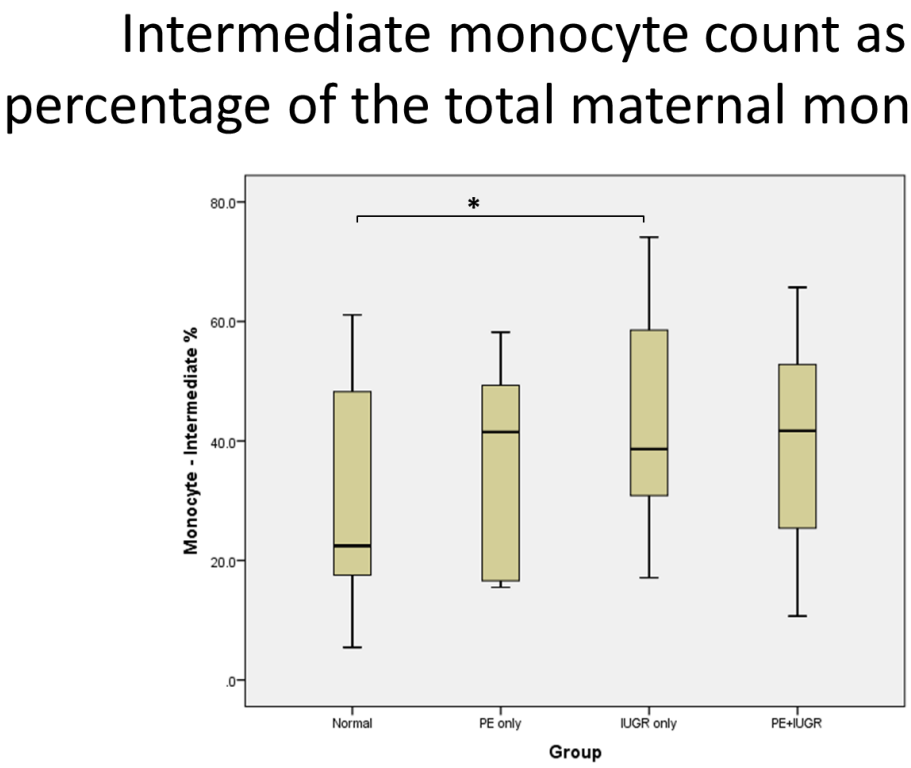

Figure 5.3 Intermediate monocyte counts as a percentage of the total monocyte count.

Comparison box plots (median and interquartiles) between the clinical groups presented. The percentage of intermediate monocytes is increased in the IUGR group as compared to Normal pregnancy ($p = 0.033$).

The gestational age of sample collection was separated into >38 weeks and < 38 weeks as the normal samples were collected in 2 groups of elective caesarean deliveries > 38 weeks and antenatal normal pregnancies < 38 weeks (Figure 5.4 A and B). There was a statistically significant reduction in the percentage of classical monocytes in IUGR compared to Normal pregnancy. The differences in distribution reached statistical significance in the < 38 weeks gestation for IUGR ($p = 0.001$) and PE+IUGR ($p = 0.021$). The data also demonstrates an increase in the intermediate monocyte subtype as a percentage of total monocytes which

showed significance for all gestations between Normal and IUGR and reached statistical significance in the <38 weeks gestation for IUGR (p = 0.007) and PE+IUGR (p = 0.030).

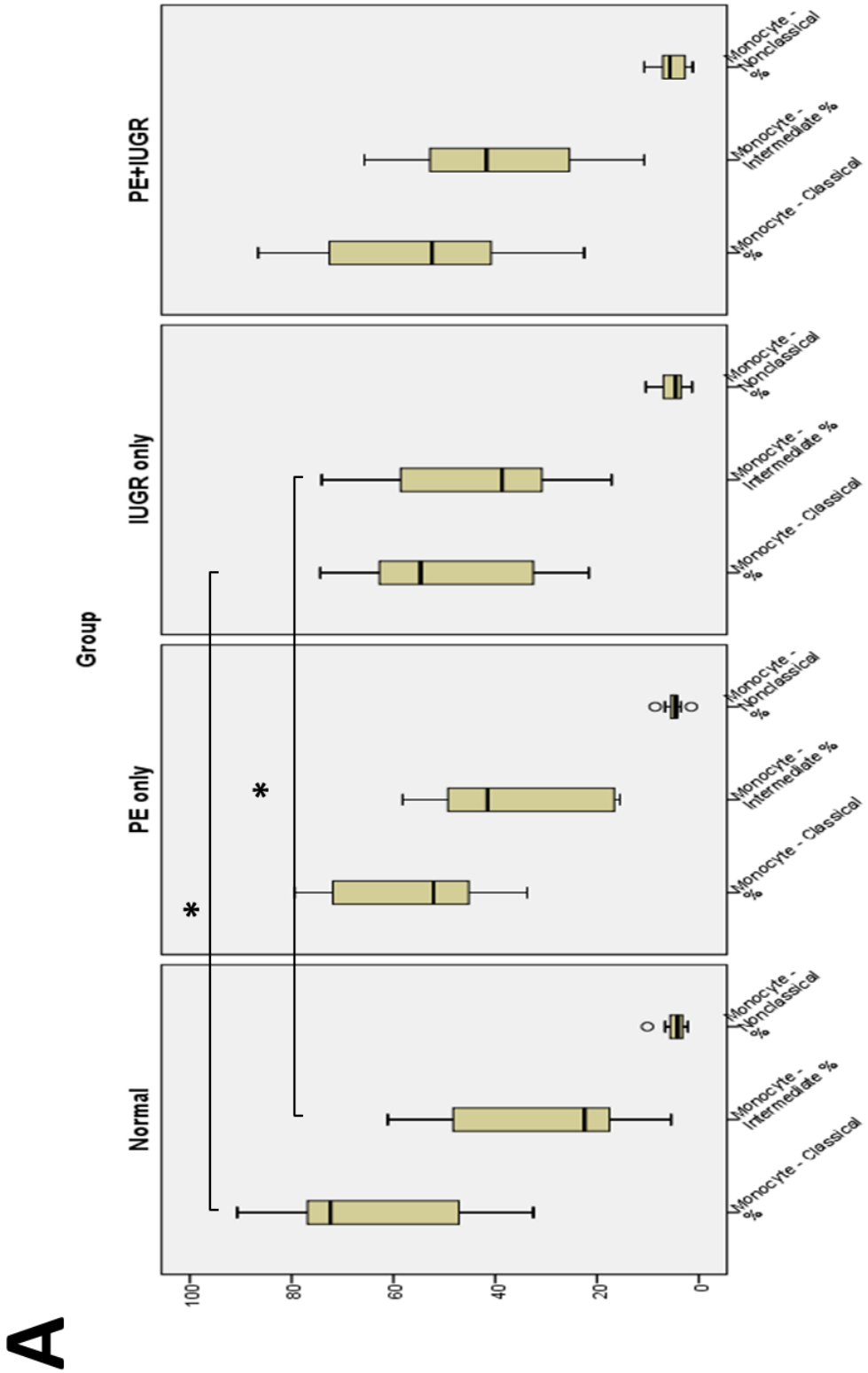

Figure 5.4 Graphical representation of the composition of monocyte subgroups across the four clinical groups.
A = All samples * Significantly different from normal pregnancy p <0.05.

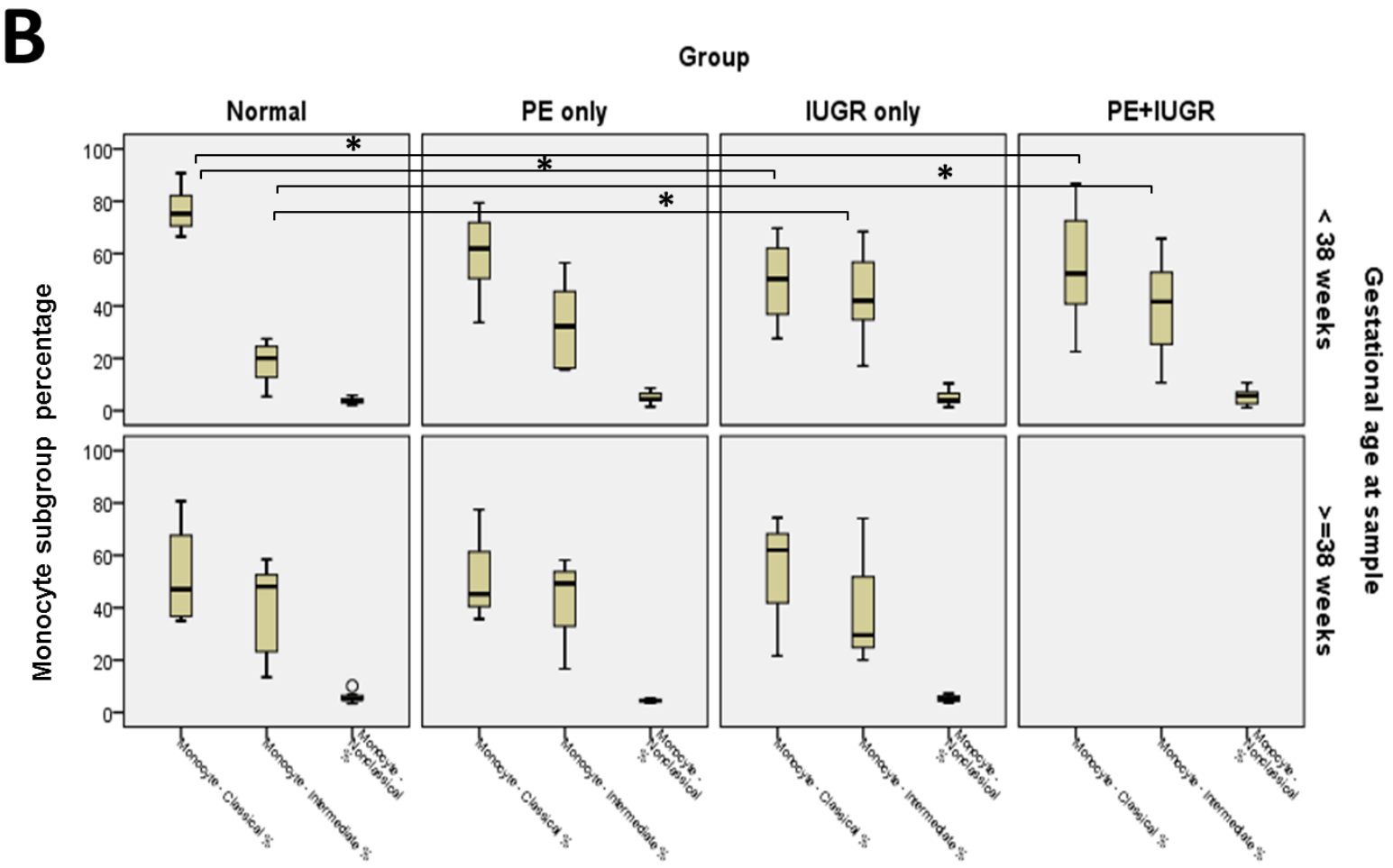

Figure 5.4 Graphical representation of the composition of monocyte subgroups across the four clinical groups. B: Separate analysis presented for gestational age <38 weeks and >38 weeks at the time of sample collection. * Significantly different from normal pregnancy p <0.05.

Figure 5.4 Graphical representation of the composite of monocyte subgroups across the four clinical groups. A: All samples. B: Separate analysis presented for gestational age <38 weeks and >38 weeks at the time of sample collection.

5.5.4 Markers of monocyte polarization

Monocyte surface expression of CD86 as a M1 polarization marker and CD163 as a M2 marker were investigated. The percentage of total monocytes expressing the marker and mean fluorescence intensity (MFI) were calculated for each marker in each clinical group. Mean fluorescence intensity ratio of CD86/CD163 was evaluated as an indicator of monocyte polarization and correlated with the clinical groups and monocyte subtypes. The data are presented in Table 5.3 and Figures 5.5 and 5.6.

5.5.4.1 Expression of CD86 as a monocyte marker of M1 polarization

The expression of maternal monocyte surface CD86 expression was detected with flow cytometry and quantified as mean fluorescence intensity using Flow Jo software. The results for expression of CD86 are presented in Table 5.3 and Figure 5.5.

There was no demonstrable difference between the clinical groups in the percentage of total monocytes expressing CD86, with 98-99% of monocytes in each clinical groups showing CD86 as a surface marker. The analysis of the percentage of monocyte CD86 expression according to monocyte subtype showed some variation with the different monocyte subtypes with a statistically significant difference in intermediate monocytes CD86 expression between normal pregnancy and PE as well as a difference in non classical monocyte CD86 expression between normal pregnancy and PE+IUGR.

	Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)
Percentage of Monocytes expressing CD86	99.1 (98.9, 99.4)	99.0 (98.7, 99.3)	99.3 (98.2, 99.5)	98.8 (98.6, 99.4)
Total monocytes CD86 MFI	2432.5 (2069.5, 3269.5)	2231.0 (2019.0, 2387.0)	3153.5 (1891.0, 3812.0)	2568.0 (1778.0, 2987.0)
Classical monocytes CD86 MFI	2628.0 (2315.0, 3309.0)	2364.0 (2143.0, 2585.0)	3338.0 (2096.0, 3916.0)	2778.0 (1973.0, 3086.0)
Intermediate monocytes CD86 MFI	3470.0 (2822.5, 4743.5)	2722.0* (2449.0, 3754.0)	3759.5 (2640.5, 4588.5)	3157.0 (2629.0, 3606.0)
NonClassical monocytes CD86 MFI	5350.5 (4411.0, 6250.5)	5073.0 (3959.0, 5904.0)	5353.5 (2159.0, 6492.5)	3828.0* (1737.0, 4882.0)
Percentage of monocytes expressing CD163	63.5 (56.1, 70.2)	65.6 (62.1, 73.3)	78.0***** (65.0, 84.7)	77.1***** (64.2, 96.5)
Total monocytes CD163 MFI	310.0 (225.5, 370.0)	377.0 (247.0, 453.0)	506.0 (241.5, 686.0)	866.0 ***** (452.0, 2717.0)
Classical monocytes CD163 MFI	427.0 (343.5, 521.0)	447.0 (322.0, 564.0)	662.0* (420.5, 770.0)	679.0*# (579.0, 3056.0)
Intermediate monocytes CD163 MFI	480.5 (371.8,641.3)	438* (365,564)	633* (325,807)	781*# (463,3231)
Non-Classical monocytes CD163 MFI	21.5 (3.0, 58.0)	0 * (0, 0)	50.5*# (0, 123.5)	222.0* # (63.0, 282.0)

Table 5.3 Maternal monocyte expression of CD86 and CD163 as markers of monocyte polarization.

Results are presented as median ± interquartile range for each continuous variable. The MFI of the isotype control was set at 0 with any MFI less than this value truncated at 0.

* Statistically significantly different from normal pregnancy. # Significantly different from PE. $p < 0.05$.

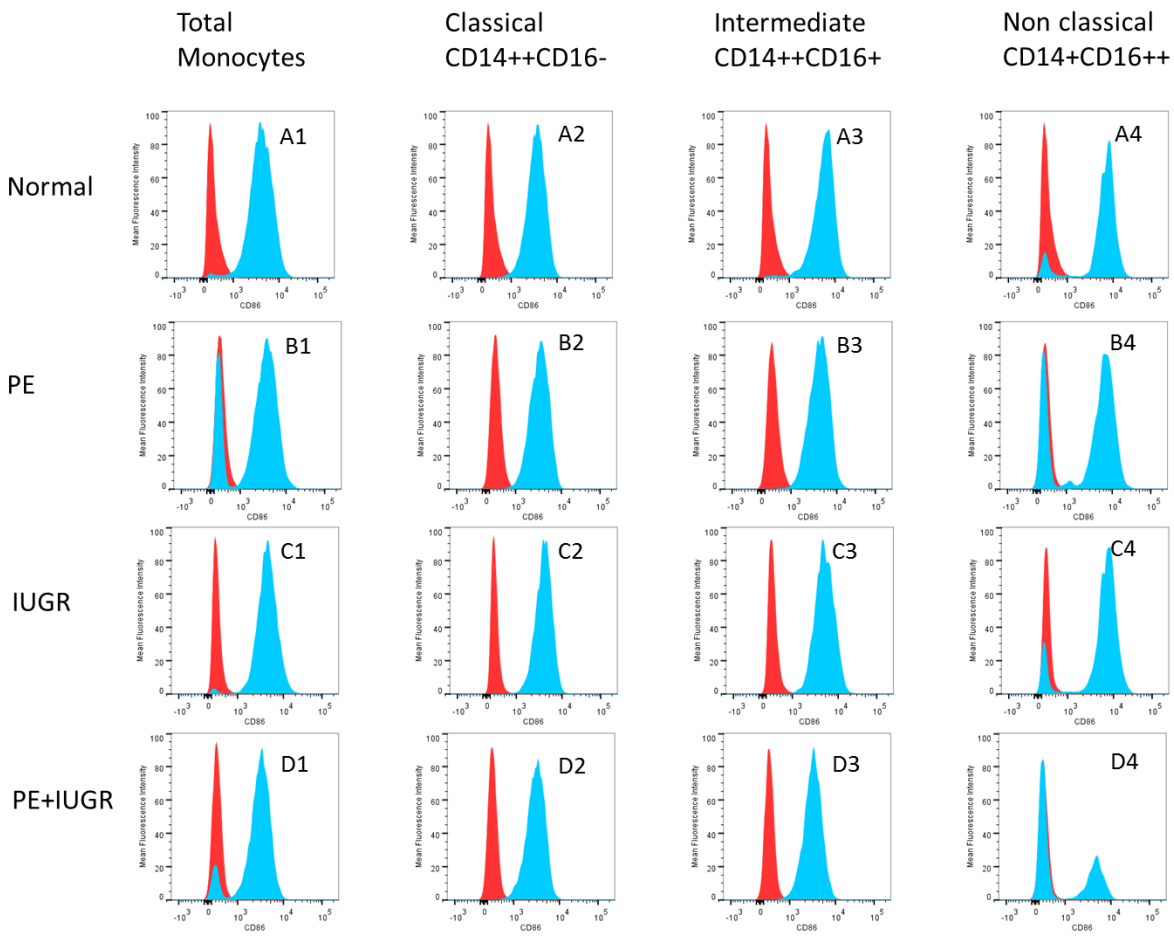

Figure 5.5 Distribution of CD86 on peripheral blood monocyte subsets in representative samples of normal pregnancy and pregnancies complicated by PE, IUGR and PE+IUGR.

Flow cytometry histogram plots of CD86 expression by different monocyte subsets are presented. Values in histograms are for MFI (Mean Fluorescence Intensity) of CD86 expression. Red colour areas represents staining with isotype matched control antibody. Blue coloured areas represent staining with CD86 monoclonal antibody.

5.5.4.2 Expression of CD163 as a monocyte marker of M2 polarization

The expression of maternal monocyte surface CD163 expression was detected with flow cytometry and quantified as mean fluorescence intensity using Flow Jo software. The results as groups presented in Table 5.3 and Figure 5.6.

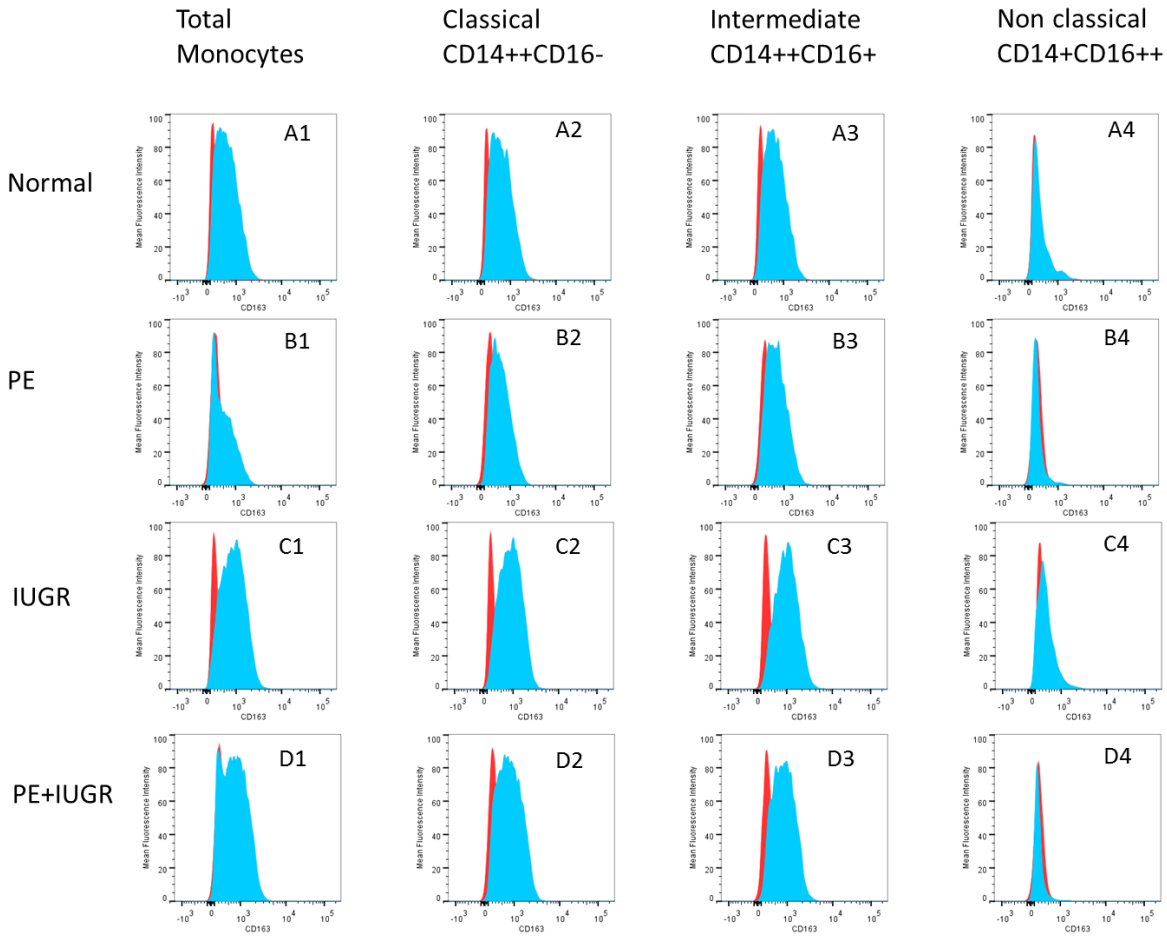

Figure 5.6 Distribution of CD163 on peripheral blood monocyte subsets in representative samples of normal pregnancy, PE and PE+IUGR.

Flow cytometry histogram plots of CD163 expression by different monocyte subsets. Values in histograms are for MFI (Mean Fluorescence Intensity) of CD163 expression. Red colour areas represents staining with isotype matched control antibody. Blue coloured areas represent staining with CD163 monoclonal antibody.

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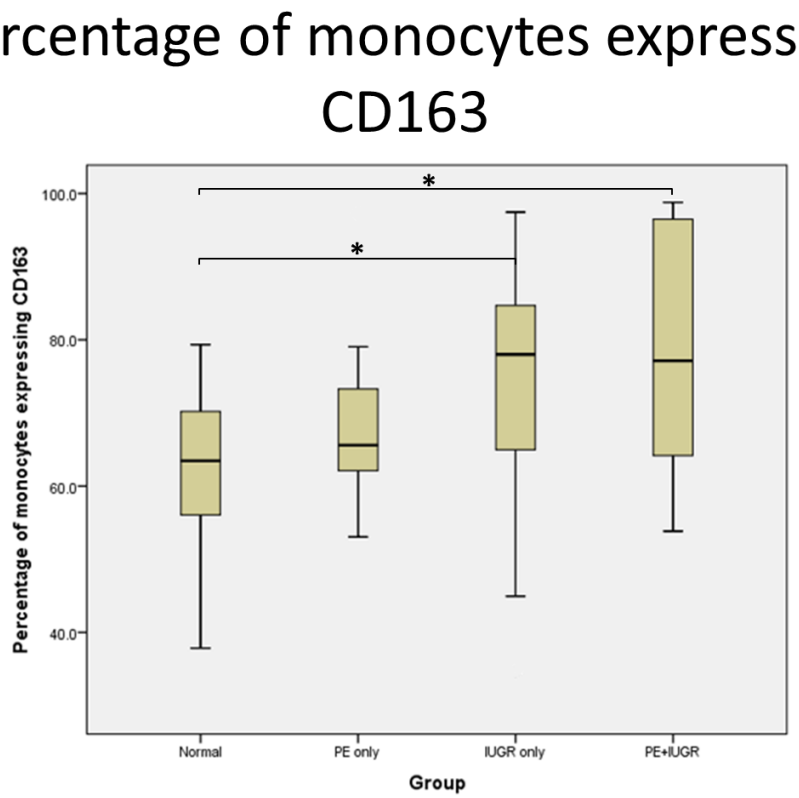

Figure 5.7 Percentage of monocytes expressing CD163.

Significantly more monocytes in the IUGR and PE+IUGR were noted to express CD163 (Figure 5.7). The expression of CD163 was increased in IUGR and PE+IUGR compared to Normal pregnancy, with a variation in CD163 expression across the monocyte subtypes (Table 5.3). * Statistically significantly difference between clinical groups $p < 0.05$.

5.5.4.3 Ratio of CD86 / CD163 Mean Fluorescence Intensity (MFI) as a measure of monocyte polarization in the clinical groups.

Ratio of CD86 / CD163 Mean Fluorescence Intensity as a measure of monocyte polarization towards M1 /M2 phenotypes was evaluated for the four clinical groups. A higher ratio would indicate polarization towards M1 phenotype.

	Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)
Total monocytes - CD86 MFI / CD163 MFI	3.41 (3.14, 3.86)	3.05 (2.92, 3.20)	3.69 (3.18, 4.10)	1.80*† (0.96, 3.15)
Classical monocytes - CD86 MFI / CD163 MFI	4.44 (4.12, 5.66)	4.09 (3.23, 4.63)	4.44 (3.79, 4.83)	2.49*† (0.95, 3.75)
Intermediate monocytes - CD86 MFI / CD163 MFI	5.13 (4.12, 6.64)	4.93 (3.29, 5.01)	4.80 (4.14, 5.90)	2.62* #† (1.00, 3.78)
Nonclassical monocytes - CD86 MFI / CD163 MFI	20.43 (16.70, 24.28)	17.74 (15.58, 22.71)	15.16* (9.64, 20.29)	12.57* #† (3.70, 15.52)

Table 5.4 Ratio of CD86 / CD163. Correlation with clinical group and monocyte subtype. Results are presented as median ± interquartile for each continuous variable unless otherwise specified. * Statistically significantly different from normal pregnancy $p < 0.05$. † Statistically significant difference from IUGR. # Statistically significantly different from PE.

The ratio of CD86/ CD163 was decreased in PE+IUGR compared to Normal pregnancy and IUGR in total monocytes, with a similar variation in CD86/CD163 expression ratio seen across the monocyte subtypes (Table 5.4). This change was seen consistently across the monocyte subtypes. The intermediate and non-classical monocytes also showed a lower CD86/CD163 ratio in IUGR+ PE compared to PE only.

5.5.5 Correlations between gestational age and distribution of monocyte subtypes and inflammatory markers in the third trimester of pregnancy.

Spearman rank correlations for gestational age at sample collection and variables associated with monocyte phenotype and polarization in normal third trimester pregnancy are presented in Table 5.5 and Figures 5.8-5.9.

	Correlation coefficient	Significance (2 tailed) p value	Interpretation
White blood cell count	-0.244	0.251	No significant correlation
Total monocytes as a percentage of total WBC %	0.094	0.662	No significant correlation
Classical monocyte s as a % of total monocytes	-0.364	0.080	Not statistically significant
Intermediate monocytes as a % of total monocytes	0.294	0.163	Not statistically significant
Non-classical monocytes as a % of total monocytes	0.414	0.044	Statistically significant trend towards increased non-classical monocytes with gestation
Percentage of monocytes expressing CD86	0.232	0.274	No significant correlation
Total monocyte CD86 MFI	0.380	0.067	Not statistically significant
Percentage of monocytes expressing CD163	0.471	0.020	Statistically significant trend towards increased CD163 expressing monocytes with gestation
Total monocyte CD163MFI	0.435	0.034	Statistically significant trend towards increased CD163 MFI with gestation
Total monocyte CD86/CD163	0.071	0.740	No significant correlation
Classical monocyte CD86/CD163	-0.122	0.571	No significant correlation
Intermediate monocyte CD86/CD163	-0.178	0.406	No significant correlation
Non-classical monocyte CD86/CD163	0.119	0.580	No significant correlation

Table 5.5 Differential distribution of monocyte subtypes and inflammatory markers with increasing gestation in third trimester of pregnancy as tested in 24 pregnancies from 26 weeks to 40 weeks of gestation. Correlation coefficients using Spearman non parametric rank correlations and a two tailed significance at $p < 0.05$ presented.

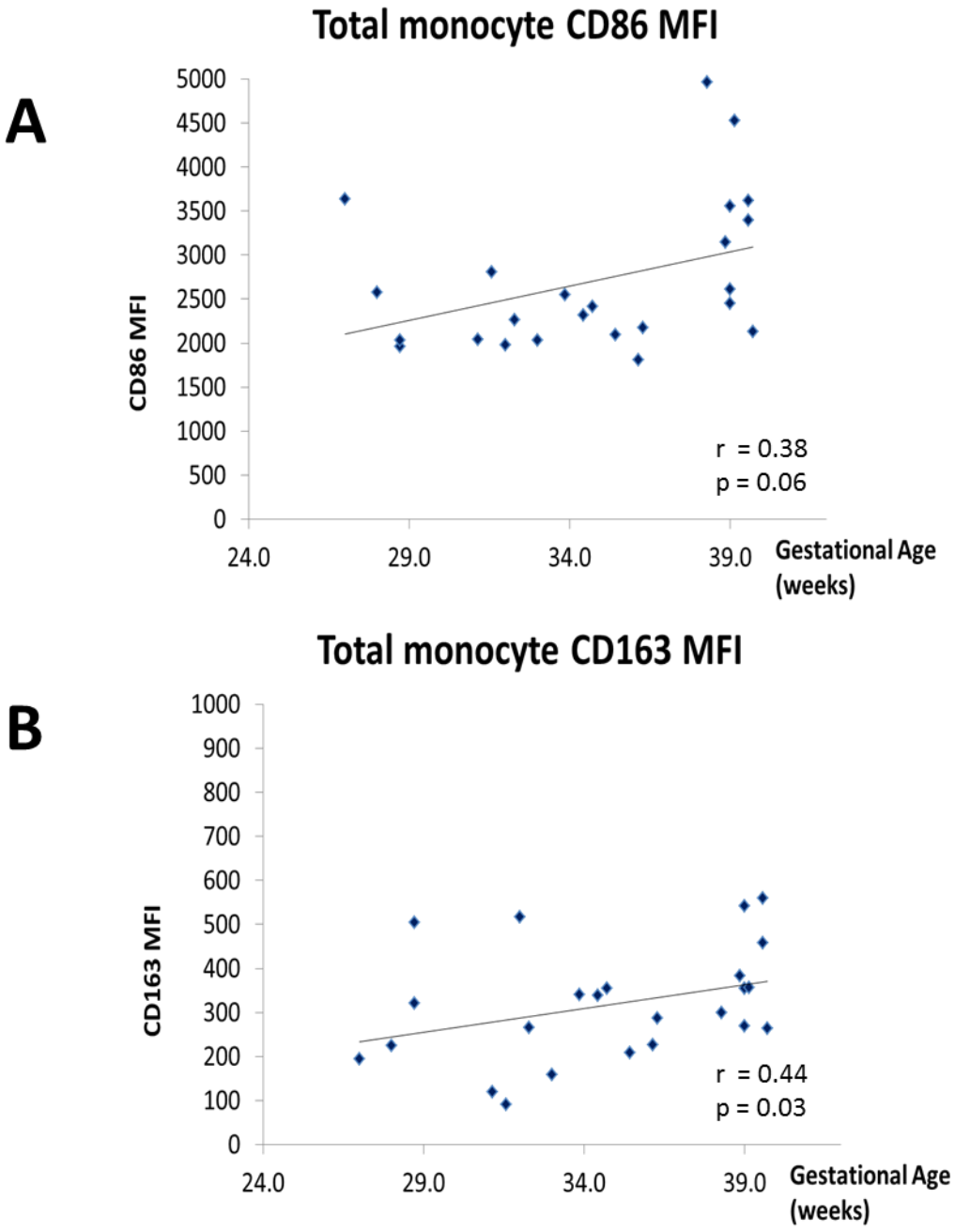

Figure 5.8 Correlation between gestational age and total monocytes Mean Fluorescence Intensity (MFI). A: CD86, B: CD163. Statistically significant trend noted towards increased CD163 MFI with gestation.

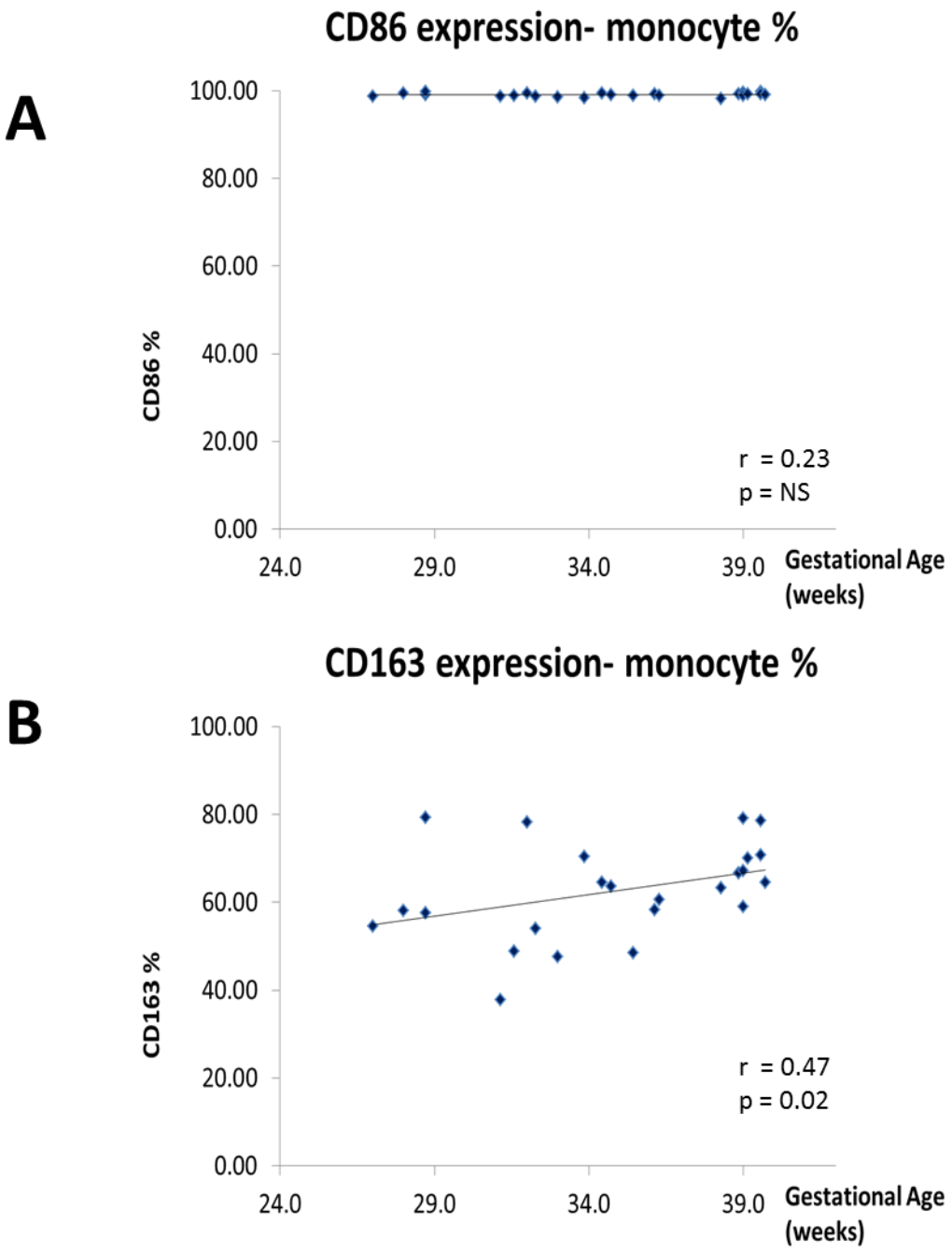

Figure 5.9 Correlation between gestational age and percentage of monocytes expressing A = CD86, B = CD163 as a surface marker. Statistically significant trend noted towards increased CD163 expressing monocytes with gestation.

5.6 Discussion

Monocytes have been shown to be a heterogeneous cell population with subset specific functions and phenotypes. The new 2010 classification of monocytes is based on the expression of CD14 (receptor for lipopolysaccharide) and CD16 (Fcγ receptor III) (262). While the differential expression of CD14 and CD16 distinguishes classical (CD14++CD16−), intermediate (CD14++CD16+), and non-classical (CD14+CD16++) monocytes, current knowledge on human monocyte heterogeneity and their functional implications is still incomplete. While pro-inflammatory monocyte function and changes in subtype distribution have been described in preeclampsia in a handful of studies (274, 306), monocytes subtype phenotypes and functional changes in IUGR have not been published before.

The differential distributions of monocyte subtypes in the maternal peripheral blood in normal pregnancies, PE, IUGR and PE+IUGR were investigated. Most studies on monocyte subsets have focused on the combined non-classical/intermediate monocytes. The present study used the recently recommended classification of monocytes into classical, intermediate and non-classical subtypes (262). To test whether monocyte polarization towards an M1 or M2 phenotypes occurs in PE and IUGR, the study examined the expression of CD86 (M1 marker) and CD163 (M2 marker) on peripheral whole blood monocytes of the mother.

In the group of 54 patients, the study was unable to demonstrate a difference in the total white cell count or the percentage of monocytes with increased gestation in third trimester or across clinical groups. Previously published studies have shown an elevated percentage of leukocytes and monocytes in healthy pregnant women as compared to non-pregnant women and an even further elevation in preeclamptic women compared to normal pregnancy (274). The results in this chapter are however consistent with other published studies showing a

stable proportion of monocytes throughout gestation (295). The variation in results may be due to smaller patient numbers and other factors associated with the pregnant state, which are not clinically evident such as subclinical infection.

Screening for subclinical infection in the mother was not carried out as part of the study. As no differences were observed in the maternal peripheral leukocyte count or the total monocyte percentage between clinical groups normal pregnancy, preeclampsia, IUGR and PE+IUGR (Table 5.2), it would be reasonable to assume that a significant infection was unlikely to have contributed to any variation in monocyte distribution.

The variation in monocyte subtypes associated with gestational age was also investigated. This may be due to the small patient numbers in this study and the clinical variation of normal pregnancy. The significant increase in non-classical monocyte population percentage with gestation is a new finding described in this study and may reflect increasing injury and repair in the later term placenta (274). This finding is consistent with a published animal study using an ATP infusion to create a model of preeclampsia (274). The authors demonstrated that the percentage of non-classical monocytes in pregnant rats increased even further with ATP infusion. This change was not seen in non-pregnant rats. They concluded that the observation of ATP stimulated numbers/activation of non-classical monocytes occurred in pregnant rats only, suggesting that non-classical monocytes are specifically altered in pregnancy and may play a role in the pathophysiology of preeclampsia.

Comparison of monocyte subgroup distribution between clinical groups showed a statistically significant decrease in classical monocytes and a significant increase in intermediate monocytes for IUGR pregnancies across all gestational ages and also for PE+IUGR <38 weeks of gestation. This is the first description of monocyte subgroup distribution in pregnancies complicated by intrauterine fetal growth restriction.

Although a similar pattern was observed with PE, the difference between normal pregnancy and PE was not statistically significant. The percentage of non-classical monocytes was not noted to be significantly different between the pathological groups. The patient numbers were not adequate in this study to perform a useful analysis of effects of fetal gender or antenatal steroid on monocyte subset distribution and should be investigated in further study.

Smaller patient numbers and the variability in gestational ages within the clinical groups may have contributed to these results (type 2 error) and further studies on larger patient groups are recommended to confirm any described trends in monocyte subgroup distribution.

One of the few published studies of monocyte subtypes in preeclampsia found that the percentage of combined non-classical /intermediate monocytes is higher during pregnancy in humans and in rats as compared to non-pregnant controls (274). These results are concordant with these findings although the changes in this study for PE did not reach statistical significance (274, 406). The increase in the combined groups was mainly due to an augmentation of the intermediate subtype. A shift towards higher numbers of combined non-classical/intermediate monocytes, in particular an increase in the intermediate subtype has been associated with several inflammatory diseases including sepsis, rheumatoid arthritis, HIV infection, atherosclerosis, atopic dermatitis and asthma (270, 278, 407, 408).

An expansion of circulatory CD14+CD16+ monocytes (intermediate + non-classical) has been demonstrated in chronic liver disease and fibrosis as well as atherosclerosis and fibrotic plaque development. These monocytes are thought to contribute to the inflammation and fibrosis associated with these conditions (409). The intermediate monocytes may also play a similar role in inflammation and fibrosis in IUGR where villous thrombosis, loss of vascularity and fibrosis of infarcted areas play a significant role in the pathology of the placenta. The modulation of monocyte subset recruitment into tissues and their subsequent

differentiation have been suggested as potential approaches for therapeutic interventions in human liver fibrosis (409). Parallel interventions in IUGR may be a therapeutic approach to reduce the inflammation and damage to placental tissue in pregnancies complicated by IUGR.

The current study also investigated the polarization of maternal and fetal monocytes into M1 (inflammatory) and M2 (healing) types in preeclampsia and fetal growth restriction. The majority of maternal monocytes expressed CD86 as a surface marker. The percentage of monocytes expressing CD163 varied between groups with a significant increase in pregnancies complicated by intrauterine fetal growth restriction.

Spearman non parametric rank correlations with gestational age revealed increased number of monocytes expressing CD163 as well as an augmentation in the CD163 MFI with increasing gestational age in normal pregnancy. The number of monocytes expressing CD86 did not change with gestation and was consistently in a high range, while the MFI of CD86 showed an increasing trend with gestational age which was not statistically significant. The high CD86 expression is likely a reflection of the increasing inflammatory status of pregnancy. The increase in CD163 with gestation may represent a response to increasing maturational changes, damage and repair in the term placenta. The gestational change in CD163 expression parallels the gestation related increase in anti-angiogenic factor expression of sFlt-1 and sEndoglin in late pregnancy (189). The ratio of CD86/CD163 mean fluorescence intensity (MFI) was used as a marker of inflammatory status within the maternal circulation. This ratio was relatively stable across gestation in third trimester with no demonstrable gestation related variations. This is consistent with the observation that both CD86 and CD163 MFI increased with gestation. The total monocyte CD86/CD163 ratio as well as the subgroup analysis showed a significant trend towards a reduced inflammatory status and polarization towards tissue remodeling monocytes (lower CD86/CD163 ratio) in pregnancies complicated by

intrauterine fetal growth restriction including IUGR only and PE+IUGR. A similar pattern was observed in the CD86/CD163 MFI ratio, confirming a shift towards M2 phenotype of monocytes in the IUGR pregnancies.

The finding of increased M2 polarization in IUGR and PE+IUGR is consistent with other studies of organ damage demonstrating the presence of predominantly M2 monocytes in severe burn patients (410) and may reflect the presence of tissue damage and a healing response. The M2 polarization is also consistent with the Th-2 immune adaptation of pregnancy. A polarization towards M2 may also promote excessive tissue remodeling and fibrosis.

This is the first study to examine monocyte subsets in PE, IUGR and PE+IUGR concurrently. Our study was limited by the small sample numbers but was strengthened by the use of umbilical artery Doppler to define growth restriction of placental origin. The variability in normal pregnancy and the difficulty in determining the disease status of the placenta by external clinical parameters lead to variability in allocation of patient groups. Differences in the pathogenesis of early onset and late onset preeclampsia may have influenced the results of this clinical group (411). The possible development of placental disease with advancing gestation also leads to the possibility that a normal preterm pregnancy may result in a significant pregnancy complication later in gestation. These factors lead to difficulties in performing and interpreting research into pregnancy and its complications.

Human monocytes are currently defined and classified by the extent of their cell surface expression of CD14 and CD16, with associated differences in function and phenotype related to the intensity of expression of these markers. With increasing interest in the function and behaviour of monocytes, it is important to have an understanding of how differing strategies of analysis can affect results and how different protocols and population backgrounds can

affect this highly morphogenic cell type (412). Differences in monocytes have been associated with differences in ethnicity and this may need to be explored as a confounding variable when interpreting study results. It is important to take into consideration that blood monocytes consist of a continuous population of cells, within which the dominant phenotype may vary dependent on the background of the study population. The potential confounding effect of steroids on monocyte populations have not been quantified.

Chapter 6 - Distribution of cord blood monocyte subtypes in preeclampsia and intrauterine fetal growth restriction

6.1 Summary

Background: Fetal monocytes circulate through the fetal and placental circulations and have the opportunity to play significant functions including roles in homeostasis, immune defense, and tissue repair. There is little available data on fetal monocyte phenotype and function. These cells may play a role in pathogenesis of pregnancy complications including preeclampsia and IUGR.

Aim: Conduct a pilot study to describe the cord blood monocyte subset phenotype in normal pregnancy and in pregnancy complications such as preeclampsia and fetal growth restriction. Compare the monocyte phenotype distribution of maternal and fetal circulations.

Methods: A prospective cross-sectional study was conducted. Cord blood samples from 27 pregnancies were collected at delivery from normal pregnancy, PE, IUGR and PE+IUGR. The distribution of fetal monocytes subtypes was characterized by CD14 and CD16 expression using flow cytometry and compared for each clinical group using a classification of classical, intermediate and non-classical subtypes. The cord blood monocyte distribution was correlated with the maternal monocyte subtype percentages.

Results: There is a trend towards an increase in the intermediate and non-classical monocyte subsets in the cord blood of PE, IUGR and PE+IUGR as compared to the normal pregnancies, which reached statistical significance for the non-classical subsets. The intermediate monocytes were the dominant monocyte subtype in the cord blood of PE, IUGR and

PE+IUGR. A statistically significant reduction in the non-classical monocyte subset was observed in the cord blood of normal pregnancy group as compared to the maternal circulation.

Conclusion: This study has documented for the first time in cord blood of pregnancies complicated by PE and IUGR pregnancies, a monocyte subset distribution with dominant intermediate subsets.

6.2 Introduction

Fetal or cord blood monocyte phenotypes have not been well described. There is no comprehensive data describing the distribution of monocyte subsets in cord blood. A study into cord blood monocyte subsets and Toll like receptor expression in normal pregnancy and pregnancies complicated by parasitic infections described a lower percentage of non-classical (CD14+CD16+) monocytes in cord blood as compared to the maternal circulation. The distribution of intermediate monocytes was not mentioned in this study (307). Human fetal and adult monocytes have been shown to be functionally distinct in response to cytokines associated with in-utero infection and preterm labour (309). Fetal monocytes have not been profiled with respect to preeclampsia or intrauterine fetal growth restriction.

6.3 Aims

- Classification of cord blood monocytes into classical, intermediate and non-classical subtypes.
- To determine whether cord blood monocyte subset phenotype is altered in pregnancy complications such as preeclampsia and fetal growth restriction.
- Determine differences in the monocyte subset distribution between maternal and fetal (cord blood) circulations.

6.4 Methods

A prospective cross-sectional case control study was conducted. Umbilical cord blood samples were collected at delivery from pregnancies between 24-40 weeks of gestation,

delivering at Westmead Hospital during the period 2013 -2014. Sample preparation, staining protocols, experiment design, flow cytometry optimization and technique as well as the gating strategy have been described in detail in Chapter 2.6.

6.5 Results

6.5.1 Neonatal demographic data of the study population

Results are presented for 54 maternal and 27 fetal cord blood samples from 24 – 40 weeks of gestation. Not all corresponding cord blood samples from the delivered pregnancies were able to be collected due to emergency delivery out of normal working hours and difficulty in harvesting cord blood from the umbilical cord in some cases. Seven of the collected cord blood samples were clotted at processing. Adequate monocytes could not be harvested from these samples to perform flow cytometric analysis. Neonatal demographic data are described as mean ± standard deviation (Table 6.1). Maternal demographic data and clinical characteristics are the same as described in Chapter 5.

	Clinical Group			
	Normal (SD)	**PE (SD)**	**IUGR (SD)**	**PE+IUGR (SD)**
Number of maternal samples	24	9	12	9
Number of fetal samples	8	4	9	6
Gestation at delivery (weeks)	39.2(0.8)	36.1(3.5)	35.4(3.6)	32.6(2.5)
Birth weight (g)	3316(505)	2749(821)	1879(670)	1509(540)

Table 6.1 Fetal demographic data of the study population.

Results are presented as mean ± SD for each continuous variable unless otherwise specified.

6.5.2 Fetal leukocyte and monocyte counts in normal and pathological pregnancies complicated by preeclampsia and intrauterine fetal growth restriction

Cord blood white cell count and the monocyte count as calculated by Symex cytometer are presented in Table 6.2.

No differences were observed in the fetal/cord blood peripheral leukocyte count between clinical groups normal pregnancy, preeclampsia, IUGR and PE+IUGR (Table 6.2). In comparison to normal pregnancy, PE+IUGR group appear to have a significantly lower number of monocytes as a percentage of the total white cell count.

	Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)
WBC (x10^9 cells/L)	10.92 (6.77, 15.44)	8.04 (3.84, 9.36)	6.36 (5.88, 8.45)	5.54 (3.34, 8.34)
Monocyte %	9.0 (7.5, 11.0)	8.5 (5.7, 11.3)	8.1 (6.7, 11.3)	6.4* (2.9, 7.2)
Monocyte - Classical %	61.8 (47.7, 69.3)	30.1* (17.6, 48.3)	54.3 (37.1, 67.9)	42.6 (21.8, 46.9)
Monocyte - Intermediate %	34.4 (26.7, 46.9)	59.3 (41.6, 75.4)	41.2 (25.6, 55.3)	48.8 (41.3, 60.5)
Monocyte - Nonclassical %	2.5 (2.2, 2.9)	5.7* (5.1, 8.2)	3.4* (3.0, 6.9)	6.7* (5.5, 10.7)

Table 6.2 Fetal leukocyte count, monocyte count and the proportion of monocyte subtypes as a percentage of total monocyte count. Results are presented as median ± interquartile range for each continuous variable unless otherwise specified. *Statistically significant difference from normal pregnancy $p < 0.05$.

6.5.3 Fetal/cord blood peripheral blood monocyte subsets

To study the changes in monocyte subsets during preeclampsia and intrauterine fetal growth restriction, surface expression of CD14 and CD16 were used to gate the total monocytes into three subsets classical CD14++CD16-, intermediate CD14++CD16+ and non-classical CD14+CD16++ using flow cytometry. The results for fetal monocyte subsets as a percentage of total fetal monocytes are presented in Tables 6.2 and Figure 6.1.

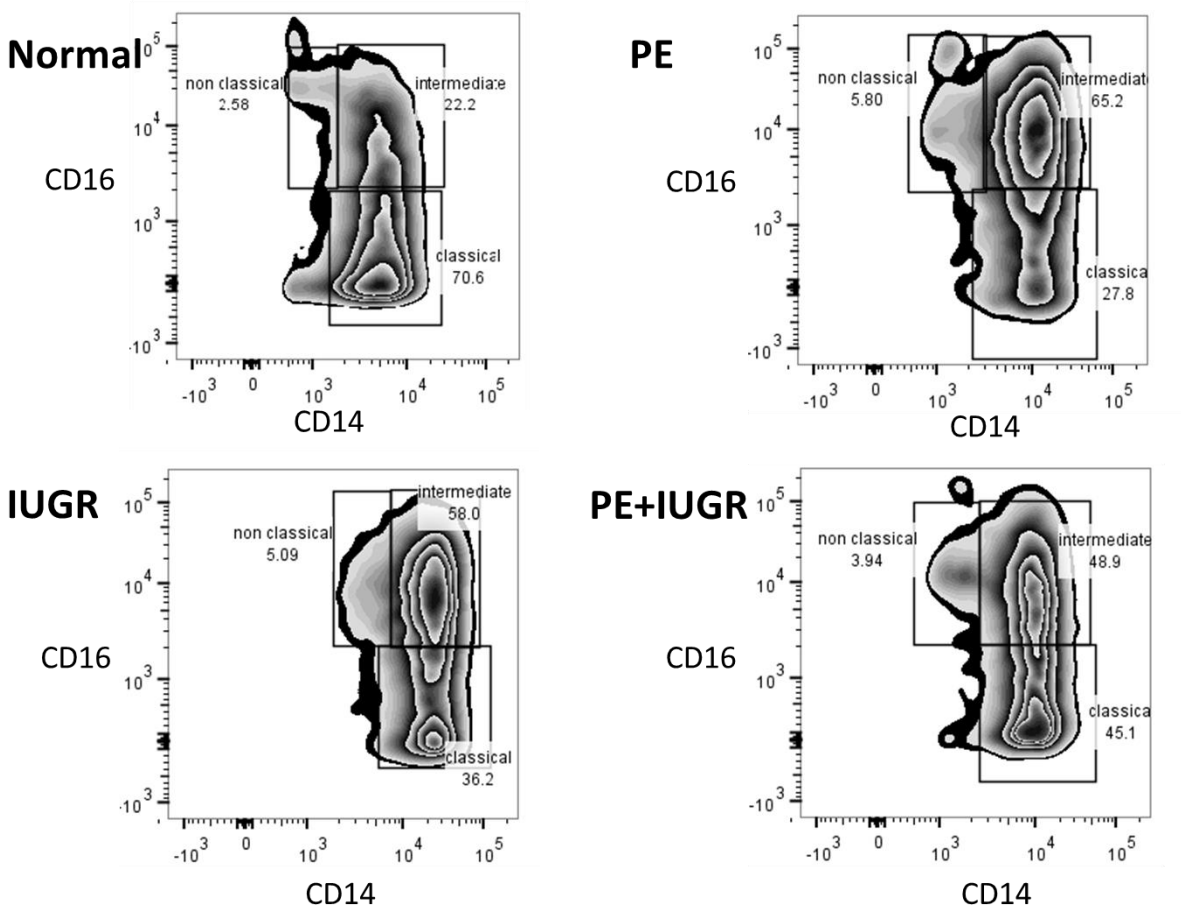

Figure 6.1 Zebra plots of a representative sample from each clinical group showing the gating strategy and distribution of monocyte subgroups. Classical monocytes CD14++CD16-, Intermediate monocytes CD14++CD16+, Non-classical monocytes CD14+CD16++. The distribution of subtypes demonstrated for Normal pregnancy, PE, IUGR and PE+IUGR. The intermediate monocytes were the dominant monocytes subtype in PE, IUGR and PE+IUGR. A trend towards a higher percentage of intermediate monocytes was seen in the pregnancies complicated by PE and IUGR although this difference did not reach statistical significance as compared to normal pregnancy.

The percentage of classical monocytes was significantly lower in preeclampsia (median 30%, interquartile 17.6%, 48.3%) as compared to normal pregnancy (median 61.8%, inter quartiles 47.7%, 69.3%,) ($p = 0.042$). Although intermediate monocytes appear to be the dominant monocyte subtype in PE, IUGR and PE+IUGR, this trend was not statistically significant in any of the clinical groups. The percentage of non-classical monocytes was noted to be significantly increased in the pathological groups PE (median 5.7% interquartiles 5.1%, 8.2%), IUGR (median3.4 % interquartiles 3.0%, 6.9 %), PE+IUGR (median 6.7% interquartiles 5.5 %, 10.7 %) as compared to normal pregnancy (median 2.5 % interquartiles 2.2 %, 2.9%).

6.5.4 Comparison of maternal and cord blood monocyte subset distributions.

A comparison was made between the maternal circulating monocyte subset distribution (as described in Chapter 5) and the cord blood monocyte subset distribution in the four clinical groups of normal; pregnancy, PE, IUGR and PE+IUGR. Comparative graphs are presented in Figure 6.2.

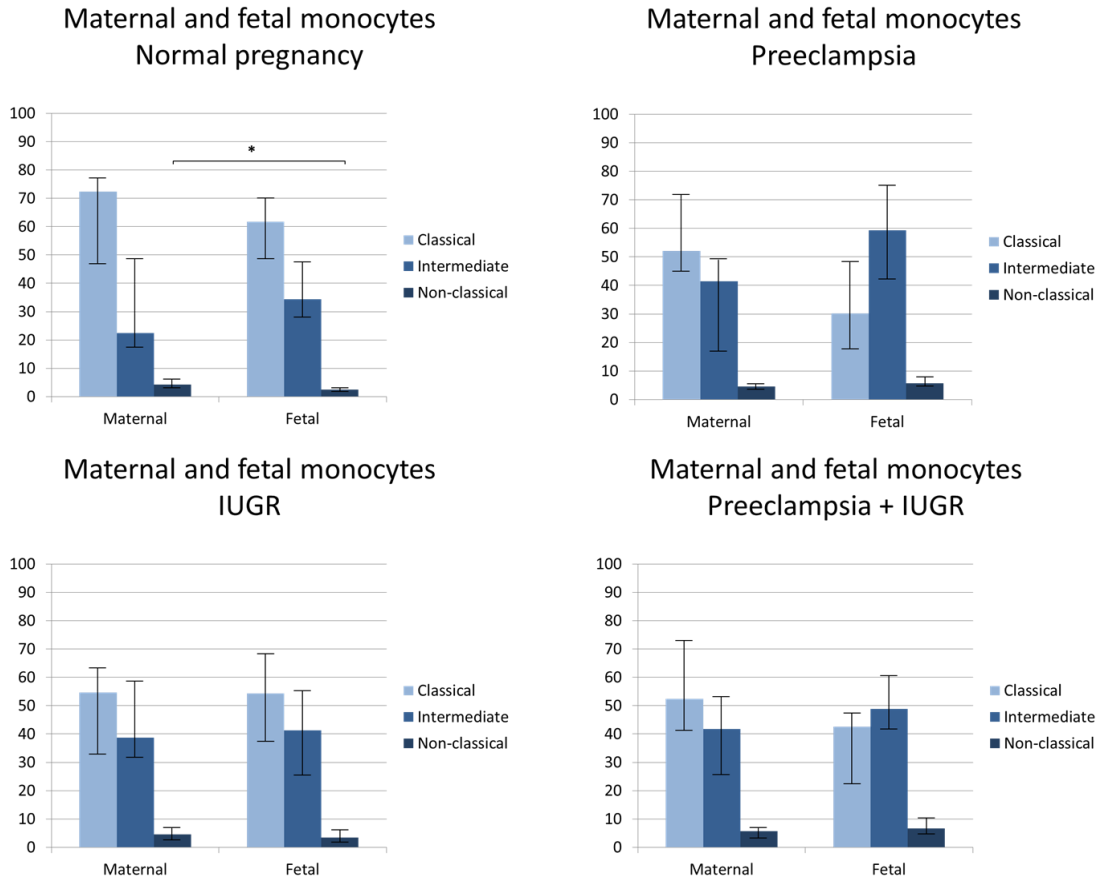

Figure 6.2 Comparison of maternal and fetal cord blood CD14+ monocytes in normal pregnancy, PE, IUGR and PE+IUGR.

The monocyte subset percentages have been compared between maternal and fetal circulation for each clinical group. The results are presented as median and interquartile. * $p < 0.05$, Mann-Whitney U test. The non-classical cord blood monocyte percentage was significantly lower than the maternal non–classical percentage in the normal pregnancy group.

6.6 Discussion

In the present study the distribution of the monocyte subsets in the fetal circulation (cord blood) was explored in normal pregnancies, PE, IUGR and PE+IUGR. The results were compared to maternal circulating monocyte subtype distribution to identify any differences between the two populations.

The gestational age at sample collection and birth weights were different between the normal pregnancies and the pathological pregnancies with gestation at cord blood collection and birth weights being significantly lower in PE, IUGR and PE+IUGR. By definition normal pregnancies will be delivered at term and this difference is difficult to overcome in the methodology of the study. The study was unable to demonstrate a difference in the total white cell count between the clinical groups. The percentage of monocytes as a percentage of total white cells in the cord blood samples (5.5-10.9%) were noted to be lower than previously published results with one study reaching up to 22.9% (413). This data finds a lower percentage of monocytes are in the PE+IUGR group. There are no published data for comparison in this clinical group.

This is the first description of the distribution of monocyte subsets in the cord blood using the nomenclature of classical, intermediate and non-classical based on CD14 and CD16 expression. Although there have been previous descriptions of monocyte subsets from cord blood of normal pregnancy, the distribution of monocyte subsets as a percentage of total monocytes has not been described using CD14 and CD16 expression.

Although the intermediate monocytes, as a percentage of the total monocytes in the cord blood samples, were the dominant subtype in cord blood in PE, IUGR and PE+IUGR (Table 6.2), reaching 59.3% in preeclampsia, 41.2% in IUGR and 48.8% in PE+IUGR as compared

to 34.4% in normal pregnancy, the difference did not reach statistical significance in this study. This is in contrast to the classical subtype being the prominent subtype in maternal circulation (Table 5.4). The small number of samples due to difficulties in sampling and processing of cord blood sample are likely to have affected the analysis of this study. The non-classical subset was seen to expand in PE, IUGR and PE+IUGR, reaching statistically significant levels as compared to normal pregnancy. This is a new distribution of monocyte subsets not previously described. These findings suggest that the innate immune signaling pathways related to monocytes are activated and functional in the fetal circulation in PE and IUGR. This study can be interpreted as a promising preliminary study to further explore the role of different monocytes subsets in the fetal circulation. Further study is recommended to characterize the cord blood monocyte phenotypes and their functional roles associated with normal pregnancy as well as pregnancies complicated by preeclampsia and intrauterine fetal growth restriction.

This study compared the maternal and the fetal monocyte subset distribution in normal pregnancy as well as pregnancies complicated by PE, IUGR and PE+IUGR. The results are limited by the small sample numbers. The study demonstrated that the non-classical percentages were lower in the cord blood as compared to the maternal circulation. These results are consistent with a previous study demonstrating a higher percentage of CD14+CD16+ monocytes in cultured cord blood monocytes as compared to cultured maternal monocytes stimulated with peptidoglycan (414). These findings are also concordant with previous description of a lower percentage of non-classical monocyte subsets described in cord blood of normal pregnancy as compared to maternal circulation (307). The differences suggest that fetal monocytes may be derived from fetal bone marrow rather than maternal bone marrow, although this was not assessed directly in this study.

Chapter 7 - Anti-angiogenic factor expression by maternal and fetal circulating peripheral monocytes

7.1 Summary

Introduction: Circulating levels of the anti-angiogenic factors sFlt-1 and sEndoglin are elevated in preeclampsia and IUGR. Whether or not excess sFlt-1 and Endoglin are produced by other cell types such as endothelial cells, monocytes/macrophages in the placenta as well as components of the maternal peripheral circulation has not been well explored.

Aims: To identify any variation of monocyte expression of Flt-1 and Endoglin across clinical groups, normal pregnancy, PE, IUGR or PE+IUGR and also with gestation, subtypes and polarization. Explore the CD86/CD163 ratio as a marker of monocyte activation with anti-angiogenic factor Flt-1 and Endoglin expression.

Methods: A prospective cross-sectional study was conducted and patients recruited from four clinical groups including normal pregnancy, PE, IUGR and PE+IUGR. Peripheral blood samples were collected from 54 pregnant women between 24-40 weeks of gestation. Twenty seven of these patients also had an umbilical cord blood sample collected at delivery. Monocyte phenotype distribution using CD14 and CD16 expression as well as surface expression of Flt-1, Endoglin, CD86 and CD163 were assessed by flow cytometry. For both maternal and fetal monocytes, the study examined Flt-1 and Endoglin expression overall and by subtype, and examined associations between monocyte anti-angiogenic factor expression and CD86/CD163 expression.

Results: Circulating monocytes from maternal and fetal circulations express Flt-1 and Endoglin as surface markers. A moderate positive correlation was noted in monocyte Flt-1 and Endoglin expression with increasing gestational age in the third trimester of pregnancy.

Flt-1 was mainly expressed by classical and intermediate monocytes. There was no overall variation in the maternal or fetal membrane bound Flt-1 expression across clinical groups other than an increase by the non-classical monocyte Flt-1 expression in preeclampsia. Surface expression of Endoglin was more prominent on intermediate and non-classical monocytes. Endoglin expression on maternal monocytes was reduced in PE+IUGR. A moderately positive correlation was noted between angiogenic factors Flt-1 and Endoglin expression and CD86/CD163 ratio indicating monocyte polarization.

Conclusion: The findings of this study suggest that maternal and fetal monocyte-derived Flt-1 and maternal monocyte Endoglin expression are unlikely to significantly contribute to the pathogenesis of PE or IUGR.

7.2 Introduction

Studies to date on preeclampsia and IUGR have focused on placental expression of Flt-1 and Endoglin, the main cell of origin being trophoblast cells (174, 178, 179). The expression of Flt-1 has been localized to only three tissues in the body, namely vascular endothelium, monocytes and trophoblast in the placenta. Little is known about the pro and anti-angiogenic factor expression by different subtypes of monocytes. This study explores whether monocyte over-expression of sFlt-1 could be an additional (extra-placental) source of sFlt-1 that contributes to the pathogenesis of preeclampsia and IUGR.

7.3 Aims

- Identify any variation of maternal and fetal monocyte expression of Flt-1 and Endoglin across clinical groups normal pregnancy, PE, IUGR or PE+IUGR and also with gestation, monocyte subtypes and polarization.

- Correlate the CD86/163 ratio as a marker of monocyte activation with anti-angiogenic factor Flt-1 and Endoglin expression.

7.4 Methods

A prospective cross-sectional case control study was conducted. Umbilical cord blood samples were collected at delivery from pregnancies between 24-40 weeks of gestation, delivering at Westmead Hospital during the period 2013 -2014. Sample preparation, staining protocols, experiment design, flow cytometry optimization and technique as well as the gating strategy have been described in detail in Chapter 2.6.

Data analysis involved the identification of the 3 main monocyte populations classical, intermediate and non-classical across the different clinical groups. Surface expression of anti-angiogenic factors Flt-1 and Endoglin and inflammatory markers CD86 and CD163 (Table 7.1) were examined. Mean Fluorescent Intensity (MFI) was recorded for the different monocyte populations using Flow Jo automatic analysis software.

7.5 Results

7.5.1 Maternal and neonatal demographic data and clinical characteristics of the study population

Results are presented for 54 maternal and 27 fetal cord blood samples from 24 – 40 weeks of gestation. Demographic data is presented in Chapter 5 (Table 5.1).

7.5.2 Differential distribution of maternal monocyte Flt-1 and Endoglin expression with increasing gestation in third trimester of pregnancy.

Spearman rank correlation was performed to identify any correlation between gestational age in normal third trimester pregnancy and the markers of interest Flt-1 and Endoglin. The percentage of monocytes expressing Flt-1 and Endoglin did not change with gestation but the Mean Fluorescence Intensity of Flt-1 and Endoglin on the monocytes showed a statistically significant increase with gestation (Table 7.1, Figure 7.1).

	Correlation coefficient	**Significance (2 tailed) p value 0.05**	**Interpretation**
Total monocyte Flt-1 MFI	0.402	0.049	Statistically significant trend towards increased Flt-1 expression with gestation
Percentage of monocytes expressing Flt-1	0.129	0.547	No significant correlation
Total monocyte Endoglin (CD105) MFI	0.457	0.025	Statistically significant trend towards increased Endoglin expression with gestation
Percentage of monocytes expressing Endoglin (CD105)	0.359	0.085	No significant correlation

Table 7.1 Differential distribution of monocyte Flt-1 and Endoglin expression with increasing gestation in normal third trimester of pregnancy, as tested in 24 pregnancies from 26 weeks to 40 weeks of gestation.

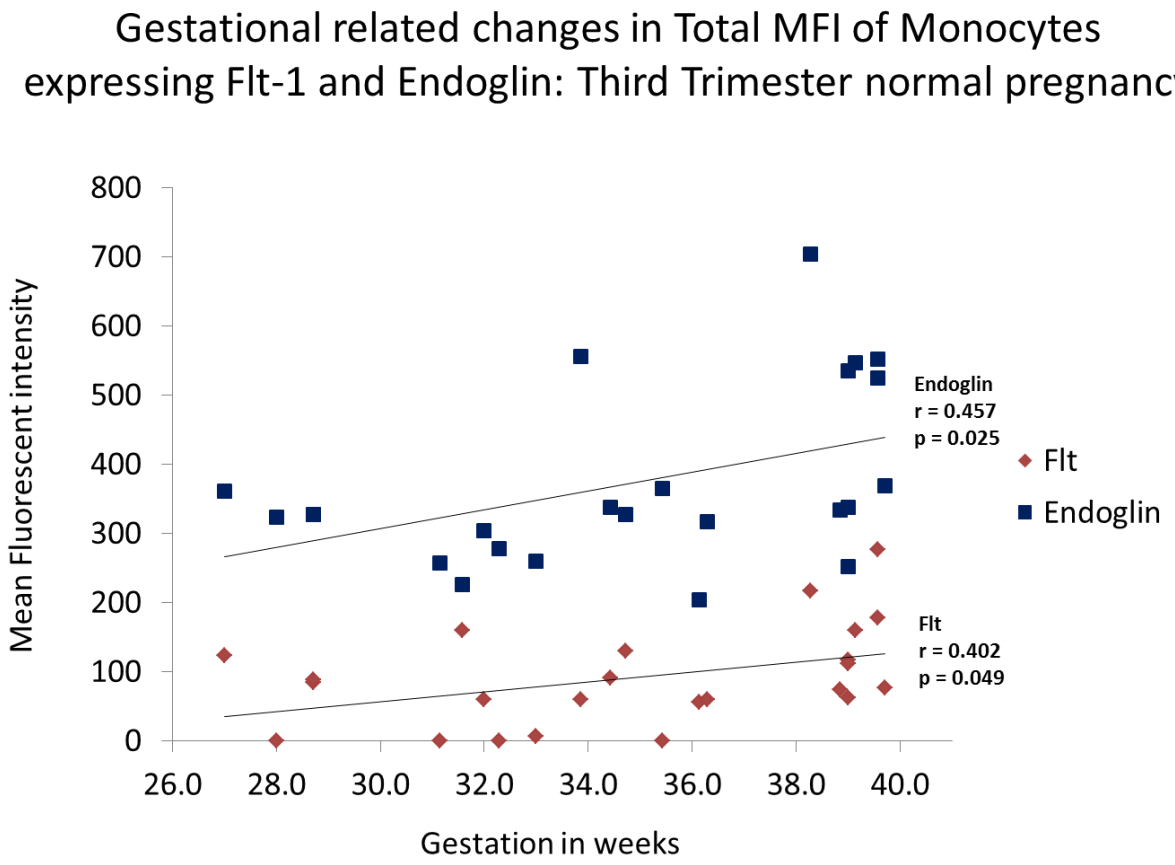

Figure 7.1 Mean fluorescent intensity of Flt-1 and Endoglin with increased gestation in normal pregnancies. There was a moderate and statistically significant correlation seen between gestational age and MFI of both Flt-1 and Endoglin.

7.5.3 Maternal monocyte Flt-1 expression

The expression of maternal monocyte surface Flt-1 expression was detected with flow cytometry and quantified as mean fluorescence intensity using Flow Jo software. The results for expression of Flt-1 are presented in Table 7.2 and Figures 7.2-7.3.

	Clinical Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)
Percentage of total monocytes expressing Flt-1	42.0 (33.4, 51.0)	37.6 (30.2, 54.9)	48.2 (25.1, 70.6)	42.4 (37.7, 44.5)
Total monocytes Flt-1 MFI	80.0 (57.0, 126.0)	49.0 (19.0, 71.0)	104.0 (33.5, 194.5)	73.0 (51.0, 102.0)
Classical monocytes Flt-1 MFI	140.0 (81.0, 181.0)	100.0 (65.0, 167.0)	185.5 (96.0, 278.0)	147.0 (102.0, 178.0)
Intermediate monocytes Flt-1 MFI	167.0 (99.0, 238.5)	128.0 (90.0, 204.0)	191.5 (99.5, 337.5)	140.0 (119.0, 212.0)
Non Classical Flt-1 MFI	52.5 (30.5, 98.0)	88.0 (48.0, 93.0)	84.0 (54.5, 103.0)	97.0 (73.0, 160.0)
Percentage of monocytes expressing Endoglin	62.7 (57.1, 76.2)	63.3 (58.8, 73.3)	74.5 (63.0, 82.1)	54.4*† (43.4, 63.6)
Total monocytes Endoglin MFI	330.0 (291.0, 446.5)	300.0 (267.0, 403.0)	362.5 (299.5, 575.5)	264.0* (75.0, 296.0)
Classical monocytes Endoglin MFI	342.0 (302.5, 403.5)	320.0 (308.0, 330.0)	454.5 (314.0, 644.0)	236.0* (216.0, 351.0)
Intermediate monocytes Endoglin MFI	653.0 (523.0, 1034.5)	483.0 (444.0, 896.0)	692.0 (468.0, 937.0)	499.0 (395.0, 595.0)
Non Classical monocytes Endoglin MFI	582.0 (526.0, 811.0)	458.0 (332.0, 678.0)	658.0 (293.0, 725.5)	464.0 (203.0, 641.0)

Table 7.2 Maternal monocyte expression of Flt-1 and Endoglin as markers of anti-angiogenic activity. Results are presented as median ± interquartile range for each continuous variable.

* Statistically significantly different from normal pregnancy $p < 0.05$. † Statistically significantly different from IUGR $p < 0.05$.

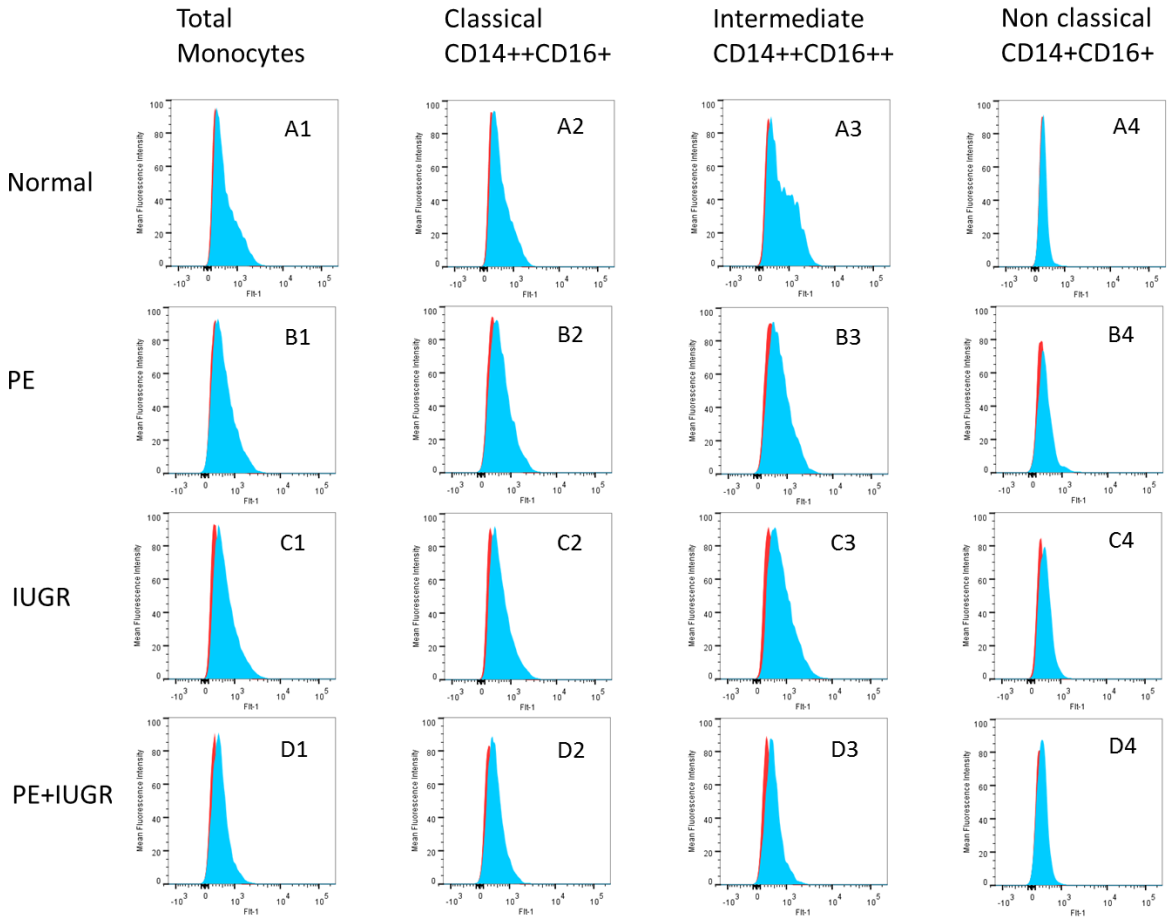

Figure 7.2 Distribution of Flt-1 on peripheral blood monocyte subsets in normal pregnancy and pregnancies complicated by preeclampsia and intrauterine fetal growth restriction or both.

A representative sample from each clinical group is presented as flow cytometry histogram plots of Flt-1 expression by different monocyte subsets. Values in histograms are for MFI (Mean Fluorescence Intensity) of Flt-1 expression. Red colour areas represents staining with isotype matched control antibody. Blue coloured areas represent staining with Flt-1 monoclonal antibody. A1-4: Normal pregnancy (Normal), B1-4: Preeclampsia (PE), C1-4: Intrauterine fetal growth restriction (IUGR), D1-4: Preeclampsia and intrauterine fetal growth restriction (PE+IUGR).

The number of total maternal monocytes expressing surface Flt-1 as a percentage of total maternal monocytes did not show any statistically significant differences between the clinical groups (Table 7.2). The results were the same in an analysis of the different monocyte subtypes classical, intermediate and non-classical. Although the total Flt-1 mean fluorescence intensity was not different across the clinical groups, differences were observed in the MFI of Flt expression in monocyte subtypes (Table 7.2 and Figure 7.4). The MFI of Flt-1 of classical and intermediate monocytes was higher than non-classical monocytes in all groups, reaching significance in normal pregnancy and IUGR.

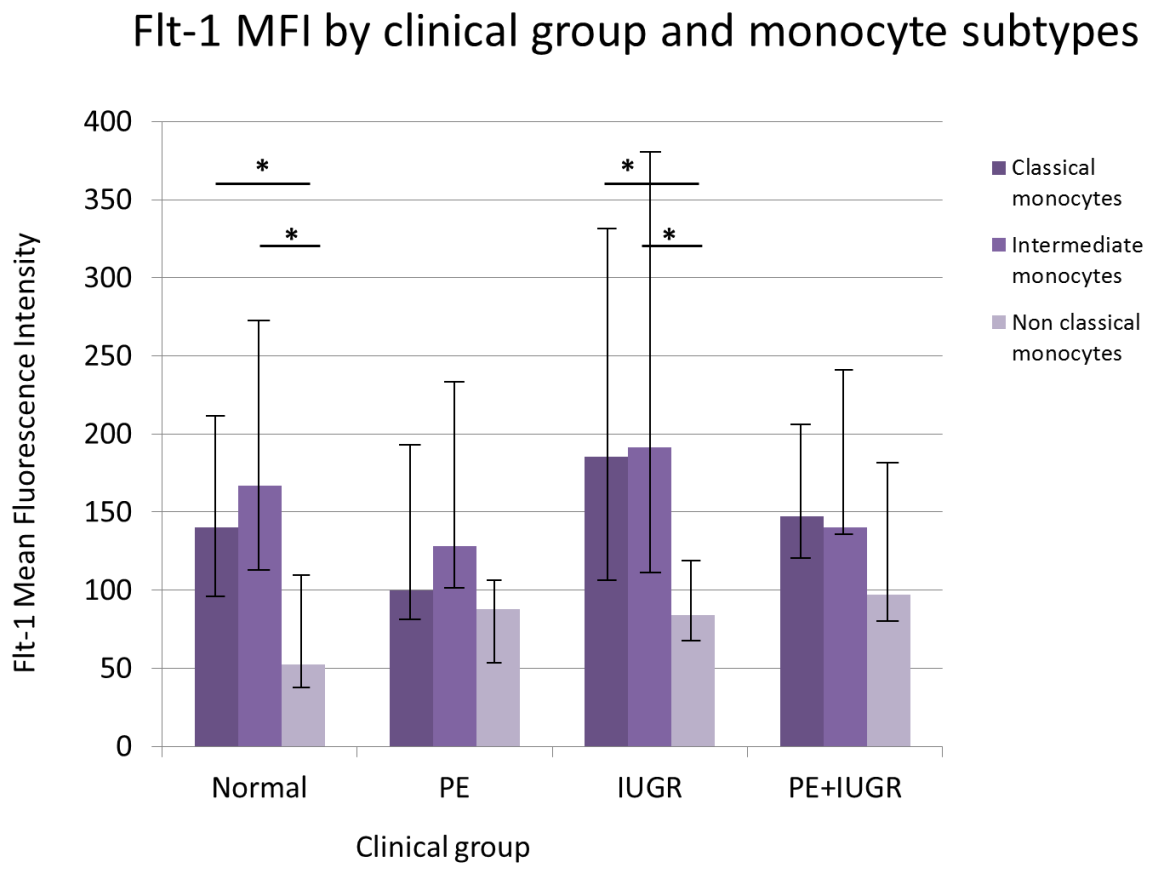

Figure 7.3 Analysis of monocyte surface Flt-1 expression (MFI) according to the subtype and clinical group. Results are presented as median and interquartile range.

* Statistically significant difference between groups $p < 0.05$.

7.5.4 Maternal monocyte Endoglin expression

The expression of maternal monocyte surface Endoglin was detected with flow cytometry and quantified as mean fluorescence intensity using Flow Jo software. The results for expression of Endoglin are presented in Table 7.2 and Figures 7.4-7.6.

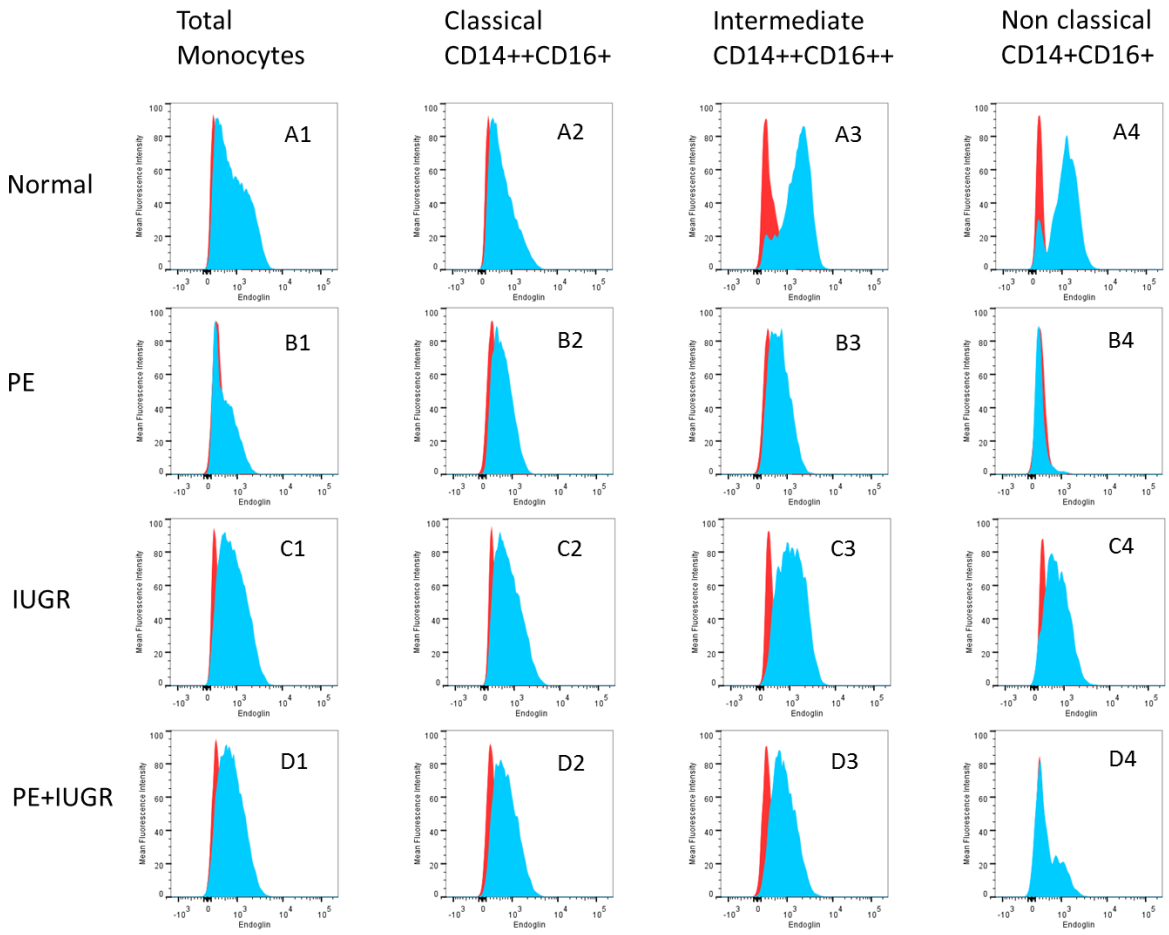

Figure 7.4 Distribution of Endoglin on peripheral blood monocyte subsets in normal pregnancy and pregnancies complicated by preeclampsia and intrauterine fetal growth restriction or both.

A representative sample from each clinical group is presented as flow cytometry histogram plots of Endoglin expression by different monocyte subsets. Values in histograms are for MFI (Mean Fluorescence Intensity) of Endoglin expression. Red colour areas represents staining with isotype matched control antibody. Blue coloured areas represent staining with Endoglin monoclonal antibody.

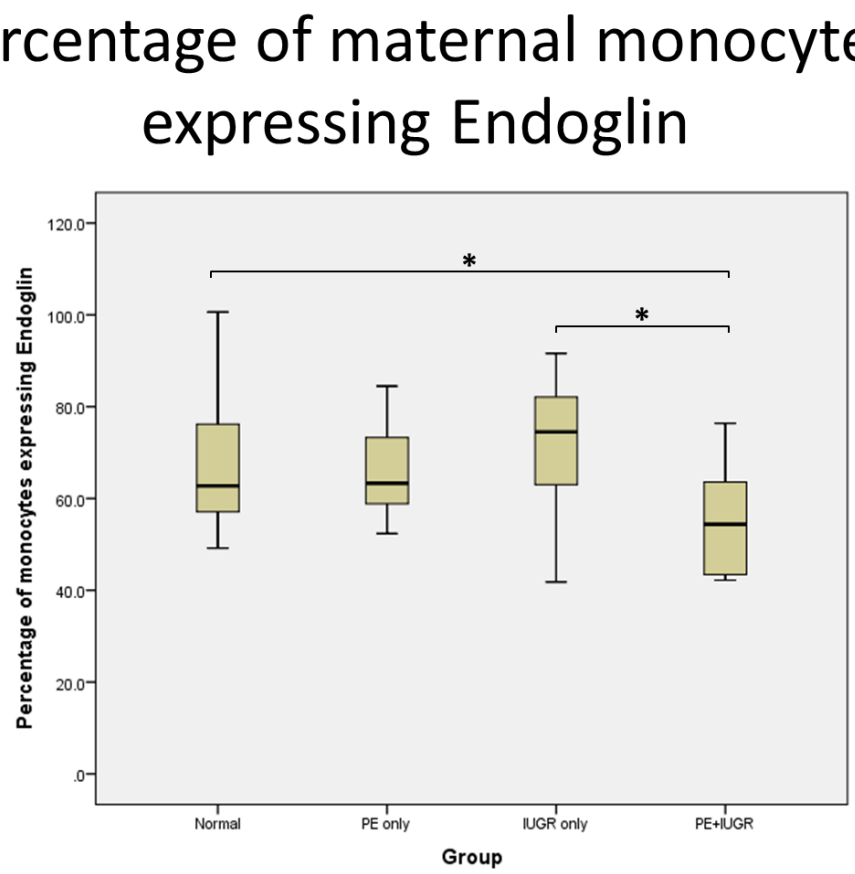

Figure 7.5 Number of maternal monocytes expressing surface Endoglin as a percentage of total maternal monocytes.

The percentage of monocytes expressing Endoglin in PE+IUGR was significantly less than normal and IUGR pregnancies. No detectable difference was noted between normal and preeclamptic pregnancies. The expression of Endoglin MFI appears to be significantly decreased in PE+IUGR for total monocytes and classical monocytes, as compared to the normal pregnancies (Table 7.2). The expression of Endoglin was significantly higher in the intermediate monocytes compared to classical monocyte subtype in all clinical groups. The MFI of non-classical monocytes for Endoglin is higher than classical monocytes in normal pregnancy (Figure 7.6).

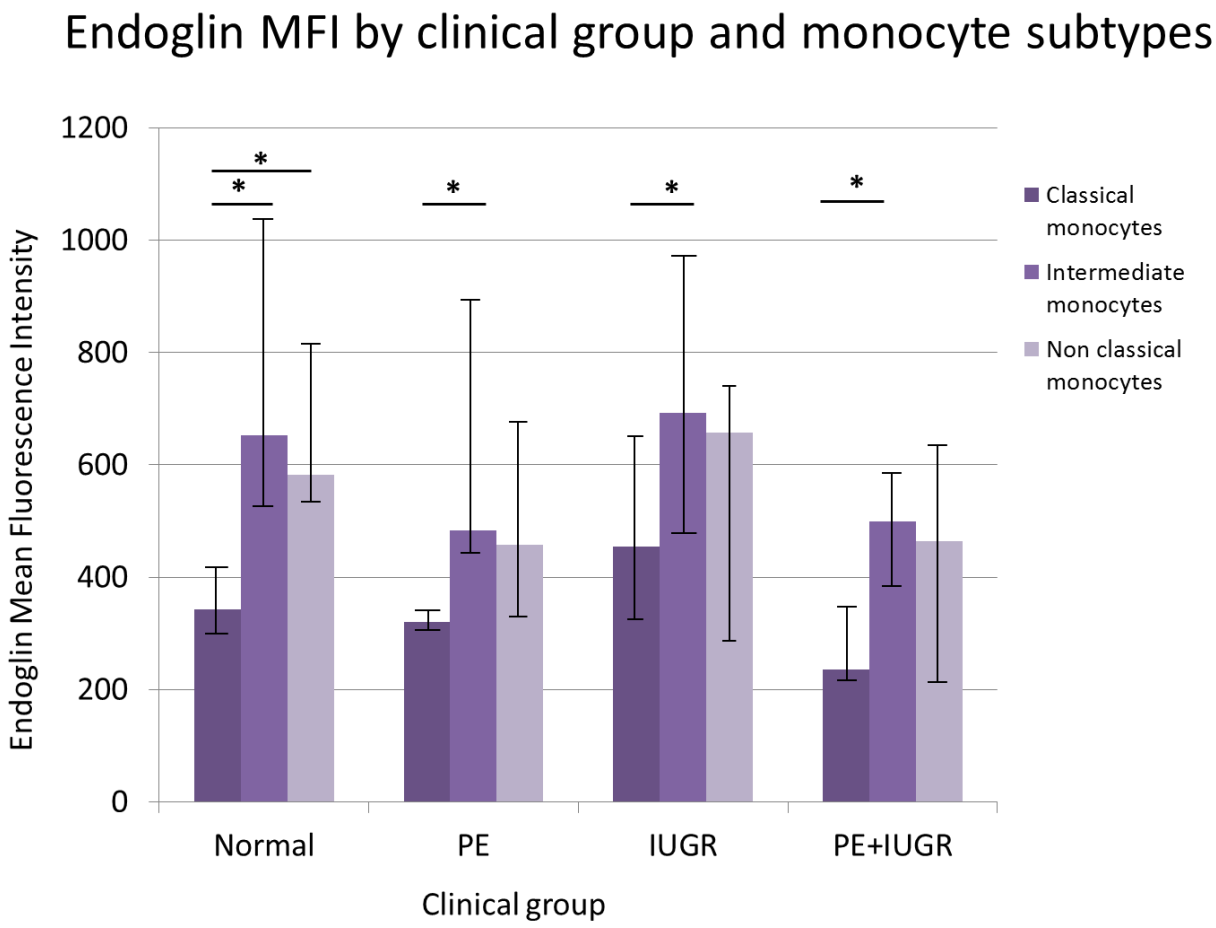

Figure 7.6 Analysis of monocyte surface Endoglin expression (MFI) according to the subtype and clinical group.

7.5.5 Maternal monocyte KDR expression

The current experiments were unable to demonstrate any surface staining for KDR on monocytes.

7.5.6 Correlation of anti-angiogenic factor Flt-1 and Endoglin expression with polarisation of monocytes into M1/M2 inflammatory phenotypes.

Spearman rank correlations were performed to investigate the relationship between monocyte Flt and Endoglin expression and polarization of monocytes into the M1/M2 phenotypes as documented by CD86/CD163 ratio. The results are summarized in Table 7.3 and displayed in Figures 7.7 – 7.9.

A moderately positive correlation was seen between Flt-1and Endoglin expressions. A strongly positive correlation was noted between Flt-1 and CD86 expression. The correlation between Flt-1 and CD163 was low (Table 7.3 and Figure 7.7).

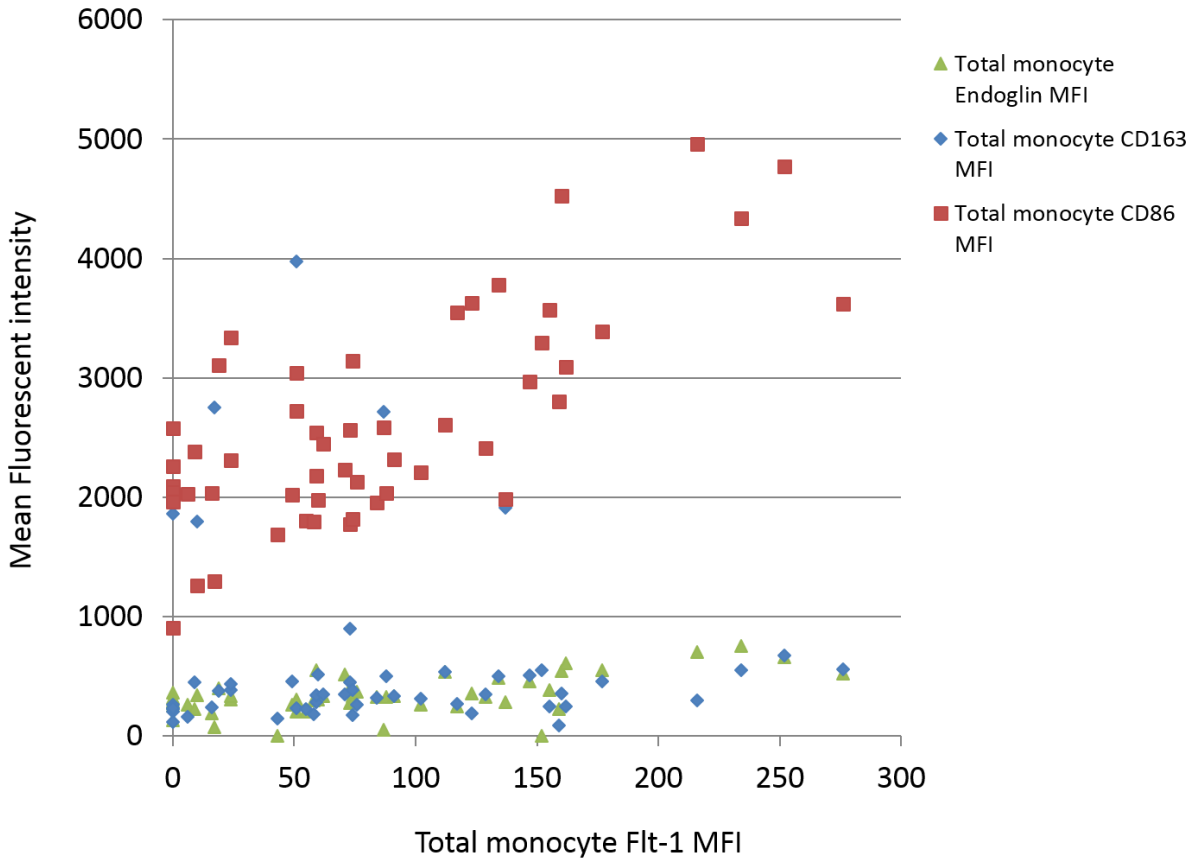

Figure 7.7 Correlations of total monocyte MFI of CD86, CD163, Flt-1 and Endoglin.

Variable 1	Variable 2	Correlation coefficient	Significance (2 tailed)	Interpretation
Total monocyte Flt-1 MFI	Total monocyte Endoglin MFI	0.547	$p < 0.001$*	Moderate positive correlation Statistically significant
Total monocyte Flt-1 MFI	Total monocyte CD86 MFI	0.643	$p < 0.001$*	Strong positive correlation Statistically significant
Total monocyte Flt-1 MFI	Total monocyte CD163 MFI	0.274	$P = 0.045$*	Low positive correlation Statistically significant
Total monocyte Flt-1 MFI	Total monocyte CD86/CD163 MFI ratio	0.412	$P = 0.002$*	Moderate positive correlation Statistically significant
Total monocyte Endoglin MFI	Total monocyte CD86 MFI	0.571	$p < 0.001$*	Moderate positive correlation Statistically significant
Total monocyte Endoglin MFI	Total monocyte CD163 MFI	0.145	$P = 0.297$	No correlation
Total monocyte Endoglin MFI	Total monocyte CD86/CD163 MFI ratio	0.045	$P = 0.001$*	Moderate positive correlation Statistically significant

Table 7.3 Spearman rank correlations for relationships between total monocyte Flt-1 MFI, Endoglin MFI, CD86 MFI, CD163 MFI and CD86/CD163 MFI ratio.

*Correlation is significant at $p < 0.05$ level (2-tailed).

A positive correlation was noted between Flt-1 and CD86/CD163 expression (Correlation coefficient 0.412, p = 0.002, Table 7.4, Figure 7.8) and between Endoglin and CD86/CD163 expression (Correlation coefficient 0.45, p = 0.001, Table 7.4, Figure 7.9)

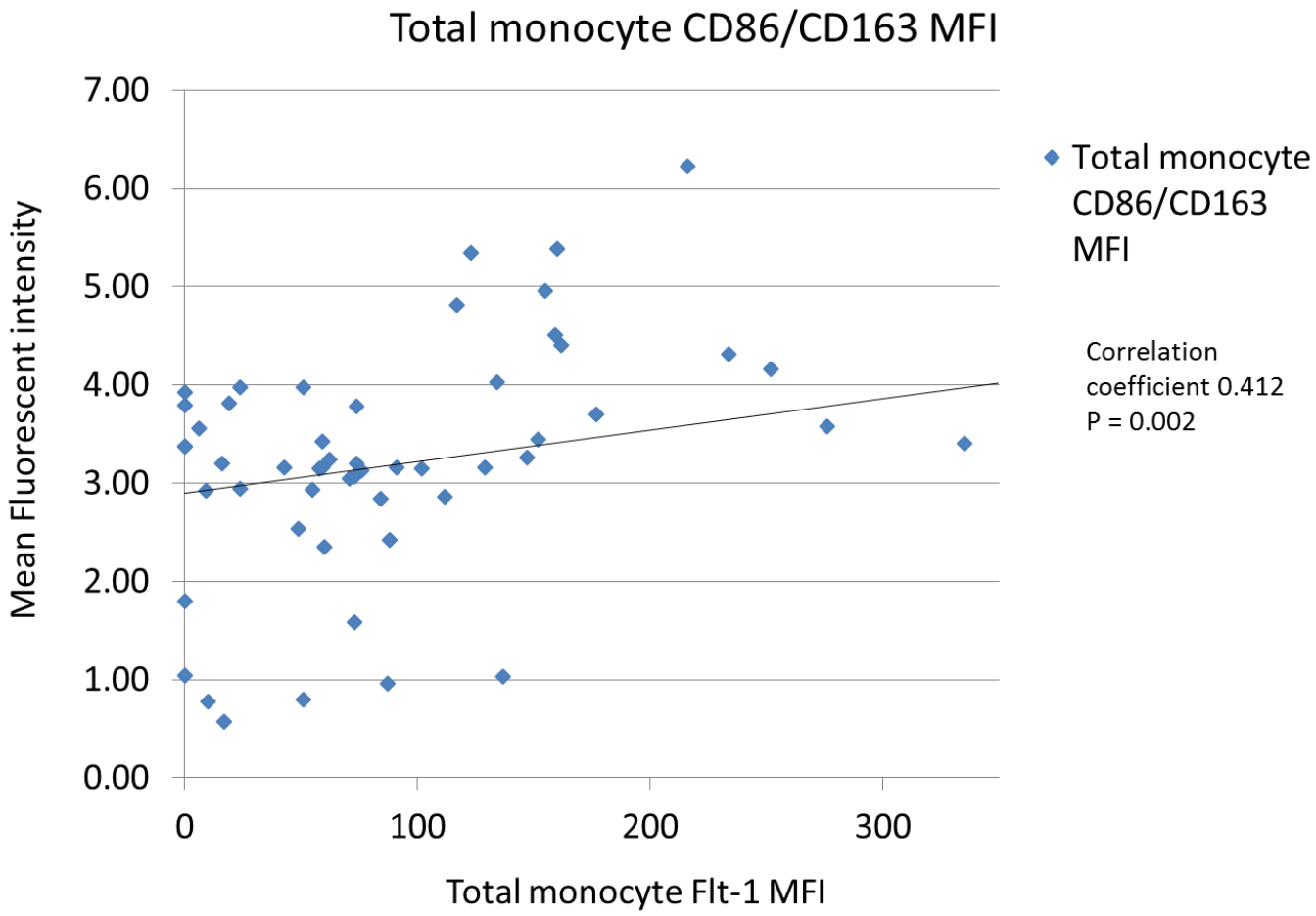

Figure 7.8 Correlations of total monocyte Flt-1 MFI and CD86/CD163 MFI ratio as an indicator of monocyte polarization into M1/M2 phenotypes.

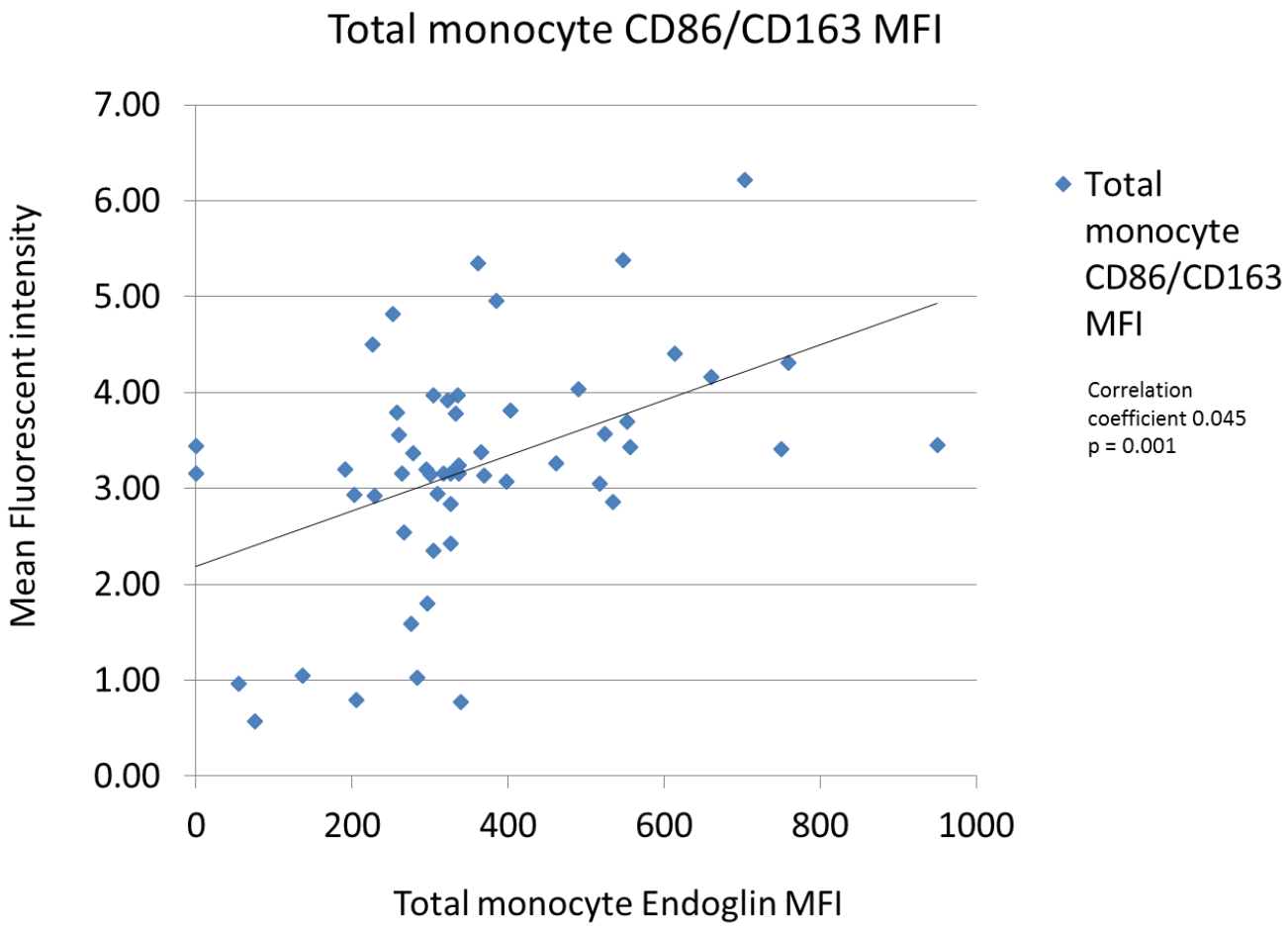

Figure 7.9 Correlations of total monocyte Endoglin MFI and CD86/CD163 MFI ratio as an indicator of monocyte polarization into M1/M2 phenotype.

7.5.7 Fetal Flt-1 expression

The expression of fetal monocyte surface Flt-1 expression was detected with flow cytometry and quantified as mean fluorescence intensity using Flow Jo software. The results for expression of Flt-1 are presented in Table 7.4. The number of total maternal monocytes expressing surface Flt-1 as a percentage of total maternal monocytes did not show statistically significant differences between the clinical groups. The results were the same for fetal monocyte Flt-1 expression in an analysis of the different monocyte subtypes classical, intermediate and non classical.

7.5.8 Fetal Endoglin expression

The expression of fetal monocyte surface Endoglin expression was detected with flow cytometry and quantified as mean fluorescence intensity using Flow Jo software. The results for expression of Endoglin are presented in Table 7.4 and Figure 7.10. Significantly more fetal monocytes in the IUGR group were noted to express Endoglin as compared to normal pregnancy, PE and PE+IUGR. A similar pattern of Endoglin expression was seen when the Endoglin expression was analyzed for monocyte subtypes.

	Clinical Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)	Median (Percentile 25, 75)
Percentage of monocytes expressing Flt-1	10.0 (6.6, 28.9)	32.3 (12.6, 49.0)	17.6 (12.7, 43.0)	30.4 (12.3, 37.7)
Total monocytes Flt-1 MFI	6.0 (0.0, 49.0)	43.5 (8.5, 87.5)	30.0 (15.0, 64.0)	34.0 (16.0, 87.0)
Classical monocytes Flt-1 MFI	58.0 (43.5, 98.0)	0.0 (0.0, 55.5)	42.0 (26.0, 137.0)	68.0 (34.0, 142.0)
Intermediate monocytes Flt-1 MFI	107.0 (68.0, 182.5)	41.5 (13.5, 128.0)	50.0 (43.0, 218.0)	157.5 (77.0, 223.0)
Non Classical Flt-1 MFI	72.0 (67.5, 140.5)	61.5 (0.0, 168.5)	48.0 (4.0, 80.0)	107.0 (71.0, 152.0)
Percentage of monocytes expressing Endoglin	49.7 (36.3, 59.3)	52.5 (39.9, 58.7)	77.9 *# (74.5, 81.9)	59.5 † (46.8, 73.5)
Total monocytes Endoglin MFI	181.0 (76.0, 331.0)	222.5 (108.5, 272.0)	469.0 *# (426.0, 490.0)	219.0 † (111.0, 280.0)
Classical monocytes Endoglin MFI	330.0 (207.0, 511.5)	235.5 (204.0, 270.5)	549.0*# (496.0, 642.0)	333.5 (259.0, 362.0)
Intermediate monocytes Endoglin MFI	375.0 (228.5, 548.0)	335.0 (229.0, 403.5)	745.0 *# (616.0, 869.0)	417.0 (269.0, 426.0)
Non Classical monocytes Endoglin MFI	279.0 (184.5, 496.0)	198.5 (76.0, 307.5)	537.0*# (457.0, 752.0)	224.0 (203.0, 332.0)

Table 7.4 Fetal monocyte expression of Flt-1 and Endoglin as markers of anti-angiogenic activity.

*Significantly different from normal pregnancy. # Significantly different from PE. †Significantly different from IUGR. Results are presented as median ± interquartile range for each continuous variable. For PE, 3 out of 4 MFI values for Flt-1 were not higher than the isotype control, resulting in a median value of 0.

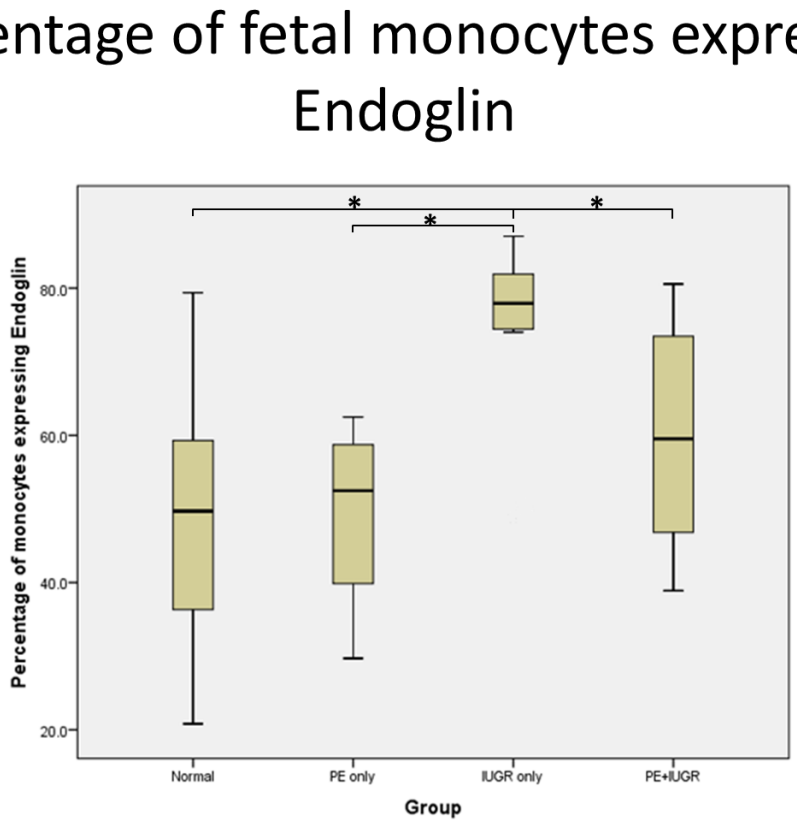

Figure 7.10 Number of fetal monocytes expressing surface Endoglin as a percentage of total fetal monocytes. The results are presented as median ± interquartile range.

* Statistically significant differences between clinical groups $p < 0.05$.

7.6 Discussion

The objective of this study was to investigate the maternal and fetal circulating monocyte expression of membrane bound Flt-1 and Endoglin in preeclampsia and intrauterine fetal growth restriction.

Monocytes and macrophages are known to differentiate into a variety of cell types such as osteoclasts in bone, Kupffer cells in liver, dendritic cells in the immune system, Hofbauer cells in the placenta and mature macrophages in a number of tissues. They are implicated in a variety of major disease processes such as inflammation, metabolic diseases, and cancer.

While there are sparse data available on monocyte function in preeclampsia including Flt-1 expression, there are no published studies exploring the role of peripheral monocytes in Endoglin expression in preeclampsia or the pregnancy complication of fetal growth restriction.

It is established that soluble Flt-1 and soluble Endoglin play a significant role in the pathogenesis of preeclampsia (155, 178, 189). This study has clearly shown that circulating monocytes from maternal and fetal circulations express Flt-1 and Endoglin as surface markers. Gestational variations in the monocyte Flt-1 and Endoglin expression in normal third trimester of pregnancy were investigated. While the number of monocytes expressing Flt-1 did not show any difference with gestation, the intensity of Flt-1 expression (MFI) showed a moderate increase with gestation in normal pregnancy (Figures 7.1 and 7.2). The percentage and MFI of Endoglin showed a moderate positive correlation with gestational age.

There was no overall variation in the membrane bound Flt-1 expression between the clinical groups of normal pregnancy, preeclampsia, intrauterine fetal growth restriction and a combination of preeclampsia and intrauterine fetal growth restriction. This data supports the notion that monocytes are unlikely to be the sole source of sFlt-1 in preeclampsia.

Endoglin expression on maternal monocytes was reduced in PE+IUGR (Figure 7.5) and significantly increased on fetal monocytes in IUGR (Figure 7.10). These results suggest that monocyte Endoglin production from the fetal/placental circulation may contribute to the total circulating maternal Endoglin. However for confirmation of this it is will be necessary to determine if Endoglin bound to fetal monocytes is secreted as soluble Endoglin, which reaches the maternal circulation , and whether there is sufficient quantity to impact on total soluble Endoglin. The authors' previous work (chapter 4) showed significantly elevated maternal sEndoglin level in women with PE+IUGR compared to normal pregnancies (189).

Little is known about the pro and anti-angiogenic factor expression by different subtypes of monocytes. The Flt-1 and Endoglin expression on circulating monocytes were further evaluated according to the monocyte subgroups. The Flt-1 MFI of classical and intermediate monocytes was noted to be higher than non-classical monocytes in all groups (Figure 7.3), reaching statistical significance in the normal pregnancy and IUGR groups. The surface expression of Flt-1 on non-classical monocytes is significantly lower than classical and intermediate in normal pregnancy and IUGR, but shows no difference in pregnancies affected by preeclampsia. This suggests an increase in Flt-1 expression by non-classical monocytes in pregnancies affected by preeclampsia.

The monocyte Flt-1 has been described to have a significant role in the chemotaxis of peripheral monocytes and tissue macrophages (147). It is possible that surface expression of Flt-1 on monocytes in preeclampsia and IUGR may have a functional role in chemotaxis and autocrine expression associated with placental injury in PE and IUGR. However this study has not supported a functional role of maternal monocyte-derived Flt-1 in PE or IUGR.

No significant variation in Endoglin expression was noted between the clinical groups other than a small reduction in classical monocytes in PE+IUGR. This data suggests that Endoglin is mainly expressed on intermediate and non-classical monocytes in normal pregnancy as well as pregnancies complicated by PE and IUGR (Figure 7.6).

The study of monocyte Flt-1 and Endoglin expression was performed in stage two of the research and on a different patient population to the study on circulating angiogenic factors. Plasma samples from the monocyte study group have been stored for future research into correlation between monocyte Flt-1 and Endoglin expression and circulating sFlt-1 and sEng levels.

The association of monocyte polarization into M1/M2 phenotypes and correlation with anti angiogenic factor Flt-1 and Endoglin expression has not been previously described in the literature. This is the first study to explore the relationship of Flt-1 and Endoglin with monocyte subtypes and M1/M2 polarization. The current study investigated whether Flt-1 and Endoglin were expressed differently by monocytes with inflammatory M1 or healing M2 phenotype. CD86 was used as a marker of M1 phenotype while CD163 was used as a M2 marker. The CD86/163 ratio was used as an indication of the inflammatory status of the monocytes in the different clinical groups. These findings demonstrated that Flt-1 and Endoglin expression is strongly associated with the inflammatory (M1) monocyte phenotype, and that Flt-1 is also (weakly) expressed by M2 monocytes.

Previous results of this study have shown that while there were no significant differences in the M1/M2 polarization of monocytes between normal pregnancy and PE (Table 5.4), a significant shift towards M2 polarization of all subclasses of maternal peripheral monocytes is evident in pregnancies complicated by IUGR. This may represent the body's response to and repair of significant placental injury. This study has demonstrated a moderate association between M1 polarization and Endoglin/Flt-1 surface expression of monocytes. The above findings as well as a reduced Endoglin expression in PE+IUGR documented in the current study suggest that monocyte surface expression of Flt-1 and Endoglin does not significantly contribute to the increased circulating Flt-1 and Endoglin noted in PE and IUGR. It is possible that M1 polarized monocytes and /or macrophages act locally within the placenta promoting the anti-angiogenic state. Further studies into placental monocytes and macrophages are required to further clarify this aspect.

The current study has contributed significantly to the literature by an exploration of Flt-1 and Endoglin expression by maternal and fetal monocytes in normal pregnancy and pregnancies complicated by preeclampsia and fetal growth restriction. The data and the interpretations are limited by small patient numbers and the cross sectional design of the study.

Chapter 8 - Maternal and fetal lipid profiles in preeclampsia and intrauterine fetal growth restriction

8.1 Summary

Introduction: Elevated lipid levels in normal pregnancy as well as in preeclampsia have been documented. Maternal lipid status in PE and PE+IUGR has not been well documented.

Aims: To characterize the serum levels of TC, HDL, LDL, Apo lipoprotein A1, Apo lipoprotein B and their ratios TC/HDL and ApoB/ApoA1 in the maternal and fetal circulations of normal pregnancy, PE, IUGR and PE+IUGR.

Methods: A prospective cross-sectional case control study was conducted in the clinical groups normal pregnancy, PE, IUGR and PE+IUGR. Maternal and fetal lipid levels were measured by enzymatic analysis and immune-turbidimetric enzymatic assays. IUGR was defined by elevated umbilical artery Doppler resistance in association with SGA.

Results: TC, HDL, LDL and TC/HDL levels did not show any significant variation between clinical groups in the maternal or fetal circulation. Apo lipoprotein levels A1 and B were not different between maternal groups but there was a statistically significant elevation in fetal ApoB levels in PE, IUGR and PE+IUGR compared to normal pregnancies. TG levels were elevated in maternal PE and fetal IUGR compared to normal pregnancies. This data also did not support a gestational variation or a difference between PE and IUGR in cholesterol levels in the maternal and fetal circulation.

Conclusion: Excessive elevation in maternal TG levels may have a role in the pathogenesis of PE and should be researched as a screening test to identify pregnant women at risk of preeclampsia as well as long term cardiovascular risk. The elevated TG and Apolipoprotein B

levels in cord blood may be a useful link in identifying newborns at risk of cardiovascular disease in later life.

8.2 Introduction

Preeclamptic women have been shown to exhibit, in the third trimester and puerperium, higher mean serum TG concentration and lower high density lipoprotein (HDL) cholesterol and Apolipoprotein A1 levels compared with healthy pregnant women in third trimester of pregnancy (364). LDL-mean particle diameter (LDL-MPD) and LDL cholesterol-Apo lipoprotein B ratio were also significantly reduced in the pathologic group. Antepartum serum triglyceride and free fatty acid concentrations were increased approximately twofold in women with preeclampsia relative to uncomplicated pregnancies in another study into lipids in preeclampsia. Total, high-density lipoprotein, and low-density lipoprotein cholesterol concentrations did not differ between groups (415). The lipid profiles in pregnancies complicated by IUGR without PE have not been well characterized. While little literature is available on the fetal lipid profile in PE, the lipid status in cord blood and neonates of IUGR pregnancies have been documented.

8.2 Aims

To examine the serum levels of TC, HDL, LDL, Apo lipoprotein A1, Apo lipoprotein B and their ratios TC/HDL and ApoB/ApoA1 in the maternal and fetal circulations of normal pregnancy, preeclampsia, intrauterine fetal growth restriction and preeclampsia with IUGR.

8.4 Methods

A prospective cross-sectional case control study was conducted. Pregnant women between 24-40 weeks of gestation, delivering at Westmead Hospital during the period 2013 -2014 were recruited. Maternal samples were taken prior to delivery and some of the pregnancies had fetal cord blood samples collected at delivery as per the methods describe in Chapter 2.7. The lipid profile including fasting serum concentrations of total cholesterol, HDL, LDL, triglycerides ApoA and ApoB were tested and recorded for each donor.

8.5 Results

8.5.1 Maternal and neonatal demographic data and clinical characteristics of the study population

Maternal fasting venous blood samples were collected from 52 women prior to delivery. Pregnancies that were delivered within one week of maternal blood sample collection had cord blood samples collected at delivery. The number of fetal samples was also limited by after hours emergency deliveries and the difficulty in collecting venous cord blood from the umbilical vein in some cases. Thirty cord blood samples from the above pregnancies were available for analysis. All women had a normal glucose tolerance test. All women were consuming an unrestricted diet and were fasting for at least 6 hours prior to the test. The normal pregnancy patients were gestationally matched to the pathological pregnancies complicated by PE and IUGR. The maternal and fetal demographic data are presented in Table 8.1.

Analysis of the effect of confounding variables using Fischer's exact test showed that PE+IUGR patients were older, were delivered earlier and had lower birth weights. There was

no significant difference in BMI between groups except the pregnancies complicated by IUGR only, where the BMI was noted to be lower as compared to the PE group. No difference was noted in the incidence of smoking and mode of delivery between the different clinical groups. The majority of preeclamptic patients received anti-hypertensive treatment whereas only half of the PE+IUGR patients were medicated. The gestational age at sample collection in the PE+IUGR group was significantly different from the other three groups of Normal, PE and IUGR. Significant differences in both gestation at delivery and birth weight seen between normal pregnancy and PE, IUGR and PE+IUGR, as well as PE+IUGR and the other three groups. No difference in gestational age was seen between PE and IUGR (Table 8.1)

	Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Mean (Standard Deviation)	Mean (Standard Deviation)	Mean(Standard Deviation)	Mean (Standard Deviation)
Number of patients	20	10	12	10
Maternal age (years)	28.8 (4.0)	27.5 (5.8)	28.3 (4.9)	33.2 (6.7)*#†
BMI	27.6 (5.4)	30.3 (8.0)	24.1 (5.3)#	28.0 (6.0)
Gestation age at sample collection	36.1 (3.8)	35.2 (3.4)	34.8 (4.2)	31.7 (2.8)*# †
Gestation age at delivery	39.3 (0.8)	36.5 (3.4)*	36.2 (2.2)*	32.0 (3.0)*# †
Birth weight (g)	3412 (388)	2897 (903)*	1831 (781)*	1398 (619)*# †
Primipara (%)	40	80	58.3	90
Smoking (%)	5.6	0.0	8.3	10
Mode of delivery (LSCS) %	75	80	75	80

Table 8.1: Maternal and fetal demographic data and clinical characteristics of the study population.

Results are presented as mean ± SD for each continuous variable unless otherwise specified. *Statistically significant difference from normal pregnancy. # Statistically significant difference from PE. †Statistically significant difference from IUGR.

8.5.2 Gestational age related changes in maternal lipid profile in normal third trimester pregnancy.

Spearman rank correlation of gestational age and the measured variables total cholesterol, HDL, LDL, triglycerides ApoA1 and ApoB for all the study patients including PE, IUGR and PE+IUGR as well as for normal group only did not show any gestational variation. The correlation coefficients and the significance (2 tailed) have been listed in Table 8.2.

	Normal pregnancy Rank correlation	Normal pregnancy 2 tailed significance	All Groups Rank correlation	All Groups 2 tailed significance
TC	-.125	.598	-.041	.772
HDL	.057	.812	.013	.930
LDL	-.218	.355	-.071	.623
TC/HDL	-.159	.502	.023	.872
ApoA1	.099	.688	-.046	.749
ApoB	-.011	.966	.034	.813
ApoB/ApoA1	-.048	.844	.071	.622

Table 8.2 Gestation related changes in lipid profile within third trimester.

Spearman rank Correlation coefficients and two tailed significance for gestational age variation in total cholesterol, HDL, LDL, triglycerides ApoA1 and ApoB and their ratiosTC/HDL, ApoB/ApoA1.

A significant correlation between gestational age and lipid parameters was not demonstrated for any of the groups for samples taken from 26 weeks to 40 weeks. This does not exclude gestation related changes in lipid profile in third trimester compared to first or second trimester.

8.4.3 Maternal lipid profile in normal and pathological pregnancies complicated by preeclampsia and intrauterine fetal growth restriction.

The maternal lipid profiles of each clinical group are summarized in Table 8.3 and Figures 8.1 and 8.2.

The results confirm that the TG levels are at higher levels in pregnancy compared to the normal population. There was a significant increase in the TG levels in preeclampsia as compared to normal pregnancy and IUGR (Figure 8.1). No significant differences noted between the clinical groups for Apolipoprotein with the majority of Apo A1 and B readings noted to be lower than the 95^{th} centile for third trimester pregnancy (Figure 8.2).

Maternal Data	Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (Quartiles 25, 75)	Median (Quartiles 25, 75)	Median (Quartiles 25, 75)	Median (Quartiles 25, 75)
Number of samples	7	5	8	10
TC mmol/L	6.6 (6.1, 7.5)	7.2 (5.9, 8.0)	6.9 (6.0, 7.5)	6.2 (5.5, 6.7)
HDL mmol/L	1.8 (1.6, 1.9)	1.8 (1.5, 2.1)	1.6 (1.3, 1.9)	1.5 (1.3, 2.1)
LDL mmol/L	3.6 (2.4, 4.6)	3.7 (2.2, 4.3)	3.6 (3.0, 4.1)	2.9 (2.6, 3.0)
TC/HDL ratio	3.8 (3.4, 4.7)	3.8 (3.3, 4.9)	4.0 (3.0, 4.7)	3.7 (2.9, 4.9)
Trig mmol/L	2.99 (2.23, 4.11)	4.21*† (3.39, 4.93)	2.62 (1.85, 3.19)	2.99 (2.19, 5.31)
Apo A1 g/L	1.91 (1.65, 2.19)	2.06 (1.87, 2.39)	1.87 (1.71, 2.02)	1.91 (1.78, 2.22)
Apo B g/L	1.44 (1.12, 1.82)	1.38 (1.19, 1.63)	1.44 (1.26, 1.53)	1.24 (1.08, 1.53)
Apo B: Apo A1	0.69 (0.60, 0.98)	0.63 (0.59, 0.81)	0.75 (0.59, 0.87)	0.67 (0.47, 0.72)

Table 8.3 Maternal lipid profile in normal pregnancy and pregnancies complicated by preeclampsia, intrauterine fetal growth restriction and a combination of PE and IUGR.

Results are presented as median ± interquartile range for each continuous variable.

*Statistically significant difference from normal pregnancy. † Statistically significant difference from IUGR.

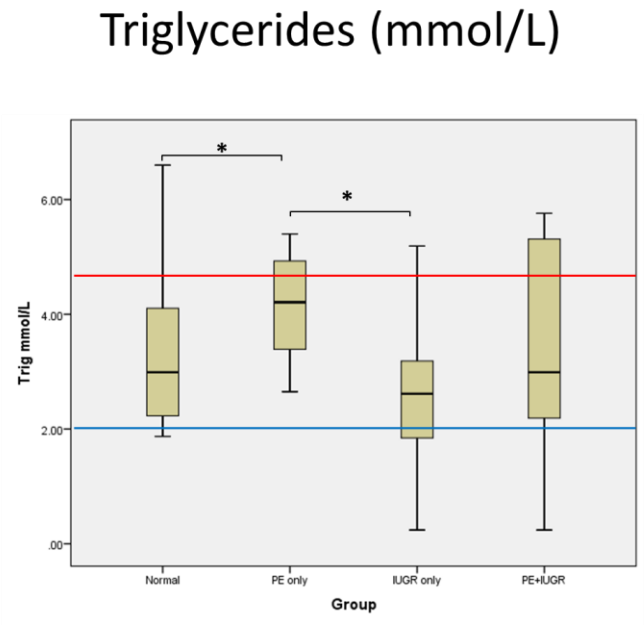

Figure 8.1 Comparison of maternal total triglyceride (TG) levels between clinical groups.Blue line indicates the 95th centile value in the commercial TG assay used for analysis for non-pregnant population. Red line indicates the 95th centile for third trimester pregnancy using reference ranges published by Piechota et al (350).

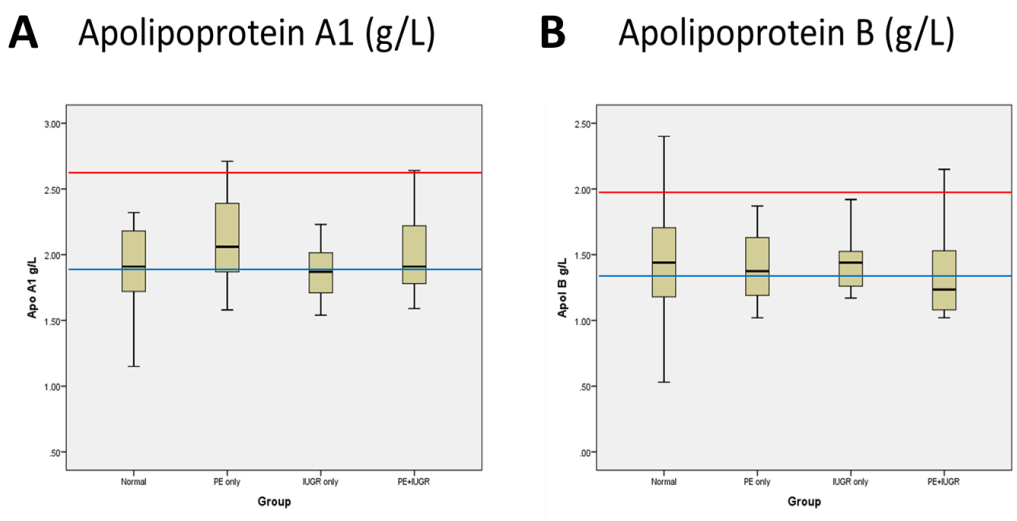

Figure 8.2 Comparison of maternal A = Apo lipoprotein A1 (ApoA1) levels and B = maternal Apo lipoprotein B (ApoB) levels between clinical groups. Blue line indicates the 95th centile value for non-pregnant population in the commercial ApoB assay used for analysis. Red line indicates the 95th centile for third trimester pregnancy using reference ranges published by Piechota et al (350). The ApoA1 and ApoB levels in all groups of pregnancy were above the non-pregnant levels.

8.5.4 Fetal lipid profile in normal and pathological pregnancies complicated by preeclampsia and intrauterine fetal growth restriction

The fetal lipid profiles of each clinical group are summarized in Table 8.4 and Figures 8.3-8.5. Although the fetal TG levels appeared to be higher in pathological groups as compared to normal pregnancy, the results did not reach significance except for PE+IUGR (Figure 8.3). No differences were noted in the cholesterol levels between groups. The Apo A1 levels were not statistically significant between groups. Significant differences were noted in ApoB levels between normal pregnancy and all three pathological groups. No significant differences seen

between PE, IUGR and PE+IUGR (Figure 8.4). The fetal Apo lipoprotein B/A1 ratios (ApoB/ApoA1) were significantly elevated in IUGR and PE+IUGR.

Fetal Data	Group			
	Normal	**PE only**	**IUGR only**	**PE+IUGR**
	Median (Quartiles 25, 75)	Median (Quartiles 25, 75)	Median (Quartiles 25, 75)	Median (Quartiles 25, 75)
Number of samples	7	5	8	10
TC mmol/L	1.5 (1.3, 2.0)	1.8 (1.5, 2.0)	1.8 (1.5, 2.2)	1.8 (1.5, 2.2)
HDL mmol/L	0.6 (0.4, 0.7)	0.7 (0.7, 0.8)	0.7 (0.5, 1.1)	0.7 (0.4, 0.7)
LDL mmol/L	0.8 (0.6, 1.0)	0.8 (0.7, 1.1)	1.0 (0.8, 1.0)	1.0 (0.9, 1.1)
TC/HDL ratio	2.5 (2.2, 3.2)	2.3 (2.2, 2.9)	2.55 (2.0, 3.4)	3.1 (2.6, 3.5)
Trig mmol/L	0.16 (0.14, 0.21)	0.35 (0.23, 0.37)	0.26 (0.12, 0.54)	0.36* (0.32, 0.43)
Apo A1 g/L	0.70 (0.66, 0.81)	0.78 (0.77, 0.81)	0.84 (0.70, 0.94)	0.70 (0.60, 0.82)
Apo B g/L	0.20 (0.20, 0.22)	0.29* (0.22, 0.33)	0.33* (0.32, 0.36)	0.33* (0.29, 0.33)
Apo B: Apo A1	0.29 (0.25, 0.30)	0.35 (0.27, 0.42)	0.37* (0.30, 0.47)	0.42* (0.35, 0.53)

Table 8.4 Fetal lipid profile in normal pregnancy and pregnancies complicated by preeclampsia, intrauterine fetal growth restriction and a combination of PE and IUGR.

Results are presented as median ± interquartile range for each continuous variable.

*Statistically significant difference from normal pregnancy.

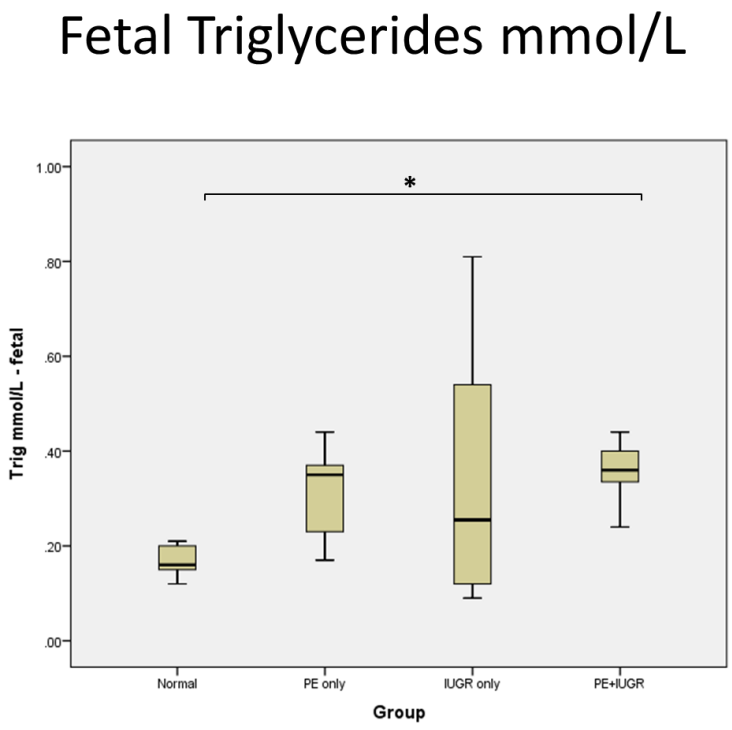

Figure 8.3 Comparison of fetal total triglyceride (TG) levels between clinical groups.

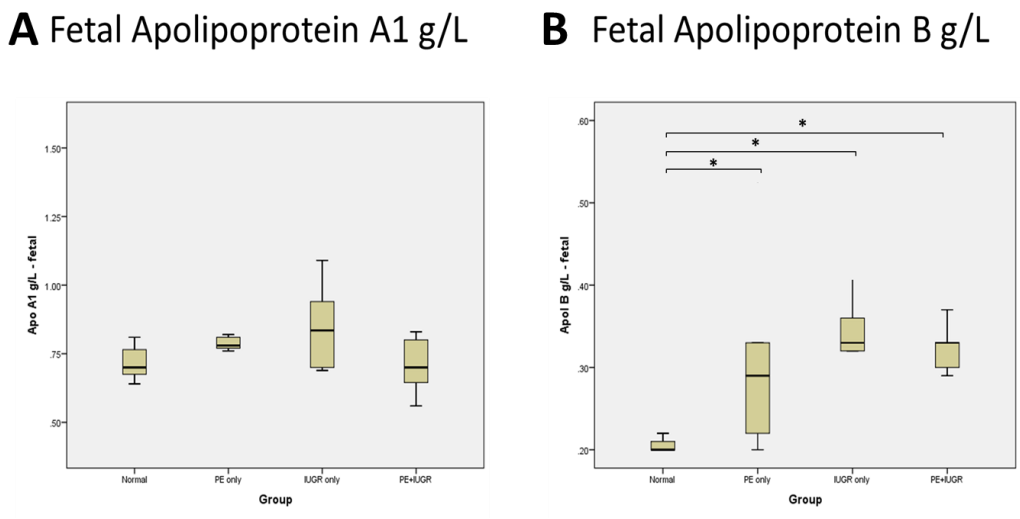

Figure 8.4 Comparison of A = fetal Apo lipoprotein A1 (ApoA1) and B = fetal Apo lipoprotein B (ApoB) levels between clinical groups.

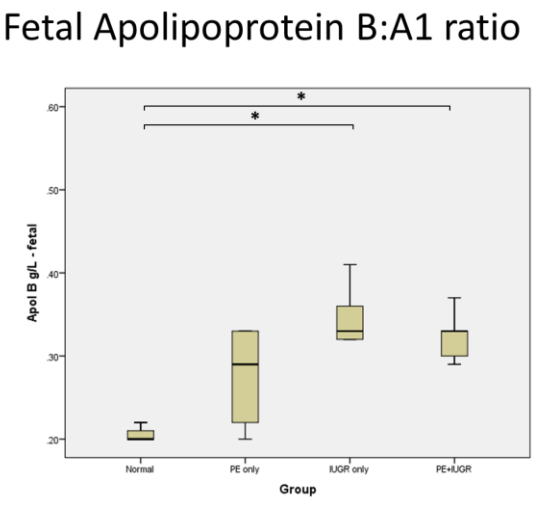

Figure 8.5 Comparison of fetal Apo lipoprotein B/A1 ratio (ApoB/ApoA1) levels between clinical groups. Significant differences noted between Normal pregnancy, IUGR as well as PE+IUGR. No significant differences seen between normal and PE as well as between the pathological groups.

8.6 Discussion

A number of previous studies have documented elevated lipids in normal pregnancy as well as in preeclampsia (350, 356). In one of the early studies on the subject, all lipids and Apo lipoproteins were found to be significantly elevated during the second and the third trimesters with a 2.7-fold triglyceride, 56% increase in ApoB, 43% increase in total TC, 36% increase in low-density lipoprotein (LDL) cholesterol and 32% increase in Apolipoprotein A1. High-density lipoprotein (HDL) cholesterol rose maximally (25%) in the second trimester (350). No prior documentation was found for the status of lipids in defined IUGR or PE+IUGR as a group. The mechanism whereby pregnancy induces hyperlipidaemia has not been fully elucidated.

These results indicate that while Apo lipoprotein levels A1 and B levels were not different between clinical groups in the maternal circulation, significant variation existed in the fetal circulations with elevated Apo lipoprotein B levels in PE, IUGR and PE+IUGR as compared to normal pregnancies. The maternal data are consistent with previous reports of no difference in ApoA1 and ApoB levels in normal and preeclamptic patients (366). The data on fetal ApoB levels confirms the findings of a previous study showing elevated Apo B levels in cordocentesis samples of growth restricted fetuses (371). This is the first documentation of such a wide variation in ApoB levels in the neonatal cord blood at delivery. Increased Apo lipoprotein B levels and an elevated Apo lipoprotein B to A1 ratio in young adults have been shown to be predictors of atherogenesis in later life. Fetal growth restriction and low growth in the first years of life has been linked to cardiovascular disease in adulthood (48, 416). The elevated Apo lipoprotein B levels in the cord blood may be a useful link in identifying newborns at risk of cardiovascular disease in later life.

The triglyceride levels were significantly higher in preeclampsia compared to normal pregnancy. The levels in IUGR were lower than normal and preeclampsia groups, reaching statistically significant differences as compared to preeclamptic group. A number of pregnancies displayed TG levels well above the 95th centiles for third trimester gestation, mainly in pregnancies complicated by preeclampsia and may represent pre-existing hyperlipidaemia or risk for hyperlipidaemia. There was a weak correlation between BMI and TG levels that was not at a statistically significant level to have a confounding effect on the findings.

Fetal total triglyceride (TG) levels in PE and PE+IUGR were higher than normal and IUGR groups, reaching statistical significance between normal and PE+IUGR. The small sample number particularly in PE, is likely to have affected the calculation of statistical significance

between groups. This is consistent with previous findings suggesting higher TG levels in fetal growth restriction (357). These findings confirm previously published data that TG levels are elevated above non-pregnant levels in normal pregnancy and even higher in preeclampsia. The study has also documented that IUGR only is not significantly associated with elevated TG levels. These data are in concordance with previous suggestions that human gestation is associated with an 'atherogenic' lipid profile that is further enhanced in preeclampsia and that this profile may be a potential contributor to endothelial cell dysfunction (364). A mechanism for serum lipid related endothelial dysfunction has not yet been confirmed.

Constitutional lipid abnormalities have been suggested as one of the maternal predisposing factors for developing preeclampsia (361). A review by Gratacos et al explored the different lines of evidence indicating that abnormal lipid metabolism is likely to be directly involved in its pathogenesis and not a mere manifestation of preeclampsia (361). It has been documented for 35 years that preeclampsia is associated with hypertriglyceridemia (347). Triglycerides and free fatty acids have been noted to be already elevated in the first and second trimester in these women (417). *In vitro* experiments have shown that lipid fraction dependent endothelial activation has been shown using preeclamptic plasma (418). The lipoprotein profile in preeclampsia parallels that of atherosclerosis with elevated LDL levels (419). Similar demographic profiles such as increased BMI, increased TG levels and elevated non-esterified fatty acid (NEFA) predispose women to preeclampsia and dyslipidaemia (420).

Ethnic differences have been described in lipid profiles in pregnancy with African/Afro-Caribbean pregnant women having lower serum concentrations of TC, LDL, HDL and TG concentrations compared with Caucasian women (421). The lipid profile in normal pregnancy in south eastern subcontinent and East Asian background has not been defined. The

multicultural nature of the patient population in this study with >50% of the population from the above mentioned ethnicities may have affected the presented results.

The study was limited by small patient numbers and the cross sectional study design. It is possible that the results may be more conclusive with larger patient numbers. The LDL levels were obtained by calculation using the Friedwald equation which assumes that the composition of lipoproteins in pregnancy is the same as in normal metabolic states and also includes intermediate density cholesterol. The calculation is generally applicable to triglyceride levels < 4.55mmol/L. The TG levels in pregnancy are often significantly higher than this range, leading to the LDL calculation to be less reliable in these patients. The Friedewald equation tends to underestimate LDL-C when triglycerides are elevated as is the case in pregnancy. The LDL levels in this study may be an underestimate

The presented research raise interesting hyportheses regarding the pathogenesis and predisposition to preeclampsia and cardiovascular disease. Further studies with larger numbers are recommended to clarify the significance of maternal, fetal and neonatal TG levels as well as fetal/neonatal Apolipoprotein B levels on the risk of maternal preeclampsia and fetal growth restriction and long term cardiovascular risk.

SUMMARY

Chapter 9 Summary of results and future directions

9.1 Introduction

The pathogenesis of preeclampsia and intrauterine fetal growth restriction, common pregnancy complications, has eluded researchers over decades. The current paradigm suggests defective placentation in early gestation and inadequate vascular remodeling of maternal spiral arteries leading to ischemia as the central cause of these pregnancy complications (34, 222, 422). Placental ischemia is thought to release soluble factors such as sFlt-1 and sEndoglin that enter the maternal circulation, resulting in endothelial dysfunction and the clinical presentation of preeclampsia (222, 397, 422). The pathogenesis of preeclampsia is thought to act at three levels- defective placentation, placental ischemia, and endothelial cell dysfunction. Of these, endothelial dysfunction is considered to be a key factor associated with pre-eclampsia (193).

Published literature to date on role of angiogenic factors suggests that while pro-angiogenic factors are essential in the development of the fetus and the placental interface with the mother, anti-angiogenic factors also plays an essential role in the regulatory control of angiogenesis during pregnancy. While the pathogenesis of preeclampsia has been extensively studied, the causative mechanisms underlying IUGR are less well understood.

The research undertaken as part of this work has looked at the placental expression of pro and anti-angiogenic factors as well as investigated the circulating levels of these biomarkers. The focus has been on the VEGF family and its receptors as well as Endoglin, a transforming growth factor β receptor, as an additional significant biomarker. A comparison of the angiogenic factor milieu has been made between normal pregnancy and pregnancies

complicated by PE, IUGR and PE+IUGR to assess differential factors that lead to maternal syndrome of preeclampsia vs the fetal syndrome of intrauterine fetal growth restriction.

The results from this assessment of circulating pro- and anti-angiogenic factor levels suggest a disparity in the clinical presentation of preeclampsia and intrauterine fetal growth restriction with concordant circulating pro and anti-angiogenic factor levels. The expression of various angiogenic factors in the maternal circulation have been analyzed and presented as potential biomarkers for preeclampsia and intrauterine fetal growth restriction. The research presented in this thesis has been cited in a recent expert opinion published on the clinical implications of sFlt-1/PlGF as a marker of preeclampsia (219).

It is unclear why some pregnant women with similar placental disease and anti-angiogenic profile, develop endothelial dysfunction and preeclampsia, some develop preeclampsia and fetal growth restriction, while others have fetal growth restriction with no signs of maternal disease.

To investigate possible causes of this disparity, the work was extended to study maternal and fetal monocytes and explore whether monocytes play a role in the contribution to the angiogenic factors and their receptors in normal and complicated pregnancies. In addition to endothelium and placental trophoblasts, monocytes are the only other tissue/cell type known to secrete sFlt-1, a significant biomarker for placental disease. As part of the work presented here, anti-angiogenic factor expression was correlated to the monocyte subtypes and activity levels. In a review by Redman et al. al. (423), preeclampsia has been described as an exacerbation of a normal maternal inflammatory response in pregnancy and that defective placentation is a predisposing factor rather than the cause of preeclampsia. Generalized intravascular inflammatory reaction involving intravascular leukocytes as well as the clotting and complement systems are thought to be significant in this process. Monocytes have been

shown to be an integral part of inflammatory response of the body. In this research maternal monocyte phenotype and activation were assessed as biomarkers for placental disease of preeclampsia and intrauterine fetal growth restriction. A pilot study was also conducted to characterize fetal monocyte phenotype and their possible association with pregnancy complications.

Previous work has suggested that the maternal metabolic syndrome or lipid status may predispose to preeclampsia (361) and may be a possible explanation for the disparity in clinical presentation of preeclampsia and intrauterine fetal growth restriction. The presented work explored the differences in maternal and fetal lipid profile between PE, IUGR and PE+IUGR.

This research contributes to the literature on the pathogenesis of preeclampsia and intrauterine growth restriction demonstrating similarities and differences between the two conditions which has lead us closer towards an understanding of their pathogenesis. While maternal lipid status appears to be a distinguishing factor in preeclampsia, low placental PlGF and KDR as well as a polarization of monocytes towards an M2 phenotype have been shown to be features of IUGR.

Maternal and fetal monocytes appear to have potential role of biomarker in preeclampsia and fetal intrauterine fetal growth restriction. This approach is in line with the revised statement of the ISSHP (2014), suggesting a move away from the traditional diagnosis of preeclampsia using hypertension and proteinuria and embracing the use of biomarkers in the diagnosis.

Recent focus on the long term consequences of preeclampsia in the mother and intrauterine fetal growth restriction in the fetus has suggested that these conditions are no longer considered solely a disease of pregnancy (424). These findings on the lipid status contribute

to this discussion and present opportunities to evaluate maternal and fetal lipid markers in the long term cardiovascular risk assessment of the mother and the fetus.

The inclusion of umbilical artery Doppler indices in the selection criteria was to ensure that the fetal growth restriction was due to placental causes and not due to constitutional factors or fetal anomalies. While previous studies into angiogenic factors have incorporated uterine artery Doppler in patient selection, no studies are currently available in the published literature on angiogenic factor expression in intrauterine fetal growth restriction due to placental causes as defined by abnormal umbilical artery Doppler waveform.

9.2 Summary of research

9.2.1 Placental pro and anti-angiogenic factors

- Standard immunohistochemical methods were used to stain placental tissue for angiogenic factors. The current study adds to the existing literature as the first description of digital image analysis techniques in the assessment of angiogenic factor expression in the placenta. This study has also shown that automated digital image analysis using software such as Aperio positive pixel algorithm can be successfully used as an alternative method to the manual reading of placental immunohistochemical staining and that this technique may improve the reproducibility and accuracy of results in placental study.

- Preeclampsia and intrauterine fetal growth restriction are associated with loss of villous architecture, vascularity and tissue. The cumulative effect of PE and IUGR

appear to lead to a more significant loss of villous architecture than either condition alone.

- The localization of VEGF, PlGF, Flt-1 and KDR in the placental tissues types was similar across the four clinical groups. The positive staining for VEGF, Flt-1, and PlGF were mainly localized to the syncytiotrophoblast layer of the placenta, while KDR staining was detected in the endothelial layer.

- While no major differences in placental VEGF and Flt-1 were noted between clinical groups, placental expression of PlGF and KDR were significantly reduced in pregnancies complicated by IUGR as compared to normal and preeclampsia only pregnancies.

- The intensity of VEGF staining has been shown to be reduced in the areas of significant pathology such as villous infarction compared to the non-infarcted areas. The results of this research study raise the possibility that changes in VEGF and Flt-1 expression may be a consequence rather than the cause of placental vascular disease and preeclampsia and that lack of PlGF and KDR may be a main cause for the development of intrauterine fetal growth restriction.

9.2.2 Circulating angiogenic factors

- Standard ELISA techniques were used to evaluate circulating angiogenic factor levels. Within the limitations of a cross sectional study, the sFlt-1 and sEndoglin levels in normal pregnancy were noted to be higher with increased gestational age while the PlGF levels decreased. No significant relationship to gestational age was seen within the pathological groups PE, IUGR and PE+IUGR as this study classified the patients on the basis of on established disease. sKDR did not reveal a gestation-related change

in any clinical group. The findings suggest that the placental disease present in preeclampsia and intrauterine growth restriction represent an accelerated process of physiological changes that may happen in normal pregnancy with advanced gestation.

- Elevated maternal sFlt-1 and sEndoglin and low PlGF levels were demonstrated in pregnancies complicated by preeclampsia and IUGR, in comparison to normal pregnancies. Higher levels of sEndoglin in PE+IUGR indicating severe disease was the only difference between PE, IUGR and PE+IUGR. This study also measured the levels of sFlt-1 in fetal umbilical artery. Plasma levels were significantly lower than maternal circulating levels and there was no demonstrable difference between normal and complicated pregnancies. This finding suggests that the fetal contribution to the overall levels of circulating sFlt-1 in the maternal circulation is negligible and unlikely to play a part in the pathogenesis of preeclampsia.

- Pro and anti-angiogenic factors and their ratios were assessed as biomarkers in identifying normal vs pathological pregnancies complicated with preeclampsia and/or intrauterine fetal growth restriction. While the sFlt/PlGF ratio appeared to have a significant ability to distinguish pregnancies affected by preeclampsia and/or fetal growth restriction (sensitivity 90%, specificity 90%), the ratios sEng/PlGF (sensitivity of 94% and a specificity of 93%) and sFlt*sEng/PlGF performed better in their predictive value (sensitivity of 94% and a specificity of 90%). These data suggests sEng to be a valuable screening or diagnostic marker of placental disease. Publication of the results presented in Chapter 4 of this work has been cited in a recent review on angiogenic factors as part of the evidence for recommending their use in a clinical setting (219).

9.2.3 Maternal and fetal monocytes subtypes and polarization in preeclampsia and intrauterine fetal growth restriction.

- In a novel approach, knowledge of monocyte function and subtypes were utilized to develop flow cytometry protocols to characterize the maternal and fetal monocyte profiles in normal and pathological pregnancies. These data suggested a statistically significant trend towards a lower circulating maternal percentage of classical monocytes and a higher percentage of intermediate monocytes in pregnancies complicated by IUGR. Similar trends in preeclampsia were not statistically significant. This is the first description of monocyte subsets in pregnancies complicated by intrauterine fetal growth restriction. The results suggest that the distribution of monocyte subsets in IUGR is similar to preeclampsia with a higher percentage of intermediate monocytes compared to normal pregnancy.

- The differences among monocyte subsets were investigated with regard to expression of monocyte/macrophage anti-inflammatory molecule, CD163 and the CD86/CD163 ratio as a marker of inflammatory (M1) and healing (M2) phenotypes. A gestational related change in M1/M2 phenotype in third trimester normal pregnancy was not detected.

- Pregnancies complicated by intrauterine fetal growth restriction showed a clear shift towards M2 (healing) monocyte phenotype. Overall no differences in polarization were noted between normal and preeclamptic pregnancies.

- Interestingly, in PE, IUGR and PE+IUGR, the intermediate subset was the dominant subset of fetal monocytes with the intermediate monocyte count as a percentage of the total monocytes reaching 59.3% in preeclampsia, 41.2% in IUGR and 48.8% in

PE+IUGR as compared to 34.4% in normal pregnancy. This is a new distribution of monocyte subsets not previously described. These findings suggest that the innate immune signaling pathways related to monocytes are activated and functional in the fetal circulation in PE and IUGR.

- The non-classical subset was seen to expand in PE, IUGR and PE+IUGR reaching statistically significant levels as compared to normal pregnancy.

- This is the first description of the distribution of monocyte subsets in the cord blood using the nomenclature of classical, intermediate and non-classical based on CD14 and CD16 expression.

9.2.4 Anti-angiogenic factor expression by maternal monocytes

- Flow cytometry experiments were designed to assess the angiogenic factor profile and polarization of monocytes. The results have clearly shown that circulating monocytes from maternal and fetal circulation express Flt-1 and Endoglin as surface markers. There was no variation in the membrane bound Flt-1 expression between the clinical groups of normal pregnancy, PE, IUGR and PE+IUGR. The maternal monocyte surface Endoglin expression was similar across clinical groups with some reduction seen in the PE+IUGR group. A significantly increased Endoglin MFI was seen in the fetal monocytes of the IUGR as compared to other clinical groups.

- The study has shown that while surface expression of Flt-1 is more prominent on classical and intermediate monocytes, Endoglin is more likely to be expressed on intermediate and non-classical monocytes.

- These findings also demonstrated a moderate correlation with M1 monocyte inflammatory phenotype and surface expression of Flt-1 and Endoglin.

9.2.5 Maternal and fetal lipid profiles in preeclampsia and intrauterine fetal growth restriction

- These results indicate that while Apo lipoprotein levels AI and B levels were not different between clinical groups in the maternal circulation, significant variation existed in the fetal circulations with elevated Apo lipoprotein B levels in PE, IUGR and PE+IUGR as compared to normal pregnancies. This is the first description of elevated ApoB levels in cord blood at delivery in PE and IUGR, and may be a useful link in identifying newborns at risk of cardiovascular disease in later life.

- The maternal and fetal triglyceride levels were significantly higher in preeclampsia compared to normal pregnancy. The levels in IUGR were lower than normal and preeclampsia groups, reaching statistically significant differences as compared to preeclamptic group. A number of pregnancies displayed TG levels well above the 95^{th} centiles for third trimester gestation, mainly in pregnancies complicated by preeclampsia and may represent pre-existing hyperlipidaemia or risk for hyperlipidaemia. Excessive elevation in maternal TG levels may have a role in the pathogenesis of PE and may also identify pregnant women at risk of preeclampsia and long term cardiovascular risk.

- Fetal total triglyceride (TG) levels in PE and PE+IUGR were higher than normal and IUGR groups, reaching statistical significance between Normal and PE+IUGR.

9.3 Future directions

The following directions are suggested to follow on from the research presented in this thesis in studying the pathophysiology of preeclampsia and intrauterine fetal growth restriction.

9.3.1 Anti-angiogenic factors

- This research has identified low placental PlGF and KDR as an important aspect of the angiogenic profile of intrauterine fetal growth restriction. The factors controlling these pro-angiogenic factors need further study in understanding the pathogenesis of IUGR. Research to date on trophoblast culture and function has focused mainly on sFlt-1 expression. Experiments using cultured trophoblast may identify whether the low PlGF and KDR are cause or effect of preeclampsia and IUGR. Exposure of trophoblast from normal pregnancy to the sera of PE or IUGR and testing the effects on PlGF and KDR expression as well as testing cultured trophoblast from normal, PE and/or IUGR pregnancies for their PlGF and KDR expression under normal and low oxygen conditions may be worthwhile.

- The presented results as well as previously published data suggest that a screening test using a composite index of sFlt-1, PlGF and sEndoglin at 24-28 weeks of gestation may be a powerful tool in identifying pregnancies at risk of adverse pregnancy outcomes such as preeclampsia and intrauterine fetal growth restriction due to placental disease but not necessarily distinguish between preeclampsia or fetal growth restriction or both (425). The validity of such a screening test including sEng should be evaluated in future studies. Longitudinal studies during pregnancy as a

screening test for the risk of preeclampsia and IUGR is suggested. If proven to be of value in altering clinical outcomes, this would be a cost effective method using minimum resources to identify where to focus antenatal care and management.

9.3.2 Maternal monocytes and placental macrophages

- The maternal immune mechanisms particularly associated with the monocyte/macrophage function and their role in implantation as well as in the peripheral circulation need to be explored. A major question to be answered is whether the mononuclear cells in pregnancy play a causative role in preeclampsia or whether any changes in their phenotype are secondary to the disease process. A prospective longitudinal study on the monocyte subset distribution from early pregnancy in a low risk population and any changes associated with PE or IUGR may help answer these questions. An animal model of preeclampsia where monocyte polarization is induced *in vivo* (by injection of IL-4/IL-13 or adoptive transfer of M2 macrophages) may help to define the causative or reactionary nature of monocyte phenotype and polarization.

- It is possible that circulating monocyte CD163 expression may be useful as a biomarker for significant placental damage and need for repair. Correlation studies with CD163 expression and clinical outcomes such as IUGR, hypoxic damage, low apgar score and stillbirth may be worthwhile to assess this as a biomarker.

- A recently published study into the trigger for inflammatory changes in monocyte subsets in normal pregnancy and preeclampsia, has suggested that monocyte activation may be induced by syncytiotrophoblast membrane micro particles (STBM) released by the placenta. The higher amounts of placental STBM circulating in

maternal blood in preeclampsia might lead to activation of an excessive maternal inflammatory reaction. The significance of STBM in monocyte activation as well as in the pathogenesis of the maternal syndrome of preeclampsia deserves further study.

- Ethnic differences have been demonstrated in monocyte subsets and may need to be explored as confounding variables when interpreting study results. While differences have been demonstrated between Caucasian and Caribbean/African individuals, the monocyte phenotypes in Southern Indian or East Asian backgrounds has not been explored. These findings would be significant when interpreting results on monocyte phenotypes from multicultural population backgrounds.

- The newly described distribution of dominant intermediate monocyte subtype in the fetal circulation suggests that fetal monocytes may play a significant role in the pathogenesis of PE and IUGR. Further study is recommended to characterize the cord blood monocyte phenotypes and their functional roles associated with normal pregnancy as well as pregnancies complicated by preeclampsia and intrauterine fetal growth restriction.

9.3.3 Maternal and fetal lipid profiles

Patients with a family history of hypercholesterolemia have a defect in the gene for Apo lipoprotein B, the component of low density lipoprotein that binds the receptor. The risk of coronary disease is seven times higher in people with this mutation than in the general population. Maternal and fetal gene polymorphisms for lipid metabolism should be investigated for their potential contribution to predisposition to preeclampsia and fetal growth restriction.

- *In vitro* studies are suggested to determine whether exposure to triglyceride, cholesterol or Apo lipoproteins affect monocyte polarization into M1/M2 phenotypes as an indicator of lipid stimulated inflammatory environment in preeclampsia. The use of CD86/CD163 may be a useful marker in monocyte/macrophage studies on M1/M2 polarization

- Lipid status in different ethnicities need to be further explored to understand not only any variations associated with different ethnicities but also the reference ranges for normal pregnancy and the hyperlipidaemia response. With increasing multicultural societies across the world, ethnic variations need to be taken into consideration in understanding processes associated with pathogenesis of disease and long term strategies for prevention of disease.

- Further study should be conducted to evaluate the value of Apo lipoprotein B levels in the cord blood samples of growth restricted fetuses to identify risk of long term atherosclerosis. Testing of Apolipoprotein B levels in childhood or adolescent/young adults and correlating with birth weight may give an indication of whether a persistent ApoB level contributes to the pathogenesis of long term cardiovascular disease.

- Further study is suggested to evaluate maternal lipid status, particularly fasting serum lipids in first trimester, second trimester and third trimester as a predictor of risk of preeclampsia as well as long term cardiovascular disease. This may require testing a large cohort of pregnant women and long term follow-up of cardiovascular status.

- Longitudinal studies in pregnancy to evaluate the benefit of such a screening method for preeclampsia in the current pregnancy and the long term benefits of identifying mothers with pre-existing hyperlipidaemia or at risk of long term hyperlipidaemia

should be separately evaluated. Screening programs into hyperinsulinaemia and gestational diabetes in pregnancy are currently utilized to identify at risk pregnant women for diabetes in pregnancy as well as long term.

Clearly much work is required to fully understand the complex pathophysiologies of preeclampsia and intrauterine fetal growth restriction. The presented research and the suggested line of inquiries will go some way towards advancing along this path.

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