ANGIOGENIC FACTORS IN PLACENTALLY-MEDIATED PREGNANCY COMPLICATIONS

by

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Abstract

Placentally-mediated pregnancy complications include pre-eclampsia, intrauterine growth restriction (IUGR), placental abruption and some causes of stillbirth. These complications are believed to arise from abnormal placental development in early gestation that leads to compromised placental function in later pregnancy which can adversely affect both mother and fetus. It is a priority in obstetrics to identify these pregnancies early and accurately so that appropriate monitoring and intervention can optimise outcomes for these mothers and babies. Novel biomarkers such as angiogenic factors in the maternal circulation may improve the prediction and/or diagnosis of these complications by adding to the information gained from tools already used in clinical practice. In this thesis, I investigated angiogenic factors in 1) the diagnosis of pre-eclampsia using new clinical immunoassays, 2) the prediction of placentally-mediated complications in a high-risk pregnancy cohort and 3) the diagnosis of placental IUGR in pregnancies with small for gestational age (SGA) fetuses. Additionally, I investigated the association between levels of circulating angiogenic factors and the presence of histopathological lesions of dysfunction in the placenta after delivery. I found that angiogenic factors, particularly low circulating placental growth factor (PIGF), had high sensitivity and specificity in the diagnosis of pre-eclampsia but all markers had poor performance as predictive markers for placentally-mediated complications. In pregnancies with SGA fetuses, low maternal PIGF discriminated between fetuses with placental IUGR (defined by the presence of histological lesions of placental dysfunction) from constitutionally small fetuses (no pathological lesions present) with high sensitivity and high negative predictive value. Additionally, low maternal PIGF in the second trimester was associated with the presence of lesions of placental dysfunction in pregnancies at high-risk for placentally-mediated complications. Low maternal PIGF was also associated with lesions of placental dysfunction as well as altered placental morphology in pregnancies with SGA fetuses. Taken together, these findings suggest that PIGF may be an antenatal marker of placental dysfunction and may provide a novel clinical tool to
identify pregnancies with placental dysfunction. This work improves our understanding of angiogenic factors in placentally-mediated complications and contributes to the growing body of evidence supporting their integration in clinical practice.
Preface

A version of Chapter 2 has been published. Benton SJ, Hu Y, Fang X, Kupfer K, Lee SW, Magee LA, von Dadelszen P. (2011). Placental growth factor as a diagnostic test for pre-eclampsia: A performance comparison of two immunoassays. *Am J Obstet Gynecol*, 205(5), 469.e1-8. This work used banked blood samples and patient data collected as part the Inflammation in Pregnancy and Pre-eclampsia Study that was designed by Dr Peter von Dadelszen. I generated the hypothesis for this current work with Dr von Dadelszen. I collected additional data from patient charts, analysed the data and wrote the manuscript. Dr Seok-Won Lee analysed the samples and Dr Ken Kupfer provided statistical support. Dr Sarka Lisonkova performed the logistic regression. All co-authors edited the manuscript. Ethics approval was granted by the University of British Columbia Children’s and Women’s Health Centre of British Columbia Research Ethics Board (Certificate H01-70062).

A version is of Chapter 3 is being prepared for publication. The EMMA Clinic was conceived by Dr Peter von Dadelszen and women were recruited by Research Assistants from 2004-2009. I generated the hypothesis for this work with Dr von Dadelszen and completed data collection that was started prior to me beginning my graduate work. I performed the angiogenic factor analyses with the support of Dr Yuxiang Hu. Placental evaluations were performed by Dr David Grynspan, our study pathologist. I was responsible for data analysis with Dr Sarka Lisonkova who performed the logistic regression and I wrote the manuscript draft which is being reviewed by the co-authors. Ethics approval was granted by the University of British Columbia Children’s and Women’s Health Centre of British Columbia Research Ethics Board (Certificate H04-70144).

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Work presented in Chapters 4 and 5 is part of an ongoing research study. I generated the hypothesis and study design with Dr Peter von Dadelszen with the assistance of Dr Jennifer Hutcheon. Under the supervision of Dr von Dadelszen and Dr Andrée Gruslin (Co-Investigator, Ottawa), I wrote the Canadian Institutes for Health Research grant for this study which was funded in January 2012. Eligible women were recruited by Research Assistants in Vancouver and Ottawa. I completed all the data collection on women recruited in Vancouver and Jinkie Quitain (Research Assistant) completed the data collection for women recruited in Ottawa. I performed all the PlGF analyses as well as all the sectioning and staining of the placental samples. Placental evaluations were performed by Dr David Grynspan. I completed all the data analysis and wrote the chapters with feedback from Dr von Dadelszen, Dr Hutcheon and my supervisory committee. Ethics approval was granted by the University of British Columbia Children’s and Women’s Health Centre of British Columbia Research Ethics Board (Certificate H12-00504).

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List of Abbreviations

AC    abdominal circumference
AEDF  absent end diastolic flow
AFI   amniotic fluid index
AFP   alpha-fetoprotein
ARDS  acute respiratory distress syndrome
ARF   acute renal failure
AST   aspartate transaminase
ATN   acute tubular necrosis
AUC   area under the curve
BPD   biparietal diameter
CD    cluster of differentiation
CI    confidence intervals
CNS   central nervous system
CVA   cerebrovascular accident
DbM   diabetes mellitus
dBP   diastolic blood pressure
DIC   disseminated intravascular coagulation
dNK   decidual natural killer
EDF   end diastolic flow
EDTA  ethylenediaminetetraacetic acid
EFW   estimated fetal weight
ELISA enzyme-linked immunosorbent assay
ER    endoplasmic reticulum
EVT  extravillous trophoblast
FL   femur length
Flt  Fms-like tyrosine kinase
GA   gestational age
HC   head circumference
hCG  human chorionic gonadotropin
H&E  haematoxylin and eosin
HELLP haemolysis, elevated liver enzymes, low platelets
HIF  hypoxia-inducible factor
HLA  human leukocyte antigen
IFN  interferon
IHC  immunohistochemistry
IL   interleukin
IUGR intrauterine growth restriction
IQR  interquartile range
KDR  kinase insert domain receptor
KW   Kruskal-Wallis analysis of variance
LV   left ventricular
mmHg millimetres of mercury
MMP  matrix metalloproteinase
mRNA messenger RNA
NPV  negative predictive value
OR   odds ratio
PAPP pregnancy associated plasma protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
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<tr>
<td>PET</td>
<td>pre-eclampsia</td>
</tr>
<tr>
<td>PI</td>
<td>pulsatility index</td>
</tr>
<tr>
<td>PIGF</td>
<td>placental growth factor</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>PRES</td>
<td>posterior reversible encephalopathy</td>
</tr>
<tr>
<td>REDF</td>
<td>reversed end diastolic flow</td>
</tr>
<tr>
<td>RI</td>
<td>resistance index</td>
</tr>
<tr>
<td>RIND</td>
<td>reversible ischemic neurological deficit</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
</tr>
<tr>
<td>sBP</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>S/D</td>
<td>systolic: diastolic</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>sEng</td>
<td>soluble endoglin</td>
</tr>
<tr>
<td>SFH</td>
<td>symphysis fundal height</td>
</tr>
<tr>
<td>sFlt</td>
<td>soluble Fms-like tyrosine kinase</td>
</tr>
<tr>
<td>SGA</td>
<td>small for gestational age</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIA</td>
<td>transient ischemic attack</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>uE</td>
<td>unconjugated estriol</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>$X^2$</td>
<td>Chi-square</td>
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</table>
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And finally, to the women who participated in these studies – your altruism and willingness to help future mothers and babies in times of uncertainty is truly inspiring and this work could not have been done without you.
Dedicated with love to my Mom and Dad and in memory of Dr Andrée Gruslin.
Chapter 1. Introduction

The placenta intimately connects mother and fetus over the course of gestation and successful pregnancy is contingent upon its proper development and function. Placentation, the process of placental development, begins as early as 5-6 days after conception as fetal trophoblast cells invade the maternal decidua (Guzeloglu-Kayisli et al., 2007; Norwitz et al., 2001). Placentation involves the remodelling of the uterine vasculature to ensure adequate blood supply into the placenta and the formation of intricate networks of vascular villi and intervillous spaces for exchange between the maternal and fetal circulations (Benirschke et al., 2012; Lisman et al., 2007).

Abnormal placentation in early pregnancy can compromise placental function in later gestation, often adversely affecting the well-being of both mother and fetus (Baergen, 2011; Benirschke et al., 2012). Pre-eclampsia and intrauterine growth restriction (IUGR) are two common placentally-mediated pregnancy complications associated with placental dysfunction and are leading causes of preterm birth, stillbirth and maternal mortality and morbidity worldwide. Despite advances in our understanding of the aetiology and pathophysiology of these complications, it still remains a clinical challenge to identify abnormal placentation and subsequent placental dysfunction antenatally. Angiogenic factors in the maternal circulation may reflect placental compromise and provide a new clinical tool for accurate and timely diagnosis of pre-eclampsia and IUGR. Work presented in this thesis investigates circulating angiogenic factors in placentally-mediated pregnancy complications and expands on current research into their utility as clinical biomarkers. The introduction of this thesis presents a review of the current understanding of pre-eclampsia and IUGR aetiology and pathophysiology, as well as clinical management of these pregnancy complications. A review of our current knowledge of angiogenic factors in pregnancy is also presented.
1.1 Pre-eclampsia

Pre-eclampsia is a pregnancy-specific, heterogeneous syndrome that is characterised by excessive systemic maternal inflammatory and coagulatory responses, superimposed on endothelial dysfunction (Steegers et al., 2010; von Dadelszen et al., 2002). It complicates approximately 3-5% of all pregnancies and is a leading cause of maternal and fetal/neonatal mortality and morbidity worldwide (Duley, 2009; Hutcheon et al., 2011, Khan et al., 2006). The most significant burden of illness to mothers and babies occurs in the developing world where access to adequate maternity care, surveillance and intervention is sub-optimal (Firoz et al., 2011). Maternal death rates due to pre-eclampsia can be as high as 15% in developing countries compared with 0.1-1.8% in developed countries (Ghulmiyyah and Sibai, 2012; Khan et al., 2006). Eclampsia and target organ damage and failure akin to that in sepsis are typical maternal complications of this syndrome (Eastabrook et al., 2011; Steegers et al., 2010). Consequences of pre-eclampsia for the fetus include IUGR, stillbirth and preterm birth (spontaneous and iatrogenic) and the associated complications and developmental sequelae of prematurity (Eastabrook et al., 2011; Xiao et al., 2003). Pre-eclampsia is also a risk factor for conditions such as chronic hypertension, cardiovascular disease and diabetes in later life (Bellamy et al., 2007; Callaway et al., 2007; Feghali and Miodovnik, 2013; Irgens et al., 2001; McDonald et al., 2008; Skjaerven et al., 2012).

1.1.1 Diagnosis

Pre-eclampsia is defined as new onset hypertension (≥140 mmHg systolic blood pressure, ≥90 mmHg diastolic blood pressure) and proteinuria (≥0.3 g per day) in a previously normotensive woman after the 20th week of gestation (American College of Obstetricians and Gynecologists (ACOG), 2013; Magee et al., 2008; National Institute for Health and Clinical Excellence (NICE), 2011). While hypertension and proteinuria are the most common clinical manifestations of pre-eclampsia, features of this disorder also include signs and symptoms that reflect the multi-organ disturbances that occur in this systemic syndrome. Signs and symptoms include severe headache, visual disturbances, right upper
quadrant pain, dyspnoea, chest pain as well as elevated liver enzymes, decreased serum albumin and increased serum uric acid (Magee et al., 2008, Royal College of Obstetricians and Gynaecologists UK (RCOG), 2010). Features of pre-eclampsia may be seen in the absence of hypertension or proteinuria. These “atypical” presentations of pre-eclampsia again represent the heterogeneous nature of the syndrome and include non-hypertensive proteinuria and hypertension without proteinuria as well as normotensive haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome (Pettit and Brown, 2012; Sibai and Stella, 2009).

1.1.2 Risk factors

Clinical risk factors for pre-eclampsia include advanced maternal age, primiparity, multiple gestation and previous history of pre-eclampsia (Magee et al., 2008; NICE, 2010; North et al., 2011; Sibai et al., 1997; Sibai et al., 2005). Women who have pre-existing medical conditions such hypertension, diabetes, vascular disease, obesity and metabolic syndrome are also at higher risk of developing pre-eclampsia likely due to exacerbation of these underlying morbidities (Baumwell and Karumanchi, 2007). However pre-eclampsia is relatively indiscriminate and can affect healthy women (Williams et al., 2008).

Autoimmune disorders and inherited thrombophilia are also associated with an increased risk of pre-eclampsia (Duckitt and Harrington, 2005; Kupferminc, 2003; Mello et al., 2005). Certain gene mutations including single nucleotide polymorphisms (SNP) may predispose women to developing pre-eclampsia either by contributing to abnormal placentation or altering maternal adaptations to pregnancy (Chappell and Morgan, 2006; Williams and Broughton Pipkin, 2011). Candidate genes include those involved in trophoblast invasion, inflammation, coagulation and endothelial cell function (Dai et al., 2013; Founds et al., 2012; Kleinrouweler et al., 2013; Morgan et al., 2013). Alterations in gene methylation may also contribute to the risk of pre-eclampsia (Wang et al., 2010; Yuen et al., 2010). No definitive correlations between pre-eclampsia and causative genes (maternal or fetal/placental) have been made to date (Roberts, 2010; Roberts and Cooper, 2001).
Pregnancy by a new partner or pregnancy by insemination with donor sperm, oocyte donation or embryo donation can increase the risk of developing pre-eclampsia (Klatsky et al., 2010; Kyrou et al., 2010; Smith et al., 1997). Longer cohabitation before pregnancy and use of non-barrier contraceptives may decrease the risk of pre-eclampsia (Einarsson et al., 2003; Verwoerd et al., 2002) but this has not been proven conclusively (Ness et al., 2004). It is believed that as pre-eclampsia is characterized by an inappropriate maternal immune response, exposure to foreign paternal antigens on the fetal trophoblasts may aggravate the maternal immune system as maternal blood is in direct contact with these cells in the intervillous spaces. Repeated exposure to paternal antigens before pregnancy may establish immune tolerance in the mother thereby reducing her risk of developing pre-eclampsia (Hawfield and Freedman, 2009; von Dadelszen et al., 2002).

Biochemical risk factors for pre-eclampsia are primarily limited to unexplained abnormal prenatal serum screening results. Maternal levels of alpha-fetoprotein (AFP), human chorionic gonadotropin (hCG), unconjugated estriol (uE3), pregnancy-associated plasma protein-A (PAPP-A) and inhibin-A are routinely measured in the first and second trimesters of pregnancy to estimate the risk of fetal aneuploidy (trisomy 13, 18 and/or 21) and open neural tube defects (Chitayat et al., 2011). When abnormal marker levels are not explained by the presence of fetal aneuploidy or neural tube defects (confirmed by amniocentesis and ultrasound), abnormal marker results have been associated with an increased risk of adverse pregnancy outcomes such as pre-eclampsia (Dugoff et al., 2005; Dugoff, 2010; Gagnon et al., 2008; Goetzl, 2010; Metcalfe et al., 2014). However, despite this association, predictive performance of these markers is low with high false positive rates (Dugoff et al., 2005; Gagnon et al., 2008). Combining abnormal serum screen markers with clinical risk factors as well as uterine artery Doppler indices (e.g., increased pulsatility index, increased resistance index, presence of notching) may improve the identification of women at increased risk of developing pre-eclampsia but again, reported predictive performances are low (Audibert et al., 2005; Gagnon et al., 2008; Lovgren et al., 2010).
1.1.3 Disease severity and subtypes

Severity of disease varies between women with pre-eclampsia. The syndrome ranges from mild (low risk of adverse outcomes) to severe (high risk of adverse outcomes) (Sibai and Kupferminc, 2005) and may be defined in terms of the gestational age at disease onset, the number of signs/symptoms present, the degree to which blood pressure, proteinuria or other laboratory tests are altered or the degree of fetal compromise (ACOG, 2013; Magee et al., 2008; NICE, 2011; Steegers et al., 2010).

It is now widely acknowledged that the syndrome of pre-eclampsia consists of two (if not several) distinct disease phenotypes. In a clinical context, these phenotypes are usually classified based on the gestational age at disease onset, typically as either early (<34 weeks gestation) or late (≥34 weeks gestation) (ACOG, 2013; Magee et al., 2008). Early- and late-onset disease are thought of as two distinct pathologies as opposed to merely a progression of disease severity due to differing initiating factors and associated risks of adverse outcomes (Ness and Roberts, 1996; Steegers et al., 2010). Early-onset pre-eclampsia is thought to result due to chronic placental ischemia and placental dysfunction arising from abnormal placentation in early gestation that leads to uteroplacental mismatch between fetal demands and placental supply (Ness and Roberts, 1996; Steegers et al., 2010; von Dadelszen et al., 2002, von Dadelszen et al., 2012). Abnormal placental pathology such as reduced placental weight, decreased fetal vessel volume, abruption, infarction and accelerated villous maturation are common findings in placentas from pregnancies complicated by early-onset pre-eclampsia and these lesions reflect compromise to the placenta (Egbor et al., 2006; Moldenhauer et al., 2003; Roberts and Post, 2008). On the other hand, placentas from women with late-onset pre-eclampsia generally exhibit benign pathology (Egbor et al., 2006). Late-onset pre-eclampsia appears to have less of a placental contribution and may arise from a lowered maternal threshold to cope with the physiological adaptations of pregnancy (Steegers et al., 2010; von Dadelszen et al., 2012). Pre-existing maternal conditions such as cardiovascular disease and metabolic syndrome may contribute to this lowered maternal threshold. As
well, late-onset pre-eclampsia appears to be also associated with fetal macrosomia and multiple gestations, indicating a disparity between fetal demands and the supply capability of the placenta despite the absence of placental dysfunction (Steegers et al., 2010; von Dadelszen et al., 2012). More recently, it has been shown that excess fetal growth seen in late-onset pre-eclampsia may be attributed to maternal obesity in women with pre-eclampsia at term (Rasmussen et al., 2014). Women with late-onset pre-eclampsia generally have less severe disease and lower risk of adverse outcomes compared to women who develop early-onset pre-eclampsia (Sibai et al., 2005; Staff et al., 2013; Steegers et al., 2010).

### 1.1.4 Aetiology

Pre-eclampsia is a multi-organ, systemic disorder characterised by exaggerated maternal inflammatory, coagulatory and endothelial responses over and above the physiological changes that normally occur during pregnancy (Redman and Sargent, 2002; von Dadelszen et al., 2002). Causes of these maternal responses are multifactorial in nature with genetic, environmental and constitutional contributions and have yet to be fully elucidated (Steegers et al., 2010; von Dadelszen et al., 2002). Despite this, it is known that the placenta is central to its development, regardless of disease subtype. Delivery of the placenta is the only definitive cure for pre-eclampsia and signs and symptoms of the syndrome usually rapidly resolve following its removal (Backes et al., 2011; Magee et al., 2008). As well, women with molar pregnancies and other gestational trophoblastic diseases develop symptoms of pre-eclampsia such as hypertension and proteinuria despite not having a viable fetus (Redman and Sargent, 2002).

### 1.1.5 Pathophysiology

A working model of pre-eclampsia pathophysiology is outlined in Figure 1.1 (von Dadelszen et al., 2012). The pathways leading to early-onset and late-onset disease are highlighted in purple and blue, respectively.
1.1.5.1 Abnormal placentation

In the proposed early-onset pathway, abnormal placentation occurs in early pregnancy. Insufficient invasion by the fetal cytotrophoblast cells into the maternal decidua and myometrium (interstitial invasion) followed by incomplete remodelling of the maternal spiral arteries by the extravillous trophoblast (EVT) cells (endovascular invasion) are believed to precipitate placental ischemia and dysfunction in later gestation (Benirschke et al., 2012; von Dadelszen et al., 2002).

Shallow invasion may result from increased trophoblast apoptosis (DiFederico et al., 1999), failed cytotrophoblast differentiation into the invasive pathway (Goldman-Wohl and Simcha, 2002; Lim et al., 1997) and/or aberrant expression of matrix metalloproteinases (MMPs) and other proteinases required for extracellular matrix degradation (Karmakar and Das, 2002; Lian et al., 2010; Plaks et al., 2013; Roth and Fisher, 1999). Failure of the trophoblasts to switch adhesion molecule expression from an epithelial phenotype to one that is endothelial in nature (termed “pseudovasculogenesis”) has also been implicated in shallow invasion and failed spiral artery remodelling (Egbor et al., 2006; Goldman-Wohl and Simcha, 2002; Irving and Lala, 1995; Pijnenborg et al., 1996; Roth and Fisher, 1999; Zhou et al., 1997a; Zhou et al., 1997b). The decidua may also affect trophoblast invasion and migration by failing to produce and/or release cytokines and other factors that stimulate trophoblast expression of proteinases, cytokines, adhesion molecules and human leukocyte antigen (HLA) molecules required for invasion and migration (Brosens et al., 2002). Improper decidual natural killer (dNK) cell signalling may limit or inhibit trophoblast migration and invasion as these cells produce cytokines, chemokines and angiogenic factors that regulate invasion (Eastabrook et al., 2008; Hanna et al., 2006; Hu et al., 2006; Moffett and Loke, 2006; Murphy et al., 2009; Santoni et al., 2008). In addition, aberrant HLA signalling between trophoblasts and dNK cells may fail to reduce dNK cytotoxicity resulting in attack on trophoblast cells, thereby reducing their numbers in the decidua (Hu et al., 2006; Moffett and Loke, 2006; Moffett-King, 2002; Santoni et al., 2008; Sargent et al., 2006a).
Immune maladaptation may also contribute to abnormal placentation. There appears to be a shift from cell-mediated type-1 immunity to antibody-mediated type-2 immunity in the uterus during pregnancy. Increased levels of type-2 cytokines (interleukin (IL)-4, -5, -6, -10) with subsequent decreases in type-1 cytokines (IL-12, IFN-γ and TNF-α) are seen in uncomplicated pregnancy, while type-1 cytokines are increased in pre-eclampsia (Dealtry et al., 2000; Saito and Sakai, 2003; Sakai et al., 2002). Unsuppressed type-1 immunity may prevent immune tolerance from developing during placentation resulting in cytotoxic T-cell attack on fetal trophoblast cells causing reduced numbers, limited invasion and abnormal placentation (Saito and Sakai, 2003). In addition, cytokines such as IFN-γ and TNF-α increase trophoblast apoptosis and inhibit trophoblast migration and may contribute to limited invasion of EVT cells and poor placentation (Bauer et al., 2004; Haider and Knofler, 2009; Hu et al., 2006; Saito and Sakai, 2003; Wang and Walsh, 1996). The specific role of the type-1/type-2 immune ratio in the generation of pre-eclampsia is still being investigated.

1.1.5.2 Uteroplacental mismatch

Insufficient trophoblast invasion and/or inadequate spiral artery remodelling results in malperfusion of the uteroplacental unit leading to a discrepancy between fetal demands and placental supply termed “uteroplacental mismatch” (Redman and Sargent, 2009; Steegers et al., 2010; von Dadelszen et al., 2012). This uteroplacental mismatch may also arise if, despite normal placentation, fetal demands outweigh the intrinsic supply capabilities of the placenta (as in macrosomia and multiple gestations). Placental ischemia-reperfusion injury, oxidative stress and endoplasmic reticulum (ER) stress cause the formation of an “intervillous soup” – a mix of pro-inflammatory cytokines, growth factors, prostaglandins, placental debris and reactive oxygen species that enter the maternal circulation by virtue of the intervillous spaces (Sargent et al., 2003; von Dadelszen et al., 2002; Walsh, 2006). These factors, released at the local level of the uterus, provoke systemic activation of inflammatory, coagulatory and endothelial pathways above that which occurs physiologically in pregnancy to generate
the maternal syndrome of pre-eclampsia (Borzychowski et al., 2006; Brenner 2004; Brewster et al., 2008; Mahmoud et al., 2003). Inflammatory, coagulatory and endothelial changes in pre-eclampsia include increased circulating levels of cytokines (IFN-γ, TNFα, IL-6, IL-8 IL-12, IL-16, IL-18) (Gu et al., 2008; Jonsson et al., 2006; Lockwood et al., 2008; Sakai et al., 2002; Sargent et al., 2006b), acute phase proteins (Qiu et al., 2004), tissue factor (Erex et al., 2008; Esmon, 2001), thrombin (Erex et al., 2008), tissue plasminogen activator and plasminogen activator inhibitor-1 (Griffin et al., 2007; He et al., 1995).

There are also increased levels of coagulation cascade factors and fibrinogen and decreased levels of anticoagulants such as activated protein C (Esmon, 2001; Lolis and Bucala, 2003). There are decreased peripheral blood lymphocytes, particularly CD4+ and CD8+ T cells; this likely results from substantial activation of lymphocytes, followed by cell death (Mahmoud et al., 2003). Additionally, neutrophil activation increases with delayed apoptosis with significantly more neutrophil adherence to the endothelium and infiltration into the intimal space of the maternal vasculature (Cadden and Walsh, 2009; Walsh, 2006). There is also increased platelet activation with decreasing numbers of platelets as the disease progresses (Redman and Sargent, 2002). Microvascular permeability increases likely due to loss of endothelial integrity (Anim-Nyame et al., 2003; Endemann and Schiffrin, 2004).

1.1.5.3 Maternal syndrome

Inflammation, coagulation and the endothelium interact, stimulate and propagate one other and eventually lead to deterioration of physiological functions in tissues/organs due to lack of inhibitory or dampening mechanisms (Endemann and Schiffrin, 2004; Levi et al., 2004; Lolis and Bucala, 2003). Clinical manifestation of signs and symptoms of pre-eclampsia reflect the end-organ consequence of these processes.

Hypertension develops as a result of a variety of molecular mechanisms that alter production of vasoactive mediators from the damaged endothelium (Baumwell and Karumanchi, 2007; Steegers et al., 2010). Glomerular capillary endotheliosis and loss of fenestrae in the endothelial lining of the
glomerular capillaries result in reduced glomerular filtration rate and proteinuria (Steegers et al., 2010). Renal dysfunction and loss of endothelial integrity contribute to oedema. Eclamptic seizures may result from cerebral haemorrhage or severe hypertension (Steegers et al., 2010). HELLP syndrome involves liver failure, platelet consumption and red blood cell lysis as a result of extreme coagulation activation (Haram et al., 2009).

1.1.6 Prevention and treatment

Interventions to prevent pre-eclampsia are limited. Low-dose aspirin may reduce the risk of pre-eclampsia in high-risk women (Magee et al., 2008; NICE, 2011; Sibai et al., 2005). Calcium supplementation is recommended for women with low-calorie diets due to the effect of low calcium on blood pressure (Magee et al., 2008). Lifestyle changes including exercise, healthy eating and stress reduction may also reduce the risk of pre-eclampsia but the extent of this has yet to be ascertained (Magee et al., 2008).

There is no steadfast treatment or cure of pre-eclampsia that favours both mother and fetus. While delivery of the placenta causes resolution of the maternal syndrome, delivery remote from term does not favour the neonate. As such, women are usually managed expectantly, having signs/symptoms treated along with close monitoring, to balance the gain of continuing gestation with the progression of the maternal syndrome (Magee et al., 2008; von Dadelszen et al., 2007).

Hypertension is the only aspect of pre-eclampsia that can be actively treated at the moment. Anti-hypertensive mediations such as nifedipine and labetalol are used to treat severe hypertension but patients require close monitoring as lowering blood pressure too quickly and too drastically can decrease uteroplacental perfusion and cause fetal compromise (McCoy and Baldwin, 2009; von Dadelszen et al., 2007). Magnesium sulphate is given for seizure prophylaxis as well as fetal neuroprotection in preterm infants (Magee et al., 2011; McCoy and Baldwin, 2009; Sibai, 2005). Steroids for fetal lung maturation are administered before 34 weeks gestation (Magee et al., 2008).
Timing and mode of delivery are assessed to best suit the situation and delivery is performed as quickly as possible once signs of maternal instability are evident.

1.2 Intrauterine growth restriction

IUGR is a pathological process that reduces the growth trajectory of a fetus. Growth restriction can result from placenta dysfunction (placental IUGR), fetal chromosomal abnormalities and/or syndromes, congenital infection or environmental factors such as poor maternal nutrition or smoking (Carlsen et al., 2010; Cox and Marton, 2009; Dessi et al., 2012; Kinzler and Kaminsky, 2007; Maruyama et al., 2007; Neerhof, 1995; RCOG, 2013). IUGR fetuses are at high risk for adverse perinatal outcomes such as preterm delivery, stillbirth, serious neonatal complications and developmental sequelae (Breeze and Lees, 2007; Garite et al., 2004; Halliday, 2009). Due to the wide range of aetiologies, the incidence of IUGR varies between populations and geographical locations (Creasy and Resnik, 2013). In North America, placental IUGR is the most common manifestation of growth restriction followed by syndromic causes (e.g., fetal aneuploidy, fetal syndromes, congenital infection).

1.2.1 Growth restriction of placental origin

Placental IUGR is of serious clinical concern as these fetuses are at extremely high risk for adverse outcomes in pre- and post-natal life (Breeze and Lees, 2007; Garite et al., 2004; Halliday, 2009). Placental dysfunction results in insufficient placental transfer of oxygen and nutrients to the fetus from the maternal circulation and disrupts normal fetal growth and development (Bryan and Hindmarsh, 2006; Lang et al., 2003; Neerhof, 1995; Steegers et al., 2010). Prolonged exposure to this deprivation can result in stillbirth or hypoxic insults leading to abnormal neurodevelopment and brain damage. Fetal hypoxia, asphyxia, sepsis and metabolic disturbances are common in these neonates, as are the complications of prematurity (e.g., intraventricular haemorrhage, bronchopulmonary dysplasia, retinopathy of prematurity and necrotising enterocolitis) (Baron and Rey-Casserly, 2010; Petersen et al., 2009; Wold et al., 2009). Placental IUGR is also a risk factor for obesity, cardiovascular disease,
hypertension and diabetes in later life (Heinonen et al., 2010; Morrison et al., 2010; Noeker, 2005; Taylor et al., 2000).

Placental dysfunction arises as a downstream consequence of pathophysiological mechanisms that alter placental growth and development. Abnormal placentation in early pregnancy, characterised by shallow trophoblast invasion and failed spiral artery remodelling, results in malperfusion of the utero-placental unit (Kingdom et al., 2012; Redline et al., 2005; Redline et al., 2008). Placental malperfusion leads to chronic ischaemia and cellular stress that reduces maternal-fetal exchange in later gestation due to impaired trophoblast function, apoptosis and necrosis. As well, malperfusion may affect the growth of the villous tree, further impairing maternal-fetal exchange by decreasing placental reserve (Kingdom et al., 2012; Redline et al., 2008).

Placental IUGR is characterised by a pattern of placental lesions that reflect injury to both the maternal and fetal components of the placenta (Figure 1.2) (Baergen, 2011; Beaudet et al., 2007; Cox and Marton, 2009; Kovo et al., 2013; Mifsud and Sebire, 2014; Redline et al., 2004a; Redline et al., 2004b; Salafia, 1997; Vedmedovska et al., 2011). Decreased placental weight with increased numbers of syncytial knots, intervillous fibrin deposition, villous agglutination and distal villous hypoplasia (large areas of intervillous space with very small terminal villi interspersed with narrow elongated intermediate villi) are typical histological findings associated with IUGR (Kingdom et al., 2012; Redline, 2008; Redline et al., 2004b). Multiple areas of villous infarction with collapsed intervillous spaces and trophoblast necrosis may arise from arterial occlusions (Kingdom et al., 2012; Redline, 2008). Loss of vascular integrity may lead to retroplacental haemorrhages and intervillous thrombi predisposing the placenta to abruptions that also contribute to reduced placental function and IUGR. In addition, deficient EVT invasion may lead to decidual vasculopathies that include atherosis and retained muscularis of the maternal spiral arteries (Redline, 2008; Redline et al., 2004b).
The fetal vascular supply may also contribute to altered placental function leading to growth restriction. Compromise to the fetal vascular supply includes obstruction to the fetal circulation from thrombosis, cord compressions or vessel wall damage (Redline et al., 2004a). These injuries result in areas of capillary degradation and avascular villi and contribute to loss of placental reserve (Redline and Pappin, 1995). Vasculitis of fetal vessels, perhaps from damaged syncytiotrophoblasts, causes a loss of vascular integrity which produces haemorrhages of fetal blood into the intervillous space and placental thrombi (Macara et al., 1996; Redline, 2008; Redline et al., 2004a).

Reduced placental reserve or exchange capacity may also arise from aetiologies intrinsic to the placental parenchyma downstream of normal placental perfusion. These include massive perivillous fibrin depositions that surround and “suffocate” placental villi. Mechanisms leading to this placental lesion are not completely understood (Redline, 2008). Activation of the maternal coagulation cascade from exposure to villous extracellular matrix leads to the formation of focal intervillous thrombi which are contributory to altered gas exchange (Redline, 2008). Villitis of unknown aetiology (i.e., of non-infectious origin) is characterised by an infiltration of maternal T-cells into the villous stroma. The cause of this inflammatory response is not known but is associated with IUGR (Redline, 2007; Redline, 2008).

Problems in the synchronization between villous stromal and vascular development are also correlated with IUGR. Examples of these villous maturation defects include distal villous immaturity, dysmorphic villi (proximal-distal villous disproportion and abnormal villous contour) and reduced vasculosyncytial membranes (Redline et al., 2004a). The most common of these lesions is distal villous immaturity and is associated with diabetes and placental overgrowth and is said to result from excessive nutrient supply (Macara et al., 1996; Redline, 2008). Chronic placental ischaemia can result in villous capillary lesions such as chorangiomas (proliferative nodules) or chorangiosis (increased capillaries per villi).
The placenta is a unique organ in that it can adapt its growth and development in response to various insults in order to sustain fetal life by continually adapting to maximise gas and nutrient exchange between the maternal and fetal circulations (Torry et al., 2004). Placentas from IUGR pregnancies exhibit varying degrees of pathological lesions (Aviram et al., 2010; Kingdom et al., 2012; Redline, 2008; Triunfo et al., 2014) and it is not known to what extent placental function must be disrupted for IUGR to manifest (Salafia, 1997). Further investigations into the pathophysiological mechanisms leading to these lesions will help correlate pathology to dysfunction and studies to this effect are ongoing.

1.2.1.1 Diagnosis

Suspicion of placental IUGR is typically triggered by ultrasound identification of a fetal abdominal circumference (AC) or estimated fetal weight (EFW) below a certain threshold, typically the <10th percentile for gestational age (Bamberg and Kalache, 2004; Figueras and Gratacos, 2014; Harkness and Mari, 2004; Maulik, 2006; RCOG, 2013). Symphysis-fundal height (SFH) can be used to screen for growth restriction, when measurements are recorded serially (Figueras and Gardosi, 2011). However, SFH is less sensitive than ultrasound at detecting fetuses with biometry below critical thresholds (Creasy and Resnik, 2013).

The use of an AC or EFW percentile cut-off to identify growth restriction presents several challenges. Firstly, by definition, a certain proportion of any fetal population will be below the threshold (Cox and Marton, 2009; Soothill et al., 1999; Zhang et al., 2010). As such, a group of fetuses below any percentile cut-off will be heterogeneous in terms of their growth trajectories and will include fetuses with normal and pathological growth patterns. A proportion of fetuses (~50-70%) with an AC or EFW <10th percentile will be small and otherwise healthy (RCOG, 2013). These constitutionally small fetuses achieve their individual growth potential and are at low or no risk for adverse outcomes.
Threshold cut-offs of AC and EFW also fail to identify fetuses who are growth restricted but have AC and EFWs above the threshold cut-offs (Cox and Marton, 2009). Serial ultrasounds to monitor AC or EFW interval growth velocity will identify the fetuses who are falling off their growth trajectories (Creasy and Resnik, 2013), however, this surveillance cannot be offered to the general obstetric population due to financial and resource constraints on healthcare systems.

1.2.1.2 Risk factors

Risk factors for placental IUGR are similar to those of pre-eclampsia and include pre-existing maternal medical conditions (e.g., active autoimmune disease) and past obstetric history such as a previous pregnancy complicated by IUGR, pre-eclampsia, stillbirth or placental abruption (Maulik, 2006; RCOG, 2013; Seed et al., 2010). However, placental IUGR can arise in healthy women with neither past nor current risk factors.

In the absence of fetal aneuploidy or syndromes, early-onset presentation of an AC or EFW <10th percentile increases the risk that the fetus may have placental IUGR. An AC or EFW percentile that falls farther from the 10th percentile or a rapid decline in interval growth velocity is also a risk factor for placental IUGR (Figueras and Gratacos, 2014). Asymmetrical presentation (also referred to as “head sparing”) may also be associated with placental IUGR (Creasy and Resnik, 2013). As with pre-eclampsia, information from prenatal screening may help to identify pregnancies at risk of placental IUGR.

Unexplained abnormal maternal serum screen results have been associated with small-for-gestational age (SGA) status at birth (Conserva et al., 2010; Dugoff, 2010; Dugoff et al., 2005; Fox and Chasen, 2009; Goetzinger et al., 2009; Pilalis et al., 2007). However, it should be noted that SGA based on birthweight is a poor proxy for growth restriction as it merely provides information about the size of an infant as opposed to its intrauterine growth pattern. Other ultrasound determinants of placental IUGR include placental length and thickness, placental homogeneity, site of umbilical cord insertion, uterine artery Doppler waveforms (i.e., notching and/or increased resistance (RI) or pulsatility (PI) index) and umbilical
artery Doppler studies (Costa et al., 2008; Gruslin and Lemyre, 2011; Krebs et al., 1996; Miller et al., 2008).

1.2.1.3 Surveillance

Fetal surveillance in placental IUGR aims to balance the risks of ongoing pregnancy (i.e., intrauterine fetal death and/or catastrophic fetal central nervous system insult) against the measurable benefits of intact perinatal survival by increased gestational age at delivery (Harkness and Mari, 2004; Mari and Hanif, 2007; Marsal, 2009; Maulik, 2006; Miller et al., 2008; RCOG, 2013). At present, expectant management is the optimal option for managing placental IUGR. Expectant management allows for interventions such as administration of antenatal steroids for fetal lung maturation (Balci et al., 2010; Roberts and Dalziel, 2006) and magnesium sulphate for fetal neuroprotection in preterm fetuses (Doyle et al., 2009; Magee et al., 2011) to improve neonatal outcomes. The pregnant woman will receive increased ongoing fetal surveillance with serial ultrasound biometry (AC, head circumference, biparietal diameter and femur length), umbilical artery, middle cerebral artery, ductus venosus and aortic isthmus Doppler assessments, amniotic fluid assessments and fetal heart rate pattern analyses to detect fetal deterioration (Cnossen et al., 2008; Gonzalez et al., 2007; Miller et al., 2008; Ott, 2000).

Decision-making about termination of pregnancy (i.e., before 24 weeks gestation or EFW < 400g), frequency of testing, out- or inpatient surveillance and timing of delivery is determined by clinical integration of the data derived from this complex surveillance, not all of which are used routinely in tertiary care facilities.

1.2.1.4 Similarities with pre-eclampsia

Placental IUGR may manifest with or without pre-eclampsia. Typically, severe early-onset pre-eclampsia presents in conjunction with growth restriction, reflecting the consequence of abnormal placentation and subsequent placental dysfunction on both mother and fetus. Normotensive placental IUGR reflects an isolated fetal burden. In this regard, placental IUGR and pre-eclampsia can be thought
of as differential responses to the same trigger: a compromised placenta (Ness and Sibai, 2006). The proposed pathway linking these complications is abnormal placentation leading to placental ischaemia and cellular stress. As insult to the placenta continues and development is further impaired, uteroplacental mismatch develops which the placenta attempts to compensate for in order to sustain fetal life. These compensatory processes result in the release of factors that may trigger a systemic maternal response (i.e., pre-eclampsia) and contemporaneously, a fetal response of growth restriction may arise as well. Alternatively, the fetal response of growth restriction may manifest without a maternal response. The reasons for these differential responses to abnormal placentation have yet to be elucidated.

Support for the hypothesis of a differential response to abnormal placentation comes from the shared placental pathology between early-onset pre-eclampsia and placenta IUGR (Egbor et al., 2006; Mifsud and Sebire, 2014; Roberts and Post, 2008). Similar histological findings include infarction, excess intervillous fibrin deposition, distal villous hypoplasia, increased syncytial knotting and villous agglutination (Mifsud and Sebire, 2014; Roberts and Post, 2008). Decidual vasculopathies are also common findings in both complications as well as fetal vasculopathies such as vascular thrombi leading to downstream avascular villi (Redline et al., 2004a; Redline et al., 2004b).

1.3 Angiogenic factors

The uteroplacental unit needs to be highly vascularised and well perfused in order to function in its exchange capacity. Vasculogenesis (formation of vascular networks from de novo production of endothelial cells) and angiogenesis (formation of new blood vessels from existing endothelium) are vital processes that occur during placentation (Benirschke et al., 2012; Reynolds and Redmer, 2001; Reynolds et al., 2010; Torry et al., 1999; Torry et al., 2004; Zygmunt et al., 2003). Both processes are initiated and regulated by complex interplay between pro- and anti-angiogenic factors expressed in the placenta throughout gestation. These angiogenic factors include, but are not limited to, vascular endothelial
growth factor-A (VEGF-A), placental growth factor (PlGF), Fms-like tyrosine kinase (Flt-1, also referred to as VEGF receptor 1) and endoglin.

1.3.1 Vascular endothelial growth factor-A

VEGF-A is homodimeric glycoprotein and a member of the vascular endothelial growth factor family. The *VEGF-A* gene is located on chromosome 6 and six isoforms of VEGF-A exist as a result of alternate mRNA exon splicing. Heparin and heparin sulphate binding abilities differ between the isoforms causing some to be tightly bound to cell surfaces while others exist as diffuse forms (Byrne et al., 2005). VEGF-A is expressed by a number of tissues including vascular smooth muscle, brain, kidney and lung (Byrne et al., 2005; Shibuya, 2013; Shinkaruk et al., 2003). In the placenta, it is expressed by trophoblasts and fetal macrophages (Hofbauer cells) (Charnock-Jones et al., 2004; Clark et al., 1996; Clark et al., 1998; Cooper et al., 1995; Shore et al., 1997).

Through its receptors, Flt-1 and the kinase insert domain receptor (KDR, also referred to as VEGF receptor 2), VEGF-A promotes vasculogenesis, branching angiogenesis, survival of vascular endothelial cells (proliferation with reduced apoptosis) and endothelial cell migration and stabilization (Andraweera et al., 2012; Vrachnis et al., 2013). It increases vascular permeability and promotes vasodilation. VEGF-A may also have roles in trophoblast proliferation and migration, especially in regards to the epithelial to endothelial phenotype transition of EVTs during spiral artery remodelling as VEGF-A has been shown to upregulate expression of endothelial intregins (Andraweera et al., 2012; Charnock-Jones et al., 2004; Crocker et al., 2001).

VEGF-A expression is up-regulated by hypoxia (Charnock-Jones et al., 2004; Lash et al., 2002) as well as by various growth factors and cytokines (Andraweera et al., 2012; Charnock-Jones et al., 2004). VEGF-A is regulated at the transcriptional level through hypoxia-inducible factor (HIF-1) and mRNA stability, with reduced oxygen levels stimulating expression in placental fibroblasts (Charnock-Jones et al., 2004).
ER stress and the unfolded protein response (UPR) also increase levels of VEGF mRNA expression (Benirschke et al., 2012).

1.3.2 Placental growth factor

PIGF is heterodimeric glycoprotein. It is a member of VEGF family and has significant sequence homology to VEGF-A. There are four known isoforms of human PlGF (-1, -2, -3, -4) which arise from alternate splicing of the primary mRNA transcript from the PLGF gene located on chromosome 14 (Ribatti, 2008; Torry et al., 1999). PlGF-2 and -4 contain a heparin-binding domain, allowing them to interact with heparin sulphate proteoglycans on cell membranes. PlGF-1 and -3 exist primarily as soluble forms due to lack of this binding domain (Torry et al., 1999). PlGF binds the Flt-1 receptor with high affinity but does not bind KDR (Andraweera et al., 2012). During pregnancy, the placenta is the major source of PlGF. It is expressed by syncytiotrophoblasts and villous cytotrophoblasts as well as EVTs in early pregnancy (Andraweera et al., 2012; Khaliq et al., 1996; Shore et al., 1997). Outside of pregnancy, it is expressed in other cell types such as heart, lung and bone marrow (Adini et al., 2002; Ribatti, 2008; Torry et al., 1999).

During placentation, PlGF stimulates formation of persistent, non-leaky blood vessels and enhances vessel stabilization through macrophage recruitment and extracellular matrix deposition (Benirschke et al., 2012; Torry et al., 1999). PlGF also stimulates vascular smooth muscle cell proliferation as well as endothelial cell proliferation and migration (Benirschke et al., 2012; Torry et al., 1999). In concert with VEGF-A, PlGF may support trophoblast differentiation and migration during decidual invasion (Andraweera et al., 2012; Ribatti, 2008), however, exact mechanisms have not been elucidated to date. PlGF may also increase the bioavailability of VEGF-A to act through the KDR receptor by binding to Flt-1 with affinity or it may exist as a heterodimer with VEGF-A for binding to Flt-1/KDR heterodimers (Andraweera et al., 2012, Torry et al., 1999). PlGF has been shown to stimulate proliferation of first trimester EVTs and may protect trophoblast cells from apoptosis, thus contributing
to their survival (Desai et al., 1999; Zhou et al., 2003). PlGF may also have pro-inflammatory actions including activation of monocytes and lymphocytes (Vrachnis et al., 2013).

Expression levels appear to be regulated by oxygen tension as decreased mRNA expression and PlGF secretion are observed in trophoblasts cultured under hypoxic conditions (Gu et al., 2008b; Lash et al., 2002; Nagamatsu et al., 2004; Shore et al., 1997). Cytokines such as IL-6 and transforming growth factor (TGF-β) may also regulate PlGF but their effect on expression in the placenta is not known.

### 1.3.3 Fms-like tyrosine kinase receptor-1

Flt-1 is a member of the VEGF family. The FLT-1 gene is located on chromosome 13 and codes for a 1316 amino acid membrane-bound protein (Vrachnis et al., 2013). As a membrane-bound receptor, Flt-1 consists of seven extracellular immunoglobulin-like domains, an intracellular consensus split tyrosine kinase domain and a single transmembrane domain (Andraweera et al., 2012; Torry et al., 1999). Alternate mRNA splicing produces soluble Flt-1 (sFlt-1), containing six immunoglobulin-like domains. Flt-1 is expressed by syncytiotrophoblasts and cytotrophoblasts as well as a number of other cell types such as endothelial cells, vascular smooth muscle cells and monocytes (Andraweera et al., 2012; Clark et al., 1996, Clark et al., 1998; Shore et al., 1997; Torry et al., 1999).

Flt-1 is a receptor for both VEGF-A and PlGF. Despite strong affinity for both, its tyrosine kinase activity is weak and the majority of VEGF-A signalling during vasculogenesis and angiogenesis is through the KDR receptor (Shinkaruk et al., 2003). It is believed that Flt-1 may act as a decoy receptor for VEGF-A and negatively regulate angiogenesis during development (Shibuya, 2006). Circulating sFlt-1 appears to have antagonistic effects on VEGF-A and PlGF, reducing their bioavailability to cause increased vascular permeability and instability (Andraweera et al., 2012). Oxygen levels may regulate its expression in trophoblasts as hypoxia has been found to upregulate mRNA expression and sFlt-1 secretion in cultured cytotrophoblast cells (Gu et al., 2008; Nagamatsu et al., 2004).
1.3.4 Endoglin

Endoglin (also named CD105) is a transmembrane glycoprotein and expressed by endothelial cells and syncytiotrophoblasts (Baumwell and Karumanchi, 2007; Romero et al., 2008). It functions as a coreceptor for TGF-β1 and -β3. TGF-β1 induces endothelial cell proliferation and migration and so endoglin functions in a pro-angiogenic capacity when bound to cell membranes. During pregnancy, it appears to be an important negative regulator of trophoblast proliferation and migration as TGFs are inhibitors of these processes (Silasi et al., 2010; Venkatesha et al., 2006).

Endoglin also exists in soluble form (sEng). The mechanisms leading to its release into the maternal circulation are unclear but may involve shedding via MMP-14 activity (Kait'u-Lino et al., 2012; Romero et al., 2008). In its soluble form, endoglin may function as an anti-angiogenic factor as it can block the pro-angiogenic effects of TGF-β1 preventing its binding and subsequent downstream signalling to surface receptors on endothelial cells (Venkatesha et al., 2006). sEng increases vascular permeability (Baumwell and Karumanchi, 2007; Venkatesha et al., 2006). Hypoxia induces both secretion and mRNA expression of endoglin in cultured trophoblast cells (Gu et al., 2008b). The exact role of sEng in placentation has yet to be elucidated (Silasi et al., 2010).

1.3.4.1 Placental vasculogenesis and angiogenesis

Vasculogenesis begins around 18 days post conception. Primitive capillary structures are seen in the developing tertiary villi as mesenchyme from the extraembryonic mesoderm invades the cores of the trophoblast sprouts that give rise to these mesenchymal villi (Benirschke et al., 2012). Haemangioblastic progenitor cells, derived locally from fetal mesenchymal cells, form haemangiogenic cords that develop into capillary lumens surrounded with flattened endothelial cells by day 28 post conception. VEGF-A from cytotrophoblasts, fetal macrophages and maternal decidual cells predominately mediates mesenchymal differentiation and formation of these tubes (Gourvas et al., 2012). VEGF-A and KDR are required for vasculogenesis as gene ablation studies in mice have shown
VEGF-/− and KDR-/− knockouts result in early embryonic lethality with failed initiation of vasculogenesis (Andraweera et al., 2012).

As placentation progresses, neighbouring endothelial tubes connect and join to vessels in the early umbilical cord around day 32 post conception to form the primitive fetoplacental circulatory network. For the rest of gestation, vasculogenesis is limited to the tips of the mesenchymal villi found in small numbers on the surfaces of immature intermediate villi (Benirschke et al., 2012; Gourvas et al., 2012).

Following vasculogenesis, vascular development in the mesenchymal villi transitions to a period of branching angiogenesis (9 to 25 weeks gestation). The capillary beds of the mesenchymal villi rapidly branch and expand through sprouting and intersusscetption (Benirschke et al., 2012; Gourvas et al., 2012). Placental expression of VEGF-A, Flt-1 and KDR are high (perhaps due to low oxygen tension) while in comparison, PlGF expression is low. Branching angiogenesis gives rise to intermediate immature villi containing highly-branching capillary webs. From weeks 15 to 32, the intermediate immature villi develop into stem villi that are characterised by highly fibrosed, centrally located arteries and veins (Benirschke et al., 2012).

Stem villi give rise to mature intermediate villi by non-branching angiogenesis. Non-branching angiogenesis in villous capillaries is characterised by an increase in PlGF, Flt-1 and sFlt-1 expression (perhaps due to increased oxygen tensions) and a decrease in VEGF-A and KDR expression (Andraweera et al., 2012; Kingdom et al., 2012). From weeks 25 onwards, elongation of the capillary loops by cell differentiation, cord formation and tubulogenesis in the mature intermediate villi produce increased number, volume and surface area of the villous capillaries. Elongation of the capillaries increases at a greater rate than the elongation of the villi resulting in coiling of the capillaries that bulge from the surface of villi, giving rise to terminal villi with maximized gas exchange area (Andraweera et al., 2012; Kingdom et al., 2012; Vrachnis et al., 2013).
In pre-eclampsia and IUGR, vascular development may be disrupted in addition to other abnormalities in placentation such as failed spiral artery remodelling. Whether this is due to altered angiogenic factor expression or aberrant cellular responses to vasculogenic or angiogenic stimuli is not currently known (Ahmad and Ahmed, 2004; Andraweera et al., 2012; Clark et al., 1998; Gu et al., 2008b; Li et al., 2005; Riddell et al., 2013; Torry et al., 2004; Tsatsaris et al., 2003; Weed et al., 2012). Uteroplacental mismatch may arise in part from decreased number and size of terminal villi should angiogenesis fail to switch from branching to non-branching (Gourvas et al., 2012). A placental vascular tree with decreased branching also results in less placental reserve and may impair uteroplacental exchange. These vascular problems may be superimposed on uteroplacental malperfusion, compounding the insult and augmenting uteroplacental mismatch. Further investigations into mechanisms that disrupt placental vascular development are ongoing.

1.3.5 Circulating angiogenic factors

Angiogenic factors are produced by the placenta and released into the maternal circulation during pregnancy. In addition to their roles during placentation, circulating angiogenic factors likely contribute to the maintenance of the maternal endothelium (Powe et al., 2011). Exogenous sFlt-1 administered to pregnant rats causes pre-eclampsia-like symptoms (hypertension, proteinuria) as well as decreased circulating concentrations of serum VEGF-A (Bridges et al., 2009; Maynard et al., 2003). In vitro studies suggest that sFlt-1 binds VEGF-A resulting in loss of VEGF-A signalling that may sensitize endothelial cells to stimulation by pro-inflammatory cytokines such as TNF-α (Cindrova-Davies et al., 2001). As well, rats infused with sFlt-1 also have increased placental and vascular superoxide production indicating that sFlt-1 may play a role in generating oxidative stress within the placenta and contributes to endothelial dysfunction seen later in the maternal syndrome (Bridges et al., 2009).

In vivo experiments with pregnant rats show that sEng administration causes modest hypertension and proteinuria. When sEng is co-administered with sFlt-1, rats develop severe proteinuria, severe
hypertension and HELLP syndrome. Thus, it is likely that sFlt-1 and sEng act in concert to elicit their pathological effects in pre-eclampsia (Venkatesha et al., 2006).

1.3.5.1 Uncomplicated pregnancy

The patterns of circulating PlGF, sFlt-1 and sEng over gestational age in uncomplicated pregnancies have been well characterised (Chaiworapongsa et al., 2011; Foidart et al., 2010; Levine et al., 2004; Levine et al., 2006; Livingston et al., 2000; Makrydimas et al., 2008; Maynard et al., 2008; Powers et al., 2012; Reuvekamp et al., 1999; Schoofs et al., 2014; Shibata et al., 2005; Torry et al., 1998; Vatten et al., 2007; Wikstrom et al., 2007).

PlGF gradually increases in the maternal circulation with advancing gestation until approximately 32-33 weeks followed by a gradual decline in circulating levels until term (Figure 1.3) (Levine et al., 2004). sFlt-1 and sEng concentrations remain relatively low during the first two trimesters of pregnancy and gradually increase until term (Figures 1.4 and 1.5) (Levine et al., 2004).

Conflicting results regarding circulating VEGF-A levels in uncomplicated and complicated pregnancies have been reported. These discrepancies likely result from immunoassays measuring either total VEGF-A or free VEGF-A. As well, free VEGF-A levels are often below the detection limits of enzyme-linked immunosorbent assays (ELISA) (Andraweera et al., 2012; Crispi et al., 2006; Lam et al., 2005; Makrydimas et al., 2008; Reuvekamp et al., 1999; Taylor et al., 2003). As such, the clinical utility of VEGF-A in the prediction and/or diagnosis of pregnancy complications is limited and it will be excluded from the remaining discussion (Conde-Agudelo et al., 2013; Kleinrouweler et al., 2012; Zhou et al., 2010).

1.3.5.2 Pre-eclampsia

Compared with uncomplicated pregnancies, women with pre-eclampsia at term (≥37 weeks gestation) have decreased concentrations of circulating PlGF (Figure 1.3) and increased circulating concentrations of sFlt-1 (Figure 1.4) and sEng (Figure 1.5) at the time of diagnosis (Chaiworapongsa et al., 2011; Levine et al., 2004; Levine et al., 2006; Livingston et al., 2000; Powers et al., 2012; Reuvekamp
et al., 1999; Schoofs et al., 2014; Shibata et al., 2005; Torry et al., 1998; Vatten et al., 2007; Wikstrom et al., 2007). More significant decreases in PI GF and increases in sFlt-1 and sEng are observed in women diagnosed with early-onset pre-eclampsia and severe pre-eclampsia compared with uncomplicated pregnancies and women with term or mild pre-eclampsia (Crispi et al., 2006; Kim et al., 2009; Levine et al., 2004; Maynard et al., 2003; Ohkuchi et al., 2007; Powers et al., 2012; Rana et al., 2007; Robinson et al., 2006; Taylor et al., 2003; Vatten et al., 2007).

In women destined to develop pre-eclampsia, PI GF concentrations are decreased prior to the onset of disease compared with gestational-age matched women who do not develop pre-eclampsia. Differences in PI GF concentrations between controls and women who eventually develop pre-eclampsia can be seen as early as the first trimester (Figure 1.3) (Cowans et al., 2010; Noori et al., 2010; Ong et al., 2001; Powers et al., 2012; Taylor et al., 2003; Thadhani et al., 2004; Tidwell et al., 2001; Tjoa et al., 2001; Vandenberghhe et al., 2011; Vatten et al., 2007). Increased sFlt-1 levels in women destined to develop pre-eclampsia are typically seen in the second trimester (Figure 1.4) (Chaiworapongs a et al., 2005; Noori et al., 2010; Powers et al., 2012; Romero et al., 2008; Vatten et al., 2007). sEng levels are also elevated in the maternal circulation before clinical symptoms of pre-eclampsia arise (Figure 1.5) and sEng levels correlate with disease severity (Chaiworapongs a et al., 2011; Levine et al., 2006; Noori et al., 2010; Romero et al., 2008; Venkatesha et al., 2006).

1.3.5.3 Intrauterine growth restriction

To date, levels of circulating angiogenic factors in placental IUGR have not been studied extensively. In most studies, SGA (usually defined as birthweight <10th or <5th percentile for gestational age at delivery and sex) has been used as a proxy for IUGR (Levine et al., 2005; Malamitsi-Puchner et al., 2005; Ong et al., 2001; Romero et al., 2008; Schlembach et al., 2007; Taylor et al., 2003). PI GF levels in the maternal circulation have been found to be decreased in women who deliver SGA infants and these changes can be seen as early as the first trimester, however, reductions are not as significant as those
seen in women who develop pre-eclampsia (Asvold et al., 2011; Cowans et al., 2010; Malamitsi-Puchner et al., 2005; Nagamatsu et al., 2004; Ong et al., 2001; Poon et al., 2008; Romero et al., 2008; Shibata et al., 2005; Taylor et al., 2003; Tjoa et al., 2001; Wallner et al., 2007). sEng is reported to be increased in women destined to deliver SGA infants (Asvold et al., 2011; Levine et al., 2006; Romero et al., 2008) while sFlt-1 concentrations in SGA pregnancies have not been found to be significantly different from sFlt-1 levels in uncomplicated pregnancies when measured in early gestation (Asvold et al., 2011; Romero et al., 2008; Shibata et al., 2005; Thadhani et al., 2004).

When IUGR is defined based on antenatal ultrasound criteria such as EFW, AC, umbilical artery Doppler and fluid assessments, PI GF levels are decreased and sFlt-1 levels are increased in these cases compared with uncomplicated pregnancy controls, particularly when IUGR onset is before 34 weeks gestation (Crispi et al., 2006; Schoofs et al., 2014). Utilizing more stringent criteria to define IUGR as opposed to birthweight alone likely reduces the inclusion of constitutionally small fetuses within the sample group, providing a better indication of angiogenic factor levels in placental IUGR.

1.4 Research objectives

The purpose of my thesis is to further investigate angiogenic factors in placentally-mediated pregnancy complications such as pre-eclampsia and placental IUGR with particular attention to the association of these markers with placental dysfunction characteristic of these complications. Pre-eclampsia and placental IUGR represent disorders that are clinically challenging to predict and diagnose and the ability to better predict and diagnose these complications would allow for streamlined care of these women and babies. As such, my research objectives are as follows:

1. Compare the performance of two new clinical immunoassays for angiogenic factor quantification in the diagnosis of pre-eclampsia.
2. Investigate the utility of angiogenic factors measured in the second trimester of pregnancy to identify women destined to develop pre-eclampsia and other placentally-mediated complications.

3. Investigate maternal PlGF concentrations in placental IUGR as well as assess the ability of PlGF to antenatally identify placental IUGR in pregnancies with SGA fetuses.

4. Investigate the association between circulating maternal angiogenic factor concentrations and histopathological lesions of placental dysfunction as well as abnormal placental morphology in placentas from high-risk pregnancies and those with SGA fetuses.

Work presented in this thesis will expand on the body of evidence supporting the clinical utility of angiogenic factors not only in the context of pre-eclampsia but more importantly, in placental IUGR. My studies will improve our understanding of the connection between angiogenic factors and pregnancy complications in a translational context.
Figure 1.1 The pathogenesis model of pre-eclampsia. Early-onset pre-eclampsia (purple boxes) begins with abnormal placentation in early pregnancy while lowered maternal threshold and/or increased fetal demands contribute to late-onset pre-eclampsia (blue boxes). Both pathways converge to result in similar manifestations of the maternal syndrome (yellow boxes). ARDS: acute respiratory distress syndrome; ARF: acute renal failure; ATN: acute tubular necrosis; CNS: central nervous system; CVA: cerebrovascular accident; DbM: diabetes mellitus; DIC: disseminated intravascular coagulation; EVT: extravillous trophoblast; IUGR: intrauterine growth restriction; LV: left ventricular; PRES: posterior reversible encephalopathy; RIND: reversible ischemic neurological deficit; SNP: single nucleotide polymorphism, TIA: transient ischemic attack; ↑: increased risk of pre-eclampsia; ↓: decreased risk of pre-eclampsia.

Adapted from von Dadelszen et al., 2012 and used with permission from the Society of Obstetricians and Gynaecologists of Canada.
Figure 1.2 Placental lesions commonly associated with intrauterine growth restriction. Light micrographs of paraffin-embedded human placental tissue stained with haematoxylin and eosin. (A) depicts placental villi from an uncomplicated pregnancy with term delivery. In contrast, in cases of IUGR, placental villi exhibit increased syncytial knotting (B, selected knots indicated by arrows), distal villous hypoplasia (C) and infarction (D, selected areas indicated by arrows). Massive perivillous fibrin deposition (E, selected areas of fibrin indicated by arrows) and chronic villitis of unknown aetiology (F, selected areas indicated by arrows) are also associated with growth restriction.

Micrographs used with permission from the personal collection of Dr David Grynspan.
Figure 1.3 Circulating maternal placental growth factor in uncomplicated pregnancies and pre-eclampsia. Concentrations of placental growth factor (PlGF) increase in the maternal circulation until approximately 32 weeks gestation then gradually decrease until delivery in uncomplicated pregnancy (blue line). In contrast, PlGF concentrations are decreased throughout gestation in women who are destined to develop pre-eclampsia (red line). Women with a clinical diagnosis of pre-eclampsia have significantly lower circulating PlGF levels compared with uncomplicated pregnancy controls (black squares).

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Figure 1.4 Circulating maternal soluble Fms-like tyrosine kinase-1 in uncomplicated pregnancies and pre-eclampsia. Concentrations of soluble Fms-like tyrosine kinase-1 (sFlt-1) increase in the maternal circulation until delivery in uncomplicated pregnancy (blue line). In contrast, sFlt-1 concentrations are increased throughout gestation in women who are destined to develop pre-eclampsia (red line). Women with a clinical diagnosis of pre-eclampsia have significantly higher circulating sFlt-1 levels compared with uncomplicated pregnancy controls (black squares).

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Figure 1.5 Circulating maternal soluble endoglin in uncomplicated pregnancies and pre-eclampsia.

Concentrations of soluble endoglin (sEng) increase in the maternal circulation until delivery in uncomplicated pregnancy (blue line). In contrast, sEng concentrations are increased throughout gestation in women who are destined to develop preterm pre-eclampsia (<37 weeks gestation) and term pre-eclampsia (≥ 37 weeks gestation; red and orange lines, respectively). Women with a clinical diagnosis of pre-eclampsia have significantly elevated circulating levels of soluble endoglin compared with controls (black squares).

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Chapter 2. Point of care assays for pre-eclampsia diagnosis

2.1 Introduction

Pre-eclampsia is a heterogeneous syndrome. Its clinical presentation can be highly variable and may not include hypertension or proteinuria. Women with non-classically defined pre-eclampsia are still at risk for adverse outcomes and represent a clinically challenging subset of women to diagnose (Sibai, 2004; Sibai and Stella, 2009; Steegers et al., 2010; von Dadelszen et al., 2011). Mimickers of pre-eclampsia (e.g., renal disease with pre-existing hypertension and/or proteinuria) can also lead to clinical uncertainty around the diagnosis of pre-eclampsia (Sibai, 2009).

More comprehensive diagnostic tools are needed to aid in identifying women with pre-eclampsia in clinical practice especially in situations where clinical signs may neither be sensitive nor specific to the diagnosis. Biomarkers of placental origin, such as angiogenic factors, may be reasonable candidates for such diagnostic tests. PlGF and sFlt-1 have emerged as two potential markers for pre-eclampsia in the last decade (Levine et al., 2004; Maynard et al., 2003; Venkatesha et al., 2006). Pre-eclampsia is associated with decreased circulating maternal levels of PlGF and increased circulating levels of sFlt-1 and changes in these factors appear to reflect the severity of pre-eclampsia disease (Levine et al., 2004; Levine et al., 2006; Taylor et al., 2003). Early-onset pre-eclampsia is associated with greater changes in PlGF and sFlt-1 compared with late-onset pre-eclampsia and uncomplicated pregnancy (Ohkuchi et al., 2007; Romero et al., 2008; Wikstrom et al., 2007). Reported sensitivities and specificities of angiogenic factors in the diagnosis of pre-eclampsia varies depending on study design and immunoassay used for marker quantification (Chaiworapongsa et al., 2011; Engeld et al., 2013; Hagmann et al., 2012; Kaufmann et al., 2012; Salahuddin et al., 2007; Srinivas et al., 2010; Stefan et al., 2007; Sunderji et al., 2010; Tripathi et al., 2008).

The technology for angiogenic factor testing has advanced to automated analysis. Automated and point of care testing are vital for biomarker analysis in a clinical setting as it allows for rapid marker
quantification with minimal variability between samples compared with conventional 96-well plate enzyme-linked immunosorbent assays (ELISA). In this study, two clinical immunoassays available for use in the diagnosis of pre-eclampsia were compared: the point of care Triage® PlGF assay (Alere, San Diego, CA, USA) and the automated laboratory-based Elecsys® sFlt-1/PlGF Ratio assay (Roche Diagnostics, Penzberg, Germany). We sought to compare their test performances in the diagnosis of pre-eclampsia to determine which could be the most suitable for application in routine clinical practice.

2.2 Methods

2.2.1 Study subjects

In this retrospective, case-control study, blood samples were collected prospectively from women following written, informed consent, between November 2004 and August 2007 at BC Women’s Hospital in Vancouver, Canada. Ethics approval was granted by the University of British Columbia Children’s and Women’s Health Centre Research Ethics Board. Women with pre-eclampsia (cases) were recruited consecutively from inpatient and outpatient services at BC Women’s Hospital (e.g., Delivery Suite, Assessment, Ultrasound, Family Practice Maternity Services, Maternal Fetal Medicine Clinic). Uncomplicated pregnancy controls were matched to cases during the recruitment period based on maternal age (±5 years), gestational age at the time of sampling (±2 weeks) and parity (0, ≥1). Women (both cases and controls) were excluded if they were in active labour at the time of eligibility or had received antenatal betamethasone within 48 hours.

Pre-eclampsia was defined as hypertension (blood pressure ≥140/90 mmHg, on at least two occasions >4 hours apart after 20 weeks gestation) and new onset proteinuria (≥2+ dipstick reading, ≥0.3 g/day by 24 hour urine collection or ≥30 mg/mmol by protein:creatinine ratio) (Magee et al., 2008). Blood pressure measurements were taken using a mercury sphygmanometer, with Korotkoff V used to determine diastolic pressure. Women were positioned in a semi-recumbent position, with a supported arm and appropriately-sized cuff (Magee et al., 2008). SGA was defined as birthweight <5th percentile.
for gestational age at delivery and sex (Kramer et al., 2001). Uncomplicated pregnancy was defined as term delivery with no documented concerns of hypertension, proteinuria, gestational diabetes or growth restriction during the current pregnancy and was confirmed after delivery. For both cases and controls, blood pressure measurements were taken at the time of blood sample collection.

A total of 128 women were included in this study: 44 women with pre-eclampsia and 84 women with uncomplicated pregnancies. For the purposes of this study, we divided the pre-eclampsia cases into two groups according to the gestational age of disease onset. We defined early-onset pre-eclampsia as pre-eclampsia diagnosed before 35 weeks gestation based on our previous experience with PlGF in pregnancy (Knudsen et al., 2012). By this definition, there were 25 women who had early-onset pre-eclampsia and 19 women had late-onset pre-eclampsia. Of the controls, 47 had a blood sample collected prior to 35 weeks gestation (early controls) and 37 had blood collected ≥35 weeks gestation (late controls).

2.2.2 Blood sample collection and angiogenic factor analysis

Venous blood was taken at the time a confirmed diagnosis of pre-eclampsia was made or during an antenatal visit for the uncomplicated pregnancy controls. Serum was collected using silicon-coated glass tubes. Plasma was collected in EDTA tubes. Samples were prepared by centrifugation and frozen at -80°C. Laboratory staff was blinded to all clinical information pertaining to the women. Samples were batch assayed to minimize any effect of inter-assay variability.

Plasma was analysed for PlGF using the Triage® PlGF assay according to the manufacturer’s instructions. Using fluorescently-labelled monoclonal antibodies against PlGF for PlGF quantification, this immunoassay utilizes a single use disposable plastic assay test cartridge in conjunction with the Triage® Meter. Briefly, 250 µL of thawed plasma (room temperature) was pipetted into the sample port of a new test cartridge. The cartridge was inserted into the meter and results were displayed in pg/mL. The cartridge contains chemistries for on-board positive and negative control systems. Control systems
at the level of the cartridge and meter ensure that the quantitative PI GF result is valid. Calibration information is supplied by the manufacturer in the form of a lot-specific EP ROM chip that is contained within each kit of devices. The measurable range of the assay is 12-3000 pg/mL. Concentrations below the detection limit were recorded as "12 pg/mL". A positive PI GF test was defined as a PI GF concentration <5th percentile for gestational age at sampling. The 5th percentile cut-offs for gestational age are derived from uncomplicated pregnancy controls and are provided in the product insert of the Triage® meter (Knudsen et al., 2012).

Serum was analysed for PI GF and sFlt-1 using the Elecsys® assay to obtain a sFlt-1/PI GF ratio, according to the manufacturer’s instructions. Detection limits for sFlt-1 range from 10-85,000 pg/mL and 3-10,000 pg/mL for PI GF. A positive ratio test was defined as a fixed (independent of gestational age) ratio of >85 as described in the product inset. In addition, gestational age dependent sFlt-1/PI GF ratios from uncomplicated pregnancy were used to derived gestational age dependent cut-offs for a positive test (above the 95th percentile for gestational age range from uncomplicated pregnancies) (Verlohren et al., 2009).

2.2.3 Statistics

Data were analysed using Prism 4.0 (GraphPad, San Diego, CA, USA), SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Descriptive data were expressed as medians with interquartile ranges. Chi-square tests were used for comparison of categorical variables while Mann-Whitney U-tests or Kruskal-Wallis analysis of variance were used for continuous variables.

Receiver operating characteristic (ROC) curves were constructed to calculate area under the curves (AUC) and used to evaluate the performance of each assay in the diagnosis of pre-eclampsia at any gestational age, <35 weeks gestation and ≥35 weeks gestation. The ROC curve for the diagnosis of pre-eclampsia at any gestational age was used to determine a fixed cut-off for a positive test on each assay which maximized sensitivity and specificity. Sensitivity, specificity, positive predictive value (PPV)
and negative predictive values (NPV) with 95% confidence intervals (CI) were calculated using product
insert cut-offs, fixed cut-offs derived from the ROC curves and gestational-age dependent ratio cut-offs
(Elecsys® only). \( P \)-values <0.05 were judged to be statistically significant. PPV and NPV were calculated
to characterize the 2x2 tables, despite the artificial prevalence in this case-control study. Also,
qualitative PI GF results below 12 pg/mL were set equal to 12 pg/mL for the purpose of statistical
analysis. This approximation was not expected to affect the reported test performance in terms of
clinical sensitivity or specificity but may have slightly under reported the significance of the difference
between groups in the Mann-Whitney analysis or in the ROC AUC analysis.

Although the primary focus of this study was to assess the performance of PlGF and the sFlt-1/PI GF ratio for the diagnosis of pre-eclampsia (by estimating the sensitivity and specificity of the tests),
analyses to account for the matched case-control study design were also carried out. Conditional logistic
regression was used to obtain odds ratios with 95% CIs expressing the relationship between a positive
test and pre-eclampsia for each assay as well as PlGF levels (log transformed given non-normal
distribution and small study size) and the sFlt-1/PI GF ratio (also log transformed) and pre-eclampsia.

Comparison of these diagnostic tests followed the STARD (Standards for Reporting of Diagnostic
Accuracy) Initiative guidelines (Bossuyt et al., 2003).

2.3 Results

2.3.1 Clinical characteristics

Demographic characteristics and outcomes of women with pre-eclampsia and their matched
controls are shown in Table 2.1. Matching criteria (maternal age, parity, gestational age at sampling) did
not differ between the groups. Higher blood pressure, more proteinuria and a trend towards lower
platelets were seen in pre-eclampsia cases compared with controls. Gestational age at delivery was
lower with pre-term birth and SGA fetuses being more common in cases compared with controls.
2.3.2 PIGF concentration

Of the 44 pre-eclampsia cases, 24 cases had PIGF levels below the detection limit of the Triage® assay (<12 pg/mL). Two uncomplicated pregnancy controls (sampled at late gestational age) had PIGF levels below the detection limit. No cases or controls had concentrations below the detection limits for either PIGF (<3 pg/mL) or sFlt-1 (<10 pg/mL) on Elecsys®. PIGF concentrations measured by each assay for each woman are shown in Figure 2.1. PIGF levels in women with pre-eclampsia (onset at any gestation) were lower on the Triage® assay compared with PIGF measured on the Elecsys® assay.

Median PIGF concentrations in maternal circulation in cases and controls measured by Triage® and Elecsys® PIGF are shown in Table 2.2. For both assays, median PIGF concentrations were lower in pre-eclampsia cases compared with controls, regardless of gestational age. A substantial difference in median PIGF concentrations between cases and controls was observed when PIGF was measured on Triage® compared with Elecsys® (28-fold difference versus 8-fold difference for pre-eclampsia <35 weeks gestation, 6-fold difference versus 3-fold difference for pre-eclampsia ≥35 weeks gestation, 16-fold difference versus 5-fold difference for pre-eclampsia at any gestational age, respectively).

2.3.3 Diagnostic performance

Triage® PIGF and Elecsys® Ratio ROC AUC results are shown in Figure 2.2. Both assays had high AUCs for diagnosing pre-eclampsia before 35 weeks gestation (both 0.99) while the discriminatory power was lower for both when diagnosing pre-eclampsia ≥35 weeks gestation or pre-eclampsia at any gestational age.

From the ROC curve for diagnosis of pre-eclampsia at any gestational age (Figure 2.2a), fixed cut-offs for both assays that optimized sensitivity and specificity were determined to be a PIGF concentration <36 pg/mL for the Triage® and an sFlt-1/PIGF ratio >20 for Elecsys®. The cut-off of >20 for Elecsys® differed from the ratio cut-off of >85 defined in the product insert (Verlohren et al., 2009).
2.3.4 Sensitivity and specificity analysis

Sensitivity and specificity for the diagnosis of pre-eclampsia were calculated for both assays using product insert cut-offs (Table 2.3). Both assays had greatest sensitivity and specificity when diagnosing pre-eclampsia before 35 weeks gestation (Triage®: sensitivity 100% [95% CI: 86, 100], specificity 96% [95% CI: 85, 99]; Elecsys®: sensitivity 64% [95% CI: 43, 82], specificity 100% [95% CI: 93, 100]). Both assays had lower sensitivity for diagnosing pre-eclampsia after 35 weeks gestation and pre-eclampsia at any gestational age.

Sensitivity and specificity were also calculated using fixed ROC-derived cut-offs (Table 2.4). When the Elecsys® cut-off was reassigned to a ratio >20, sensitivity increased for the diagnosis of pre-eclampsia at any gestational age, pre-eclampsia <35 weeks gestation and pre-eclampsia ≥35 weeks gestation. Specificity was not improved, however. Improvement in sensitivity was only significant in diagnosing pre-eclampsia at any gestational age as these confidence intervals did not overlap (cut-off ratio >85: 59% [95% CI: 43, 74] versus cut-off ratio >20: 87% [95% CI: 75, 96]). The ROC-derived fixed cut-off improved the sensitivity of Triage® when diagnosing pre-eclampsia at any gestational age and after 35 weeks gestation; however, specificity decreased. Sensitivity was lost for the diagnosis of pre-eclampsia before 35 weeks gestation but a small increase in specificity was seen using a fixed PlGF concentration of <36 pg/mL (96% [95% CI: 85, 99] to 100% [95% CI: 92, 100]), however, these confidence intervals overlapped and the 95% CI of one estimate included the point estimate of the other indicating not statistical difference between them (i.e., P-value >0.05).

The Triage® PlGF test provided gestational-age dependent cut-offs to define a positive test. Using this approach, sensitivity and specificity of the Elecsys® assay using gestational-age dependent cut-offs for a positive test were calculated (Table 2.5). A slight increase in sensitivity in diagnosing pre-eclampsia before 35 weeks gestation was observed, however, confidence intervals overlapped and included the sensitivity point estimate obtained using the product insert cut-off of a ratio >85 indicating
no significant differences between the fixed and gestational-age cut-offs (68% [95% CI: 47, 85] versus 64% [95% CI: 43, 82]). Specificity was unchanged.

Conditional logistic regression analyses assessing the relationship between a positive PIGF test on Triage® and pre-eclampsia yielded an undefined odds ratio. Modelling log transformed PIGF values showed that the odds ratio for pre-eclampsia per unit increase in log PIGF was 0.133 (95% CI: 0.042, 0.419, P-value =0.0006) indicating a substantial decrease in pre-eclampsia rates with unit increase in log PIGF levels. Conditional logistic regression analyses assessing the relationship between a positive log sFlt-1/PIGF ratio test on Elecsys® and pre-eclampsia also yielded an undefined odds ratio. Modelling log transformed ratio values showed that the odds ratio for pre-eclampsia per unit increase in the log sFlt-1/PIGF ratio was 5.235 (95% CI: 2.056, 13.33, P-value =0.0006) indicating a substantial increase in pre-eclampsia ratio per unit increase in the log sFlt-1/PIGF ratio.

2.4 Discussion

We compared, for the first time, the performance of two commercially available automated immunoassays, Triage® PIGF and Elecsys® sFlt-1/PIGF ratio, in the diagnosis of pre-eclampsia. Both assays had high AUC results (0.99) for the diagnosis of pre-eclampsia before 35 weeks gestation; however, at the respective product insert cut-offs, Triage® had higher sensitivity, without loss of specificity, compared with Elecsys®. The 95% CIs of the sensitivities of both assays did not overlap, indicating a statistically significant difference in the diagnostic sensitivity. Conditional logistic regression analyses also showed a strong inverse relationship between log transformed PIGF levels and pre-eclampsia and a strong relationship between log transformed sFlt-1/PIGF ratio values and pre-eclampsia.

As expected, PIGF concentrations were decreased in women with pre-eclampsia compared with controls when measured on either assay. However, Triage® had greater differentiation of median PIGF concentrations between cases and controls compared with Elecsys®, with the greatest differentiation
seen in pre-eclampsia before 35 weeks gestation. Our results are consistent with previous reports that PIGF and sFlt-1 are altered in pre-eclampsia compared with uncomplicated pregnancy (Levine et al., 2004; Taylor et al., 2003; Venkatesha et al., 2006; Wikstrom et al., 2007). Previous reports using these angiogenic markers to diagnosis pre-eclampsia have demonstrated potential, but studies directly comparing the available assays for this purpose are limited. In one previous study, the sFlt-1/PlGF ratio measured on Elecsys® showed greatest diagnostic power for both pre-eclampsia and early-onset pre-eclampsia, compared with Elecsys® sFlt-1 or Elecsys® PlGF alone. Using serum samples, Verlohren et al. (2010) reported an AUC of 0.95 for the sFlt-1/PlGF ratio to diagnosis pre-eclampsia at any gestational age and an AUC of 0.97 for the diagnosis of early-onset pre-eclampsia (defined as onset before 34 weeks gestation). Using the ratio cut-off of >85 derived from the AUC curves, the authors report 89% sensitivity and 95% specificity for the test to diagnose early-onset pre-eclampsia. In our study, the Elecsys® ratio test had 64% [95% CI: 42, 82] sensitivity in the diagnosis of pre-eclampsia with onset before 35 weeks gestation. We did use a slightly different definition for early-onset pre-eclampsia (onset <35 weeks), but using the same definition of early-onset pre-eclampsia as <34 weeks gestation, the sensitivity of Elecsys® was 60% [95% CI: 36, 81] (data not shown). Apart from the difference in the definition of early-onset pre-eclampsia, we cannot explain the differences in test sensitivities between the two studies as all other reported methods were the same.

In our comparison, we found that Triage® PlGF had greater sensitivity with a small drop in specificity compared with Elecsys® sFlt-1/PlGF ratio in diagnosing pre-eclampsia before 35 weeks gestation when following product insert cut-offs. We compared both assays using fixed cut-offs derived from our ROC results. For Triage®, the only significant change observed was an increase in sensitivity in diagnosing pre-eclampsia at ≥35 weeks gestation (47% [95% CI: 24, 71] to 95% [95% CI: 74, 100], P-values <0.05). When the cut-off of Elecsys® was reassigned from a ratio of >85 to >20, sensitivity increased (64% to 92%, <35 weeks gestation; 53% to 84%, ≥35 weeks). In a previous study by Ohkuchi et
a cut-off ratio of >45.3 for Elecsys® gave 100% sensitivity and 95% specificity for diagnosis of early-onset pre-eclampsia (defined as onset before 34 weeks gestation). Authors commented that the fixed cut-off selected may need to be refined. Using a gestational age dependent reference range may be more appropriate due to the physiological fluctuations of these markers with advancing gestation (Levine et al., 2004; Levine et al., 2006). When gestational-age dependent cut-offs were used to define a positive test with Elecsys®, a minor increase in sensitivity without any change to specificity was observed. A re-assignment of the cut-off may raise the sensitivity of the assay.

We limited variability in our study by comparing test results of both assays by measuring samples from the same cases and controls. However, intrinsic differences in the assays themselves are a source of limitation. Firstly, we have used different matrices (plasma and serum) from the same patient to perform each assay to follow product specifications. A recent publication has indicated the need for standardization of immunoassays for PlGF as detectable concentrations vary between serum and plasma (Ogge et al., 2010). While differences in the performance of each assay may be due to the utilization of the different matrices, we believe that the variations arise due to the different target epitopes of the antibodies and not the matrix. A recent evaluation of Elecsys® PlGF using plasma samples produced similar results as other studies analysing serum samples with the Elecsys® platform (Ohkuchi et al., 2010). It is likely that Triage® would produce similar findings. As well, we have compared the assays according to their marketed purpose, the diagnosis of pre-eclampsia. Since the intended purpose is the same, performance comparison is justified and warranted as each has the potential to be implemented into clinical practice. Data comparing these assays, highlighting strengths and weaknesses, is needed for informed decision making on the part of hospital administrators and clinicians. We believe we have made such a comparison in a transparent fashion as each assay’s specific instructions were followed and performed on the blood samples collected from the same woman.
One additional limitation to this study is the Triage® results below the detection limit of the assay. Twenty-four of the 44 pre-eclampsia cases had PIGF measurements below the detection limit of 12 pg/ml. All of these cases were clearly differentiated from gestational-age matched uncomplicated controls. In addition, two normal pregnant controls had PIGF measurements below the detection limit, but these women had blood samples collected at late gestational ages (36^{+1} and 36^{+4} weeks, respectively) where PIGF is known to decline rapidly, as already discussed. Sample collection was standardized and is an unlikely source of variability as all samples were also run on the Elecsys® assay with no problems. Also, the Triage® meter contains internal control mechanisms to ensure sample integrity and ensure interfering substances are not responsible for decreased PIGF measurements. Therefore, we do not feel the qualitative readout of concentrations <12 pg/ml was a limitation in the current study.

In this study, our samples were obtained from women with confirmed pre-eclampsia, diagnosed according to conventional parameters (hypertension with proteinuria). There was no clinical uncertainty around their diagnoses. We recognize that inflated diagnostic test properties were obtained since we used samples from confirmed pre-eclampsia cases. Also, our control group was composed of women with completely uncomplicated pregnancies and this may contribute to inflation of the diagnostic results (Myers et al., 2013). This inflation is likely equal in both assays and as our primary focus was to compare one approach with the other, we do not feel this inflation confounded our results. It would also be beneficial to run the analysis on women recruited consecutively, rather than matching cases with controls, from a heterogeneous population of women with suspected pre-eclampsia and uncomplicated pregnancies. Such research would provide a better judgment regarding the assays’ application in routine clinical use.

In this study, we have found that the single analyte assay (Triage®) diagnosed pre-eclampsia before 35 weeks gestation with higher sensitivity compared with the ratio assay (Elecsys®) at the given
product insert cut-offs. Investigation into why these assays perform differently in terms of cut-offs is warranted using a larger sample size. It would also appear from this study that gestational-age dependent cut-offs for a positive test may perform better than fixed cut-offs. As well, as Triage® relies on only a single biomarker measurement, as opposed to two biomarkers like Elecsys®, chances of an incorrect result are minimized. Although PI GF and sFlt-1 both change in pre-eclampsia, reliance on both markers for a diagnosis may increase the probability for false negative and false positive results as there is inherent arithmetic instability in the ratio used.

Balancing the danger of false positives with the danger of false negatives in test performance is also warranted. In pre-eclampsia, the greatest danger lies in the false negative aspect. While a false positive woman would undergo increased (unnecessary) monitoring, greater concern would lie in sending a false negative women home with a missed diagnosis. A useful diagnostic test for pre-eclampsia would aim to maximize sensitivity as opposed to specificity. As well, because of the heterogeneity of pre-eclampsia, we believe an aid-to-diagnosis will have the greatest clinical impact in situations with uncertainty surrounding the diagnosis because of atypical presentation. It is unlikely a definitive test to diagnose all forms of pre-eclampsia will ever exist. Instead, reliable diagnosis will likely involve concurrent laboratory and examination methods. A multivariable approach for inclusion of point of care testing in clinical practice, in conjunction with other clinical and laboratory parameters, would be particularly beneficial.

In summary, this study compared the performance of two different point of care immunoassays in the diagnosis of pre-eclampsia. When comparing the sensitivity and specificity of the two tests using the cut-offs provided by the manufacturer for a positive test result, Triage® (gestational-age dependent cut-off) had higher sensitivity, without loss of specificity, in the diagnosis of pre-eclampsia before 35 weeks gestation compared with Elecsys® (fixed cut-off), despite similar AUCs. It would appear from this study that gestational-age dependent cut-offs for a positive test may perform better than fixed cut-offs.
Future directions for this work include validating the results in a larger group of women with atypical presentations of pre-eclampsia as well as in women with suspected pre-eclampsia (Chaiworapongsa et al., 2011; Chappell et al., 2013). Moreover, identifying optimal cut points and the best gestational-age window for when testing should be used in routine clinical practice is also needed.
Table 2.1 Clinical characteristics and pregnancy outcomes of the study groups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PET &lt;35 weeks (n=25)</th>
<th>Early controls(\circ) (n=47)</th>
<th>PET ≥35 weeks (n=19)</th>
<th>Late controls(\square) (n=37)</th>
<th>P-value (X² or KW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>36 [31, 40]</td>
<td>34 [32, 35]</td>
<td>33 [31, 37]</td>
<td>33 [30, 36]</td>
<td>0.32</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>15 (60)</td>
<td>25 (53)</td>
<td>16 (84)</td>
<td>31 (84)</td>
<td>0.0074</td>
</tr>
<tr>
<td>GA at sampling (weeks)</td>
<td>32.3 [28.5, 33.3]</td>
<td>31.9 [27.1, 33.7]</td>
<td>37.1 [35.6, 38.4]</td>
<td>36.9 [36.1, 38.3]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Multiple pregnancy</td>
<td>3 (12)</td>
<td>1 (2)</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>0.10</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
<td>160 [157, 182]</td>
<td>116(\ast\ast\ast) [108, 125]</td>
<td>160 [150, 175]</td>
<td>118(\ast\ast\ast) [110, 122]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
<td>107 [100, 115]</td>
<td>76(\ast\ast\ast) [70, 85]</td>
<td>100 [100, 110]</td>
<td>70(\ast\ast\ast) [70, 84]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Proteinuria(\circ)</td>
<td>25 (100)</td>
<td>0 (0)</td>
<td>19 (100)</td>
<td>0 (0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelets (x10⁹/L)</td>
<td>186 [168, 227]</td>
<td>221 (n=23) [172, 258]</td>
<td>158 [124, 216]</td>
<td>214 (n=20) (\ast) [185, 257]</td>
<td>0.0067</td>
</tr>
<tr>
<td>Uric Acid (uM)</td>
<td>347 [296, 410]</td>
<td>[380] [335, 410]</td>
<td>--</td>
<td>--</td>
<td>0.30*</td>
</tr>
<tr>
<td>AST (uM)</td>
<td>32 [28, 44]</td>
<td>--</td>
<td>33 [26, 57]</td>
<td>--</td>
<td>0.92*</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA at delivery</td>
<td>32.9 [31.6, 34.4]</td>
<td>39.7(\ast\ast\ast) [38.1, 40.3]</td>
<td>37.4 [36.1, 39.0]</td>
<td>39.7(\ast\ast\ast) [39.0, 40.3]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pre-term Birth (&lt;37^{\circ}) weeks)</td>
<td>24 (86)</td>
<td>0 (0)</td>
<td>5 (25)</td>
<td>0 (0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Birthweight (g) (n=fetuses)</td>
<td>1650 [1400, 1785]</td>
<td>3496(\ast\ast\ast) [3185, 3790]</td>
<td>2730 [2225, 3310]</td>
<td>3428(\ast) [3055, 3710]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SGA &lt;5th percentile</td>
<td>6 (21)</td>
<td>0 (0)</td>
<td>6 (30)</td>
<td>0 (0)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Mann Whitney U-test

\(\circ\) Uncomplicated pregnancy with blood sample collected <35 weeks gestation.

\(\square\) Uncomplicated pregnancy with blood sample collected ≥35 weeks gestation.

\(\circ\) Defined as either ≥2+ dipstick reading, ≥0.3 g/day by 24 hour urine collection or ≥30 mg/mmol by protein:creatinine ratio.
Data are expressed as median [interquartile range] or n (%).

Data were first compared across all columns to calculate the Kruskal-Wallis or $\chi^2$ P-value. Between-column comparisons were performed with pre-eclampsia <35 weeks gestation and pre-eclampsia ≥35 weeks gestation as the comparator groups to their matched uncomplicated pregnancy controls. §P<0.05, §§P<0.01, §§§P<0.001 (Dunn’s test, versus pre-eclampsia <35 weeks gestation); †P<0.05, ††P<0.01, †††P<0.001 (Dunn’s test, versus pre-eclampsia ≥35 weeks gestation).

AST: aspartate transaminase; GA: gestational age; KW: Kruskal-Wallis analysis of variance; sBP: systolic blood pressure; dBP: diastolic blood pressure; PET: pre-eclampsia; SGA: small for gestational age, $\chi^2$: Chi-square.
Table 2.2 Maternal concentrations of PlGF as measured by each immunoassay in women with pre-eclampsia and uncomplicated pregnancies.

<table>
<thead>
<tr>
<th>Gestational age at sampling</th>
<th>Pre-eclampsia</th>
<th>Uncomplicated pregnancy</th>
<th>P-value (MW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triage® PlGF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any gestation</td>
<td>12.0 [12.0, 20.6]</td>
<td>194.1 [86.4, 451.9]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;35 weeks</td>
<td>12.0 [12.0, 17.1]</td>
<td>334.3 [203.5, 688.6]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≥35 weeks</td>
<td>14.7 [12.0, 22.3]</td>
<td>83.9 [30.5, 173.3]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Elecsys® PlGF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any gestation</td>
<td>83.2 [45.1, 151.2]</td>
<td>394.0 [220.4, 667.8]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;35 weeks</td>
<td>62.5 [39.2, 123.5]</td>
<td>522.1 [363.4, 849.9]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≥35 weeks</td>
<td>90.9 [58.4, 194.2]</td>
<td>228.5 [133.4, 394.0]</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are expressed as median [interquartile range].

MW: Mann-Whitney test.
Table 2.3 Sensitivity and specificity of each immunoassay in diagnosing pre-eclampsia using product insert cut-offs for a positive test result.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity [95% CI]</th>
<th>Specificity [95% CI]</th>
<th>PPV [95% CI]</th>
<th>NPV [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triage® PlGF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any gestation</td>
<td>77 [62, 89]</td>
<td>95 [88, 99]</td>
<td>89 [75, 97]</td>
<td>89 [80, 95]</td>
</tr>
<tr>
<td>&lt;35 weeks</td>
<td>100 [86, 100]</td>
<td>96 [85, 99]</td>
<td>93 [76, 99]</td>
<td>100 [92, 100]</td>
</tr>
<tr>
<td>≥35 weeks</td>
<td>47 [24, 71]</td>
<td>95 [81, 99]</td>
<td>82 [48, 98]</td>
<td>78 [63, 89]</td>
</tr>
<tr>
<td><strong>Elecsys® Ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any gestation</td>
<td>59 [43, 74]</td>
<td>100 [96, 100]</td>
<td>100 [87, 100]</td>
<td>82 [74, 89]</td>
</tr>
<tr>
<td>&lt;35 weeks</td>
<td>64 [43, 82]</td>
<td>100 [93, 100]</td>
<td>100 [79, 100]</td>
<td>84 [72, 92]</td>
</tr>
<tr>
<td>≥35 weeks</td>
<td>53 [29, 76]</td>
<td>100 [91, 100]</td>
<td>100 [69, 100]</td>
<td>80 [66, 90]</td>
</tr>
</tbody>
</table>

* Positive test defined as PlGF concentration <5<sup>th</sup> percentile for gestational age derived from in normal pregnancies (Knudsen et al., 2012; Saffer et al., 2013).

^ Positive test defined as ratio > 85 (Verlohren et al., 2009).

CI: confidence interval; NPV: negative predictive value; PPV: positive predictive value.
Table 2.4 Sensitivity and specificity of each immunoassay in diagnosing pre-eclampsia using fixed cut-offs derived from receiver operating characteristic curves.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity [95% CI]</th>
<th>Specificity [95% CI]</th>
<th>PPV [95% CI]</th>
<th>NPV [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triage® PlGF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35 weeks</td>
<td>84 [64, 95]</td>
<td>100 [92, 100]</td>
<td>100 [84, 100]</td>
<td>92 [81, 98]</td>
</tr>
<tr>
<td>≥35 weeks</td>
<td>95 [74, 100]</td>
<td>73 [56, 86]</td>
<td>64 [44, 81]</td>
<td>96 [82, 100]</td>
</tr>
<tr>
<td><strong>Elecsys® Ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any gestation</td>
<td>89 [75, 96]</td>
<td>86 [76, 92]</td>
<td>76 [63, 87]</td>
<td>94 [85, 98]</td>
</tr>
<tr>
<td>&lt;35 weeks</td>
<td>92 [74, 99]</td>
<td>100 [92, 100]</td>
<td>100 [85, 100]</td>
<td>96 [86, 100]</td>
</tr>
<tr>
<td>≥35 weeks</td>
<td>84 [60, 97]</td>
<td>68 [50, 82]</td>
<td>57 [37, 76]</td>
<td>89 [72, 98]</td>
</tr>
</tbody>
</table>

* Positive test defined as PlGF concentration < 36 pg/mL derived from ROC curves.

^ Positive test defined as ratio > 20 derived from ROC curves.

CI: confidence interval; NPV: negative predictive value; PPV: positive predictive value, ROC: receiver operating characteristic.
Table 2.5 Sensitivity and specificity of Elecsys® sFlt-1/PIGF Ratio test in diagnosing pre-eclampsia using gestational-age dependent cut-offs.

<table>
<thead>
<tr>
<th>Pre-eclampsia onset</th>
<th>Sensitivity [95% CI]</th>
<th>Specificity [95% CI]</th>
<th>PPV [95% CI]</th>
<th>NPV [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any gestation</td>
<td>57 [41, 72]</td>
<td>100 [96, 100]</td>
<td>100 [86, 100]</td>
<td>82 [73, 86]</td>
</tr>
<tr>
<td>&lt;35 weeks</td>
<td>68 [47, 85]</td>
<td>100 [92, 100]</td>
<td>100 [80, 100]</td>
<td>85 [73, 94]</td>
</tr>
<tr>
<td>≥35 weeks</td>
<td>42 [20, 67]</td>
<td>100 [91, 100]</td>
<td>100 [63, 100]</td>
<td>77 [63, 88]</td>
</tr>
</tbody>
</table>

CI: confidence interval; NPV: negative predictive value; PPV: positive predictive value.
Figure 2.1 Maternal placental growth factor concentrations as measured by the Triage® PlGF test and the Elecsys® PlGF test for each patient sample. Triage® PlGF concentrations are plotted on the x-axis and Elecsys® PlGF concentrations are plotted on the y-axis. Axes were Log10 transformed. The dotted black line represents the detection limit of Triage® PlGF at 12 pg/mL. The green line represents as the detection limit for Elecsys® PlGF at 10 pg/mL.
Figure 2.2 Receiver operating characteristic curves for each assay in the diagnosis of pre-eclampsia.

Calculated area under the curves (AUC) of Triage® PI GF (green line) and Elecsys® Ratio (blue line) in the diagnosis of (A) pre-eclampsia at any gestational age, (B) pre-eclampsia<35 weeks gestation and (C) pre-eclampsia ≥35 weeks gestation.
Chapter 3. Prediction of placentally-mediated pregnancy complications

3.1 Introduction

Identifying women who will develop pre-eclampsia and other placentally-mediated complications in later gestation is a priority in obstetrical care. Stratifying women based on risk allows for streamlined management with appropriate surveillance and intervention and identifies women who would benefit most from specialist referral and preventative treatment (Magee et al., 2008). Currently, identifying women at increased risk for developing pre-eclampsia and other placentally-mediated complications is primarily based on clinical risk factors such as maternal age and past obstetrical history. Clinical risk factors alone have poor to modest power to screen for pre-eclampsia and current predictive models report area under the receiver operating characteristics curves (AUC ROC) between 0.65-0.80 (Doyle et al., 2005; North et al., 2011; Seed et al., 2011). Unexplained abnormal serum markers and uterine artery Doppler (bilateral notching, increased RI and/or PI) are also used for identifying women at higher risk of developing pre-eclampsia. However, alone or in combination, these parameters yield low sensitivities with high false positive rates given the variable rates of pre-eclampsia even in women with risk factors.

Angiogenic factors such as PlGF, sFlt-1 and sEng present in the maternal circulation may have potential as predictive markers for pre-eclampsia and intrauterine growth restriction/small for gestational age as circulating levels are known to change prior to the onset of disease. Despite early changes in circulating angiogenic factors, their value as predictive markers is poor with sensitivities and specificities ranging from 35-99% (Crispi et al., 2008; Espinoza et al., 2007; Ghosh et al., 2013; Kleinrhouweler et al., 2012; Kusanovic et al., 2009; McElrath et al., 2012; Myers et al., 2013; Moore Simas et al., 2007; Polliotti et al., 2003; Su et al., 2001; Vandenberghe et al., 2011; Vatten et al., 2007). The highest predictive performances are reported for early-onset disease, likely reflecting the placental contribution of this disease subtype. Studies evaluating the performance of these markers to predict
other important placentally-mediated complications such as stillbirth and placental abruption are limited (Smith et al., 2007).

In this study, we evaluated the performance of circulating angiogenic factors measured in the second trimester to predict placentally-mediated pregnancy outcomes (pre-eclampsia, stillbirth, placental abruption and SGA) in a high-risk pregnancy cohort. In addition, we performed an exploratory secondary analysis to investigate if alterations in circulating angiogenic factors are associated with typical placental lesions of placental dysfunction, as these lesions are prominent pathological features of pre-eclampsia, IUGR and other placentally-mediated complications.

3.2 Methods

3.2.1 Study subjects

In this prospective cohort study, women were recruited consecutively from the EMMA Clinic at BC Women’s Hospital between 2004 and 2009. The EMMA Clinic is a referral clinic for the province of British Columbia that assigns an integrated estimated risk score for developing placentally-mediated pregnancy complications (pre-eclampsia, IUGR, placental abruption, stillbirth) based on clinical information, maternal serum screening results and uterine artery Doppler tests. Recommendations on surveillance and management are made based on this risk assignment (Magee et al., 2008). Indications for referral to the clinic include past obstetric history (e.g., pre-eclampsia, IUGR, stillbirth), unexplained abnormal serum screen results (e.g., elevated AFP) and/or other significant medical history (e.g., renal disease). Women participating in this study represent a high-risk pregnancy group. Ethics approval was granted by the University of British Columbia Children’s and Women’s Health Centre Research Ethics Board.

Women with singleton pregnancies seen in the EMMA Clinic between 15–23 weeks gestation were approached to participate in the study. In total, 296 women attended the EMMA clinic during the recruitment period and 255 (86%) women agreed to participate and provided written informed consent.
Maternal age, parity and reasons for referral to the clinic did not differ between the women who did and did not consent to participate in the study (data not shown).

### 3.2.2 Blood sample collection and angiogenic factor analysis

Following informed consent, a maternal venous blood sample was collected in the normal fashion. Serum was collected using silicon-coated glass tubes and plasma was collected in EDTA tubes. Blood was centrifuged and aliquots of serum and plasma were frozen at -80°C. Laboratory and clinical staff were blinded to all patient information. Samples were batch assayed to minimize any effect of inter-assay variability.

Plasma was analysed for PlGF using the Triage® PlGF assay according to the manufacturer’s instructions (Alere, San Diego, CA, USA). The measurable range of the assay is 12-3000 pg/mL. Concentrations below the detection limit were recorded as "12 pg/mL". Plasma concentrations of sFlt-1 and sEng were measured using conventional 96-well ELISA (R&D, Minneapolis, MN, USA). Samples were assayed in duplicate and an averaged concentration was obtained. Samples were diluted so that values fit within the linear portion of the standard curve. The minimum detectable concentration for sFlt-1 was 13.3 pg/mL and 0.03 ng/mL for sEng.

### 3.2.3 Outcome of interest

Maternal demographics, obstetrical and medical history, pregnancy outcomes as well as neonatal outcomes were collected by chart review following delivery. Women were excluded if they elected for termination of pregnancy before 24 weeks gestation or if they were lost to follow-up.

Our primary outcome of interest was a composite outcome defined as the development of one or more of the following complications: early-onset pre-eclampsia, stillbirth, placental abruption and/or delivery of an SGA live birth (used as a proxy for IUGR). Early-onset pre-eclampsia was defined as hypertension (blood pressure ≥140/90 mmHg, on at least two occasions >4 hours apart after 20 weeks gestation) and new onset proteinuria (≥2+ dipstick reading, ≥0.3 g/day by 24 hour urine collection or ≥30
mg/mmol by protein:creatinine ratio) diagnosed before 34 weeks gestation (Magee et al., 2008). An outcome of placental abruption was based on physician notes indicating the presence of abruption at the time of delivery. SGA status was defined as birthweight <10th percentile for gestational age and sex (Kramer et al., 2001).

The primary outcome of interest was evaluated according to abnormal or normal angiogenic factor concentrations at the time of sampling (i.e., between 15–23 weeks gestation). A PIGF concentration was defined as abnormal if it was <5th percentile for gestational age (Saffer et al., 2013). A PIGF concentration ≥5th percentile for gestational age was defined as normal. sFlt-1 and sEng concentrations were defined as abnormal if they were >95th percentile for gestational age. A normal sFlt-1 and sEng concentration was defined as a concentration ≤95th percentile for gestational age. A woman was included in the abnormal group if one or more of the markers was abnormal.

3.2.4 Statistics

Data were analysed using Prism 5.0 (GraphPad, San Diego, CA, USA) and SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Descriptive data were expressed as means with standard deviations for normally distributed data and medians with interquartile ranges for non-normally distributed data. Continuous variables were compared with unpaired Student’s t-tests (parametric) or Mann-Whitney U-tests (non-parametric). Categorical variables were described using counts and proportions and compared with Chi-square tests. P-values <0.05 were judged to be statistically significant.

ROC curves were constructed to calculate AUC with 95% CIs and used to evaluate the performance of each marker to predict the composite outcome as well as each individual outcome. To account for the physiological changes of circulating angiogenic factors with advancing gestation, marker concentrations were converted into a percentile for gestational age at sampling. Percentiles were derived from marker concentrations in women who did not develop any pregnancy complications (i.e., from women with uncomplicated pregnancies in this cohort). In addition, the performance of each
analyte to predict the composite outcome using a gestational-age dependent percentile cut-off for a positive test result was evaluated using sensitivity, specificity, positive and negative predictive values (PPV, NPV) with 95% CIs. For PlGF, a positive result was defined as a PlGF concentration <5\textsuperscript{th} percentile for gestational age. For sFlt-1 and sEng, a positive test result was defined as a concentration >95\textsuperscript{th} percentile for gestational age.

Logistic regression analysis was used to examine the relationship between the composite outcome and the following exposure variables: PlGF concentration, sFlt-1 concentration, sEng concentration and other covariates including maternal age, gestational age at sampling, unexplained serum screen and uterine artery notching. Odds ratios and adjusted odds ratios (OR) with 95% CIs were calculated. For modelling purposes, a logarithmic scale (log transformation) was used for PlGF concentrations to approximate a normal distribution. In addition, dichotomous indicators including PlGF concentrations <5\textsuperscript{th} percentile and sFlt-1 and sEng concentrations >95\textsuperscript{th} percentiles of normal concentrations for gestational age were modelled in logistic regression analysis for their association with the composite outcome.

3.2.5 **Exploratory study**

In addition to the evaluation of angiogenic factors to predict the composite outcome, we undertook an exploratory secondary analysis to investigate the relationship between second trimester marker concentrations and abnormal placental pathology at the time of delivery.

Placental pathology reports were collected from the hospital charts of the women recruited in the study. Of the 246 women, 52 (21\%) women had a pathology report available. Each report was evaluated by our Study Pathologist, blinded to all clinical and outcome information apart from gestational age at delivery, for placental lesions according to a standard classification system (Redline et al., 2003; Redline et al., 2004a; Redline et al., 2004b; Redline, 2008). Each lesion was graded as follows: maternal malperfusion (distal villous hypoplasia and/or infarction with accelerated villous maturation: 0[absent],
1[mild], 2[moderate], 3[severe]); chronic villitis of unknown aetiology (0[absent], 1[focal], 2[multifocal], 3[diffuse]); fetal stromal-vascular maldevelopment (distal villous immaturity and dysmorphology: 0[absent], 1[mild], 2[moderate], 3[severe]); perivillous fibrin deposition (0[absent], 1[focal], 2[patchy], 3[diffuse]); fetal vascular obstruction (0[absent], 1[focal], 2[multifocal], 3[diffuse]) and chronic marginal separation (0[absent], 1[marginal hematoma], 2[diffuse chorionic haemosiderosis]). Our placental phenotype of interest was lesions of maternal malperfusion with fetal stromal-vascular maldevelopment as these lesions reflect placental dysfunction. The placental phenotype of interest was defined as a grading ≥1 in maternal malperfusion with an associated grading ≥1 in fetal stromal-vascular maldevelopment (Redline, 2008).

Logistic regression was used to examine the association between circulating angiogenic factors (PlGF, sFlt-1 and sEng) and the placental outcome, adjusting for other covariates including maternal age, gestational age at sampling, unexplained serum screen results and uterine artery notching. Independent variables were log transformed for modelling. Similar to the analysis of the composite adverse outcome, percentile cut-offs were also used to examine dichotomous indicators of low concentration (PlGF) or high concentration (sFlt-1, sEng) of the markers.

3.3 Results

3.3.1 Clinical characteristics

Of the 255 women recruited in the study, 9 were excluded from analyses because of elective termination of pregnancy or being lost to follow up. Overall, 246 women had complete outcome information and were included in the analysis.

Of the 246 women, 7 (2.8%) developed early-onset pre-eclampsia. Four of these cases were associated with SGA status at delivery. One woman with early-onset pre-eclampsia also had a placental abruption. There were 4 (1.6%) stillbirths. One stillborn fetus was SGA at delivery. There were 5 (2.0%) cases of isolated placenta abruption. There were 46 (18.7%) SGA live births. Two of these SGA cases
were associated with placental abruption. Overall, 58 (23.6%) women developed one or more components of the composite outcome (Table 3.1).

Of the 246 women, 38 (15%) had one or more abnormal angiogenic factor concentrations at the time of sampling. The remaining 208 (85%) women had normal angiogenic factor levels. Demographic characteristics and outcomes of the women grouped according to abnormal or normal angiogenic factor concentrations at the time of sampling are presented in Table 3.2. Baseline characteristics between the groups did not vary in terms of maternal age, gestational age at sampling, nulliparity, pre-pregnancy weight or pre-existing hypertension ($P$-value $>0.05$ for each characteristic). As expected, median circulating angiogenic factor concentrations were different between the groups ($P$-value $<0.0001$ for PlGF and sFlt-1, $P$-value $=0.002$ for sEng). In the abnormal marker group, most women had an abnormal PlGF concentration (i.e., PlGF $<5^{\text{th}}$ percentile, 25 of out 38 (66%)). Women with abnormal marker levels delivered earlier and had smaller babies than women with normal marker levels at the time of sampling ($P$-value $<0.0001$ for both). There was not a significant difference in the rate of adverse pregnancy outcomes in women with abnormal markers as a group compared with women with normal marker levels ($P$-value $>0.05$ for each outcome).

### 3.3.2 Association between marker concentrations and the composite adverse outcome

Rates of the composite outcome by marker concentration at the time of sampling are shown in Table 3.3. There was an increased prevalence of the composite outcome in women with PlGF $<5^{\text{th}}$ percentile for gestational age at the time of sampling compared with women who had PlGF $\geq 5^{\text{th}}$ percentile at sampling (56% vs 20%, $P$-value $=0.0004$). The rate of the composite outcome did not differ significantly between women with sFlt-1 $>95^{\text{th}}$ percentile or $\leq 95^{\text{th}}$ percentile for gestational age at sampling (31% vs 23%, $P$-value $=0.75$). This finding was also found for sEng (35% vs 23%, $P$-value $=0.24$) (Table 3.3).
In the multivariable logistic regression analysis, a PlGF concentration <5th percentile for gestational age was associated with 4-fold higher odds of the composite adverse outcome, independent of other covariates including maternal age, gestational age, uterine artery notching between 20–24 weeks and unexplained abnormal serum screen result (adjusted OR=3.78, 95% CI: 1.39, 10.23). An increase in log PlGF concentration was associated with a significant decrease in odds of the composite adverse outcome, (adjusted OR=0.53, 95% CI: 0.37, 0.75). Log concentrations of sFlt-1 and sEng >95th percentile for gestational age at sampling were not significantly associated with the composite adverse outcome (adjusted OR=2.09, 95% CI: 0.49, 8.91 and adjusted OR=0.92, 95% CI: 0.26, 3.28, respectively).

3.3.3 Predictive performance

AUC ROC results for each analyte to predict the development of the outcomes of interest, either alone or as a composite, are shown in Table 3.4. No marker had an AUC above 0.80 (desired AUC for a good clinical test) although PlGF had an AUC of 0.74 to predict early-onset pre-eclampsia. Sensitivities and specificities were calculated for each marker to predict the composite adverse outcome using cut-offs <5th percentile (PlGF) or >95th percentile (sFlt-1 and sEng) for gestational age at sampling in uncomplicated pregnancy (Table 3.5). All markers yielded low sensitivities and specificities at these cut-offs.

3.3.4 Exploratory secondary analysis

Of the 246 women in the cohort, 52 (21 %) women in the study had a placental pathology report available and where included in the analysis. A total of 18 (34%) placentas in this group had the placental outcome of interest (grade ≥1 for maternal malperfusion with grade ≥1 for fetal villous-stromal maldevelopment).

Women were grouped based on abnormal or normal angiogenic factor concentrations at the time of sampling. There were 11 (21%) women who had one or more abnormal marker concentrations and 41 (79%) women had normal marker concentrations. Demographics characteristics and outcomes of the
groups are shown in Table 3.6. Baseline characteristics between the groups did not vary in terms of gestational age at sampling, nulliparity, pre-pregnancy weight or pre-existing hypertension (all P-values >0.05). Median circulating angiogenic factor concentrations were different between the groups (P-value <0.0001 for all markers). In the abnormal marker group, most women had an abnormal PlGF concentration (i.e., PlGF <5th percentile, 10 of out 11 (91%)). Women with abnormal marker levels delivered earlier and had smaller babies than women with normal marker levels at the time of sampling (P-value <0.0001 for both) but there was not a significant difference in the number of SGA infants between the groups. There was not a significant difference in the rate of adverse pregnancy outcomes in women with abnormal markers as a group compared with women with normal marker levels (P-value >0.05 for each outcome). Trimmed placental weight was significantly reduced in the abnormal marker group compared with the normal marker group (P-value =0.003).

Rates of the placental outcome by markers levels at the time of sampling are shown in Table 3.7. The placental outcome occurred in 8 (80%) women with PlGF <5th percentile compared with 10 (24%) women with PlGF ≥5th percentile (P-value =0.0019). In the multivariable analysis, an increase in log PlGF concentration was associated with a significant decrease in the odds of the placental outcome (adjusted OR=0.37, 95% CI: 0.15, 0.93). A PlGF concentration <5th percentile was associated with approximately 20-fold higher odds of the placental phenotype, after adjustment for other covariates (adjusted OR=19.83, 95% CI: 2.69, 146.25). Wide confidence intervals reflect the small sample size of this exploratory analysis.

The placental outcome occurred in 4 (66%) women with sFtI-1 >95th percentile and in 5 (100%) women with sEng >95th percentile and in 12 (31%) and 13 (28%) women who had concentrations of sFtI-1 and sEng ≤95th percentile, respectively. In multivariable analysis, the odds of the placental outcome increased by 53% per one unit increase in log sFtI-1 concentration, however, this association was of borderline statistical significance (adjusted OR=1.53, 95% CI: 1.00, 2.34). An sFtI-1 concentration >95th
percentile was not significantly associated with the placental outcome (adjusted OR=7.45, 95% CI: 0.87, 63.95). Similarly, the odds of the placental outcome increased by 11% per unit increase in log sEng concentration but the association was also of borderline statistical significance (adjusted OR=1.11, 95% CI: 0.97, 1.26). sEng > 95th percentile was significantly associated with the placental outcome (P-value =0.003), however, the odds ratio (crude and adjusted OR) was undefined due to low numbers.

3.4 Discussion

In this study, we investigated angiogenic factors measured in the second trimester of pregnancy and their association with placentally-mediated pregnancy complications in a cohort of high-risk women. Women with decreased circulating PlGF at the time of sampling had significantly higher odds of developing the composite outcome (early-onset pre-eclampsia, placental abruption, stillbirth and/or SGA live delivery). PlGF values below the 5th percentile for gestational age at sampling were associated with approximately a 4-fold increased odds of the composite outcome. Maternal sFlt-1 and sEng were not significantly associated with the composite outcome. Additionally, as shown by the AUCs, no marker predicted the composite outcome with adequate power (all AUCs less than 0.70) and the corresponding sensitivities and specificities were low. PlGF did have an AUC 0.74 in the prediction of early-onset pre-eclampsia when this outcome was considered alone.

Our results are similar to previous studies investigating angiogenic factors and the prediction of pregnancy complications. PlGF levels, measured in the second trimester, have been reported to be decreased in women who later develop pre-eclampsia as well as those who deliver an SGA infant (Cowans et al., 2010; Kusanovic et al., 2009; Malamitsi-Puchner et al., 2005; Noori et al., 2010; Ong et al., 2001; Poon et al., 2008; Powers et al., 2012; Romero et al., 2008; Shibata et al., 2005; Taylor et al., 2003; Thadhani et al., 2004; Tidwell et al., 2001; Tjoa et al., 2001; Vandenberghe et al., 2011; Vatten et al., 2007; Wallner et al., 2007). sFlt-1 and sEng have been reported to be increased in the second trimester in the circulation of women who eventually develop pre-eclampsia (Chaiworapongs et al.,
As an extension to these previous reports, we also included other important pregnancy complications in our definition of the composite outcome such as stillbirth and placental abruption to reflect the heterogeneity of outcomes in high-risk women. In terms of the predictive performance of angiogenic factors, previous studies using a composite outcome are limited and have focused on the prediction of early-onset pre-eclampsia and IUGR/SGA delivery (Espinoza et al., 2007; Ghosh et al., 2013; Moore Simas et al., 2007; Polliotti et al., 2003; Su et al., 2001; Vandenberghe et al., 2011). High predictive performance has been reported. For example, a large study reported 82% sensitivity and 65% specificity for PlGF, measured between 20–22 weeks gestation, to predict pre-eclampsia with onset before 32 weeks gestation (Ghosh et al., 2013). This study also reported 84% sensitivity and 67% specificity of PlGF to predict IUGR onset before 32 weeks gestation (defined as EFW or AC <10th percentile with abnormal umbilical artery PI >95th percentile) (Ghosh et al., 2013). Another study found sFlt-1 measured between 22–26 weeks gestation had an AUC of 0.90 for predicting pre-eclampsia before 34 weeks gestation in a group of high-risk women (Moore Simas et al., 2007). A nested case-control study of PlGF and sFlt-1 measured at 24 weeks gestation predicted early-onset of pre-eclampsia and/or IUGR (birthweight <10th percentile with umbilical artery PI >95th percentile before 32 weeks) with 84% and 37% sensitivity, respectively, at 95% specificity (Crispi et al., 2008). In our study, we observed an AUC of 0.74 and 0.53 for PlGF and sFlt-1 to predict early-onset pre-eclampsia, respectively. Additionally, we found an AUC of 0.67 and 0.55 for PlGF and sFlt-1 to predict an SGA live birth. The differences between our findings and the higher performance indices reported in other studies may be due differences in study design and a larger sample size is needed to confirm these findings.

It has been suggested that prediction of pre-eclampsia may be feasible with multivariable modelling and like others, our findings suggest that PlGF may be a candidate predictor (Audibert et al., 2010;
Boucoiran et al., 2013; Crispi et al., 2008; Di Lorenzo et al., 2012; Myers et al., 2013). In one study, multivariable analysis showed that the best prediction of preterm pre-eclampsia required information on clinical risk factors (i.e., mean arterial pressure, sister with history of pre-eclampsia, previous fertility treatment) and PlGF measured at 15 weeks gestation (AUC 0.84, 45 % sensitivity at 95% specificity) (Myers et al., 2013). Another study reported that PlGF between 11–14 weeks gestation combined with maternal characteristics had an AUC of 0.73 to predict the onset of pre-eclampsia (Boucoiran et al., 2013). Chronic hypertension, β-hCG and PlGF combined predicted early-onset pre-eclampsia with 67% sensitivity at a false positive rate of 5% in study measuring these analytes in the first trimester of pregnancy (Di Lorenzo et al., 2012). Another study reported a screening model of clinical characteristics, PAPP-A, inhibin-A and PlGF could predict early-onset pre-eclampsia with 75% sensitivity and 90% specificity (Audibert et al., 2010). Further studies evaluating PlGF in the prediction of pre-eclampsia and other placentally-mediated pregnancy complications are warranted. It would be particularly interesting to determine if PlGF levels correlate with adverse maternal and/or fetal outcomes in the women that do developed these complications. Future studies should also consider the optimal gestational age for PlGF testing.

In our exploratory secondary analysis, we evaluated circulating second trimester angiogenic factor levels and placental lesions to investigate if these markers are associated with abnormal placental pathology. Studies investigating associations between placental pathology and angiogenic factor concentrations are limited. In one study, authors found that in women with late-onset pre-eclampsia who had placental lesions associated with maternal malperfusion, PlGF levels were decreased while sEng and sFlt-1 levels were increased compared with normotensive controls and women with late-onset pre-eclampsia without placental lesions (Soto et al., 2012). In our study, maternal PlGF concentration <5th percentile measured in the second trimester was associated with a 20-fold increase in the odds of placental lesions of maternal malperfusion and fetal villi maldevelopment (Redline et al., 2004a; Redline
et al., 2004b). Additionally, an sEng concentration >95th percentile was also significantly associated with the placental lesions (P-value =0.033) but we were unable to define an odds ratio due to the small number of women who had an sEng concentration >95th percentile. The association between sFlt-1 levels >95th percentile and the placental phenotype was not statistically significant (P-value =0.17) and was of borderline statistical significance after adjustment for other covariates (the adjusted OR per one unit increase was OR=1.53, 95% CI: 1.00, 2.34).

While our sample size is small, these findings suggest that angiogenic factors, particularly PlGF, may act as early markers of placental dysfunction in later pregnancy. A biomarker that predicts specific placental lesions/phenotypes could help to identify those women at risk pregnancy complications associated with these lesions (i.e., placentally-mediated complications). For example, it is believed that lesions of maternal malperfusion reflect placental dysfunction arising from abnormal placentation in early pregnancy. If abnormal placentation could reliably be detected antenatally, surveillance, treatment and interventions could be adjusted accordingly in anticipation of adverse outcomes arising from abnormal placentation. Further studies are warranted to elucidate these associations (Salafia et al., 1997).

In summary, angiogenic factors had low performance for predicting a composite outcome of early-onset pre-eclampsia, stillbirth, placental abruption and SGA delivery in this cohort of high-risk women. PlGF appears to show promise for the prediction of early-onset pre-eclampsia and future directions should include multivariable modelling with other predictors that are known risk factors for placentally-mediated pregnancy complications. Also, decreased PlGF in the second trimester of pregnancy was associated with a placental phenotype related to placental dysfunction. As such, PlGF may have potential for distinguishing placental phenotypes antenatally, although a large, well-characterised cohort would be required to demonstrate such an effect.
Table 3.1 Pregnancy outcomes of the women in the cohort.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>No composite outcome</th>
<th>Early-onset pre-eclampsia</th>
<th>Placental abruption</th>
<th>Stillbirth</th>
<th>SGA live birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>No composite outcome</td>
<td>139 (56.5%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early-onset pre-eclampsia*</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Placental abruption</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Stillbirth</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SGA live birth</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>139 (56.5%)</td>
<td>7 (2.8%)</td>
<td>8 (3.3%)</td>
<td>4 (1.6%)</td>
<td>46 (18.7%)</td>
</tr>
</tbody>
</table>

* Defined as onset before 34 weeks gestation.

SGA: small for gestational age.
Table 3.2 Demographic characteristics and outcomes of the women with abnormal and normal angiogenic factor concentrations at sampling.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Abnormal markers (n=38)</th>
<th>Normal Markers (n=208)</th>
<th>P-value (Student’s t-test, MW or X²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA at sampling (weeks)</td>
<td>18.4 ± 1.9</td>
<td>18.7 ± 1.8</td>
<td>0.37</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>33 ± 6</td>
<td>34 ± 5</td>
<td>0.30</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>19 (50)</td>
<td>88 (42)</td>
<td>0.38</td>
</tr>
<tr>
<td>Pre-pregnancy weight (kg)</td>
<td>66 ± 17</td>
<td>68 ± 21</td>
<td>0.67</td>
</tr>
<tr>
<td>Pre-existing hypertension</td>
<td>3 (8)</td>
<td>23 (11)</td>
<td>0.60</td>
</tr>
<tr>
<td>Past obstetrical history*</td>
<td>5 (13)</td>
<td>40 (19)</td>
<td>0.37</td>
</tr>
<tr>
<td>Unexplained abnormal serum marker **</td>
<td>26 (68)</td>
<td>120 (58)</td>
<td>0.35</td>
</tr>
<tr>
<td>Uterine artery notching present between 20–24 weeks gestation</td>
<td>15 (39)</td>
<td>72 (35)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>At sampling</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIGF (pg/mL)</td>
<td>13.2 [12.0, 43.1]</td>
<td>72.0 [39.1, 124.0]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PIGF &lt;5th percentile</td>
<td>25 (66)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>sFlt-1 (ng/mL)</td>
<td>4.0 [2.1, 6.1]</td>
<td>2.0 [1.4, 2.7]</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>sFlt-1 &gt;95th percentile</td>
<td>16 (42)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>sEng (ng/mL)</td>
<td>15.6 [13.2, 21.7]</td>
<td>13.2 [10.3, 16.5]</td>
<td>0.0023</td>
</tr>
<tr>
<td>sEng &gt;95th percentile</td>
<td>17 (45)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA at delivery (weeks)</td>
<td>35.3 ± 5.7</td>
<td>38.1 ± 2.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pre-term birth (&lt;37° weeks)</td>
<td>14 (36)</td>
<td>49 (24)</td>
<td>0.085</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>2487 ± 1122</td>
<td>3114 ± 677</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Composite adverse outcome</td>
<td>13 (34)</td>
<td>43 (22)</td>
<td>0.09</td>
</tr>
<tr>
<td>Early-onset pre-eclampsia</td>
<td>3 (8)</td>
<td>4 (2)</td>
<td>0.077</td>
</tr>
<tr>
<td>Placental abruption</td>
<td>2 (5)</td>
<td>6 (3)</td>
<td>0.36</td>
</tr>
<tr>
<td>Stillbirth</td>
<td>2 (5)</td>
<td>2 (1)</td>
<td>0.053</td>
</tr>
<tr>
<td>Characteristic</td>
<td>Abnormal markers (n=38)^◊ (Mean ± SD, n (%) or median [IQR])</td>
<td>Normal Markers (n=208) (Mean ± SD, n (%) or median [IQR])</td>
<td>P-value (Student’s t-test, MW or X^2)</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------------------------------------</td>
<td>----------------------------------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGA &lt;10^{th} percentile</td>
<td>9 (24)</td>
<td>38 (18)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

◊ Defined as one or more marker <5^{th} percentile (PIGF) or >95^{th} percentile (sFlt-1, sEng) for gestational age at sampling.

* Included pre-eclampsia, stillbirth and/or IUGR in a previous pregnancy.

** Elevated AFP, low first trimester or elevated second trimester hCG, low uE3 and/or low PAPP-A.

GA: gestational age; IQR: interquartile range; SD: standard deviation; SGA: small for gestational age.
Table 3.3 Rates of the composite adverse outcome by angiogenic factor concentration at the time of sampling.

<table>
<thead>
<tr>
<th>Adverse Outcome</th>
<th>PI GF &lt;5(^{\text{th}}) percentile (n=25)</th>
<th>PI GF ≥5(^{\text{th}}) percentile (n=220)</th>
<th>Fisher’s exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>14</td>
<td>44</td>
<td>0.0002</td>
</tr>
<tr>
<td>No</td>
<td>11</td>
<td>176</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>sFlt-1 &gt;95(^{\text{th}}) percentile (n=16)</th>
<th>sFlt-1 ≤95(^{\text{th}}) percentile (n=213)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>No</td>
<td>11</td>
<td>165</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>sEng &gt;95(^{\text{th}}) percentile (n=17)</th>
<th>sEng ≤95(^{\text{th}}) percentile (n=229)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>6</td>
<td>52</td>
</tr>
<tr>
<td>No</td>
<td>11</td>
<td>177</td>
</tr>
</tbody>
</table>

◊ One plasma sample missing.

☒ Seventeen plasma samples missing.
Table 3.4 Area under the receiver operating characteristic curves for each marker to predict adverse pregnancy outcomes.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>PIGF  [95% CI]</th>
<th>sFlt-1 [95% CI]</th>
<th>sEng  [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composite adverse outcome (n=58)</td>
<td>0.67 [0.57, 0.75]</td>
<td>0.55 [0.47, 0.64]</td>
<td>0.57 [0.48, 0.66]</td>
</tr>
<tr>
<td>Early-onset pre-eclampsia* (n=7)</td>
<td>0.74 [0.58, 0.90]</td>
<td>0.53 [0.21, 0.85]</td>
<td>0.68 [0.50, 0.85]</td>
</tr>
<tr>
<td>Placental abruption (n=8)</td>
<td>0.68 [0.50, 0.86]</td>
<td>0.61 [0.41, 0.81]</td>
<td>0.57 [0.32, 0.82]</td>
</tr>
<tr>
<td>Stillbirth (n=4)</td>
<td>0.65 [0.29, 1.00]</td>
<td>0.51 [0.17, 0.85]</td>
<td>0.62 [0.48, 0.76]</td>
</tr>
<tr>
<td>SGA delivery (n=47)</td>
<td>0.67 [0.58, 0.76]</td>
<td>0.55 [0.46, 0.64]</td>
<td>0.61 [0.44, 0.77]</td>
</tr>
</tbody>
</table>

* Defined as onset before 34 weeks gestation.

CI: confidence interval; SGA: small for gestational age.
Table 3.5 Sensitivities and specificities of each marker to predictive the composite adverse outcome based on a gestational-age dependent cut-off.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity [95% CI]</th>
<th>Specificity [95% CI]</th>
<th>PPV [95% CI]</th>
<th>NPV [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIGF (&lt;5th percentile for GA)</td>
<td>24.1 [13.9, 37.2]</td>
<td>93.6 [89.1, 96.7]</td>
<td>53.9 [33.4, 73.4]</td>
<td>80.0 [74.1, 85.1]</td>
</tr>
<tr>
<td>sFlt-1 (&gt;95th percentile for GA)</td>
<td>6.9 [1.9, 16.7]</td>
<td>94.2 [89.8, 97.0]</td>
<td>26.7 [7.8, 55.1]</td>
<td>76.6 [70.6, 81.9]</td>
</tr>
<tr>
<td>sEng (&gt;95th percentile for GA)</td>
<td>10.3 [3.9, 21.1]</td>
<td>94.1 [89.8, 97.0]</td>
<td>35.3 [14.2, 61.7]</td>
<td>77.3 [71.3, 82.6]</td>
</tr>
</tbody>
</table>

CI: confidence intervals; GA: gestational age.
Table 3.6 Demographic characteristics and outcomes of the women according to angiogenic factor concentration at the time of sampling in the exploratory study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Abnormal markers (n=11)</th>
<th>Normal markers (n=41)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SD, n (%))</td>
<td>(Mean ± SD, n (%))</td>
<td>(Student’s t-test, MW or χ²)</td>
</tr>
<tr>
<td>GA at sampling (weeks)</td>
<td>18.3 [16.7, 20.1]</td>
<td>19.0 [16.3, 20.0]</td>
<td>0.84</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>33 ± 6</td>
<td>34 ± 5</td>
<td>0.74</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>7 (64)</td>
<td>24 (59)</td>
<td>1.00</td>
</tr>
<tr>
<td>Pre-pregnancy weight (kg)</td>
<td>64 ± 8</td>
<td>66 ± 17</td>
<td>0.80</td>
</tr>
<tr>
<td>Pre-existing hypertension</td>
<td>1 (9)</td>
<td>8 (20)</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>At sampling</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIGF (pg/mL)</td>
<td>12.0 [12.0, 12.0]</td>
<td>40.6 [28.2, 117.5]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PIGF &lt;5th percentile</td>
<td>10 (91)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>sFlt-1 (ng/mL)</td>
<td>5.7 [2.9, 8.2]</td>
<td>2.2 [1.3, 2.8]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sFlt-1 &gt;95th percentile</td>
<td>7 (64)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>sEng (ng/mL)</td>
<td>23.7 [17.1, 25.6]</td>
<td>13.7 [10.6, 17.0]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sEng &gt;95th percentile</td>
<td>5 (45)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Outcome characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placental outcome°</td>
<td>8 (73)</td>
<td>10 (24)</td>
<td>0.0048</td>
</tr>
<tr>
<td>GA at delivery (weeks)</td>
<td>31.2 ± 5</td>
<td>37.0 ± 2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>1458 ± 1097</td>
<td>2599 ± 615</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SGA &lt;10th percentile</td>
<td>6 (55)</td>
<td>16 (39)</td>
<td>0.49</td>
</tr>
<tr>
<td>Early-onset pre-eclampsia</td>
<td>2 (18)</td>
<td>1 (2)</td>
<td>0.11</td>
</tr>
<tr>
<td>Stillbirth</td>
<td>1 (9)</td>
<td>1 (2)</td>
<td>0.38</td>
</tr>
<tr>
<td>Trimmed placental weight (g)</td>
<td>284 ± 144</td>
<td>402 ± 100</td>
<td>0.003</td>
</tr>
</tbody>
</table>

◊ Defined as one or more marker <5th percentile (PIGF) or >95th percentile (sFlt-1, sEng) for gestational age at sampling.
Placental outcome was defined as histological evidence of maternal malperfusion (graded ≥1) with fetal stromal-vascular maldevelopment (graded ≥1).

GA: gestational age; SD: standard deviation; SGA: small for gestational age.
Table 3.7 Rates of the placental outcome by angiogenic factor concentration at the time of sampling.

<table>
<thead>
<tr>
<th>Placental outcome◊</th>
<th>PlGF &lt;5\textsuperscript{th} percentile (n=10)</th>
<th>PlGF ≥5\textsuperscript{th} percentile (n=41)\textsuperscript{○}</th>
<th>Fisher’s exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>8</td>
<td>10</td>
<td>0.0019</td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

| sFlt-1 >95\textsuperscript{th} percentile (n=6) | sFlt-1 ≤95\textsuperscript{th} percentile (n=39)\textsuperscript{□} |
|-----------------------------------------------|-------------------------------------------------|------------------|
| Yes                                           | 4                                              | 12               | 0.17             |
| No                                            | 2                                              | 27               |                  |

| sEng >95\textsuperscript{th} % percentile (n=5) | sEng ≤95\textsuperscript{th} percentile (n=47) |
|-------------------------------------------------|-------------------------------------------------|------------------|
| Yes                                              | 5                                              | 13               | 0.003            |
| No                                               | 0                                              | 34               |                  |

◊ Placental outcome was defined as histological evidence of maternal malperfusion (graded ≥1) with fetal stromal-vascular maldevelopment (graded ≥1).

○ One plasma sample missing.

□ Seven plasma samples missing.
Chapter 4. Maternal PIGF in the identification of placental intrauterine growth restriction

4.1 Introduction

Fetuses antenatally identified as being small for gestational age (typically EFW or AC< 10th percentile for gestational age on ultrasound) attract increased clinical attention due to the potential for adverse outcomes as a result of poor in utero growth (Harkness and Mari, 2004; Miller et al., 2008; RCOG, 2013; Maulik, 2006; Mari and Hanif, 2007; Marsal, 2009). However, fetuses with AC <10th percentile can be divided into two main populations, those who are constitutionally small (small but healthy fetuses) and those who are pathologically growth-restricted. Constitutionally small fetuses achieve their growth potential and are at low or no for adverse outcomes (RCOG, 2013). On the other hand, growth restriction due to placental dysfunction puts the fetus at risk of preterm delivery, stillbirth, serious neonatal complications and developmental sequelae (Breeze and Lees, 2007; Garite et al., 2004; Halliday, 2009).

Constitutionally small fetuses and fetuses with placental IUGR are difficult to differentiate antenatally. As such, constitutionally small (and healthy) fetuses may be followed as though they are IUGR, adding unnecessary burden to families and healthcare systems. These fetuses will be at risk for adverse events such as iatrogenic preterm delivery should they be misdiagnosed as placental IUGR. The ability to tailor assessment and surveillance to accurately determine the presence or absence of placental IUGR would represent a significant advance in antenatal care. Biomarkers present in maternal circulation that reflect placental functional status may provide this vital piece of additional information. Additional surveillance tools would help to streamline and improve care for the high-risk fetus (placental IUGR) and avoid unnecessary monitoring, health care costs and parental anxiety for the low-risk fetus (constitutionally small).
Angiogenesis is a process that may be disrupted during placentation which contributes to placental dysfunction in later gestation. PI GF is a key factor in placental angiogenesis and is present in maternal circulation and placental tissues during pregnancy (Clark et al., 1996; Makrydimas et al., 2008). In uncomplicated pregnancy, PI GF levels gradually increase until the end of the second trimester, then decrease gradually until delivery. Decreased maternal PI GF is characteristic of pre-eclampsia, another placentally-mediated pregnancy complication (Levine et al., 2006; Maynard et al., 2003; Romero et al., 2008; Taylor et al., 2003). Studies investigating maternal PI GF concentrations in placental IUGR are limited. Studies have reported decreased PI GF concentrations in the circulation of women who deliver SGA infants. However, these studies used varying birthweight cut-offs to define SGA and failed to categorize pregnancies based on the cause of SGA (pathological or physiological). As well, most research has only investigated PI GF alterations in pregnancies delivering SGA fetuses in association with pre-eclampsia, not in normotensive women. The relationship between maternal levels of PI GF and fetuses with and without placental IUGR remains to be further elucidated.

We propose that differences in maternal PI GF concentrations discriminate between fetuses with placental IUGR and constitutionally small fetuses as PI GF may reflect compromise to placental function. In this study, we sought to characterize PI GF in pregnancies complicated by placental IUGR (defined by placental pathology) and those with constitutionally small fetuses. We postulate that a positive PI GF test (low PI GF in the maternal circulation) as measured on a new point of care rapid assay differentiates fetuses with placental IUGR from constitutionally small fetuses.

4.2 Methods

4.2.1 Study subjects

In this study, blood samples were prospectively collected from women with singleton pregnancies antenatally diagnosed with IUGR following written informed consent, between November 2004 and August 2007 at BC Women's Hospital in Vancouver, Canada. Ethics approval was granted by
the University of British Columbia Children’s and Women’s Health Centre Research Ethics Board. Eligible women were consecutively recruited from inpatient and outpatient services at BC Women’s Hospital. Women were excluded if they were in active labour at the time of eligibility or were within 48 hours of antenatal betamethasone administration.

The antenatal diagnosis of IUGR was defined as a fetal AC <10\textsuperscript{th} percentile for gestational age on ultrasound. Maternal blood samples were collected at this time but were not included in the study until after delivery. Inclusion required either an infant birthweight <5\textsuperscript{th} percentile for gestational age at delivery and sex (Kramer et al., 2001) or birthweight <10\textsuperscript{th} percentile with either uterine artery Doppler notching at 22\textsuperscript{nd} – 24\textsuperscript{th} weeks gestation, absent/reversed umbilical artery end diastolic flow or oligohydramnios [amniotic fluid index <50 mm] documented during the pregnancy.

A total of 19 confirmed cases were available for inclusion in this study. Two were excluded from the primary analysis due to IUGR being attributed to fetal congenital anomalies confirmed after delivery (one Treacher-Collins syndrome, one Cornelia de Lange syndrome). One additional case was excluded due to IUGR of unknown origin. During this pregnancy, the women had an appendectomy at 10 weeks gestation, followed by cerclage for cervical incompetence at 12 weeks. Preterm spontaneous rupture of membranes occurred at 29\textsuperscript{st} +2 weeks followed by delivery of a live fetus three weeks later (birthweight <10\textsuperscript{th} percentile). Placental pathology showed chorioamnionitis and funisitis. No infection was noted. The remaining 16 cases were sub-grouped based on the presence or absence of placental IUGR.

Placental IUGR (n=9) was defined as IUGR with an abnormal placental pathology report consistent with placental dysfunction (Redline et al., 2004a; Redline et al., 2004b). Lesions included decidual necrosis and/or vasculopathies, increased fibrin deposition, fetal vascular obstruction, increased calcification, infarction, intervillous thrombi, advanced villous maturation, distal villous hypoplasia and villous dysmorphology. A constitutionally small fetus (n=7) was defined as a fetus antenatally diagnosed with “IUGR” and with a no evidence of significant placental pathology as indicated in the pathology report.
Although not included in the primary analysis, the three syndromic IUGR cases all had normal placental pathology reports. Placental pathology status was determined by a perinatologist blinded to all aspects of the woman’s history, pregnancy outcomes and PlGF concentration (apart from gestational age at delivery).

A total of 79 non-smoking women served as uncomplicated pregnancy controls. These women had no documented concerns of hypertension, proteinuria, gestational diabetes or IUGR during their pregnancy. Controls were matched for maternal age (±5 years), gestational age (±2 weeks) and parity (0, 1, ≥2).

### 4.2.2 Sample collection and PlGF analysis

Maternal venous blood was collected antenatally in EDTA tubes in the standard fashion at the time the antenatal diagnosis of IUGR was made. Plasma was obtained through centrifugation and samples were frozen at -80°C. Laboratory staff was blinded to all clinical information of the women. Plasma was analysed for PlGF using the Triage® PlGF assay according to the product insert (Alere, San Diego, CA, USA). The measurable range of the assay is 12-3000 pg/mL. Concentrations below the detection limit were recorded as "12 pg/mL". A positive test was defined as a PlGF concentration <5th percentile for gestational age derived from uncomplicated pregnancies as described in the product insert (Knudsen et al., 2012). Samples were batch assayed to minimize any effect of inter-assay variability.

### 4.2.3 Statistics

Data were analysed using Prism 4.0 (GraphPad, San Diego, CA, USA) and SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Descriptive data are expressed as medians with interquartile ranges for non-normally distributed data. Chi-square test or Fisher’s exact test was used for comparison of categorical variables. Kruskal-Wallis analysis of variance or Mann-Whitney U-test was used for continuous variables.

The performance of a positive PlGF test for identifying placental IUGR was evaluated using a 2x3 contingency table (positive versus negative tests for each group) and a test of association (the Freeman-
Halton test for the complete 2x3 table and the Fisher exact test of the relevant 2x2 sub-table).

Sensitivity, specificity, PPV and NPV for a positive PIGF test to identifying placental IUGR from constitutionally small fetuses were calculated with 95% CIs as a secondary analysis. We performed this analysis with and without the three syndromic IUGR cases excluded from the primary analysis. PPV and NPV were calculated to characterize the 2x2 tables, despite the artificial prevalence in this case-control study. Also, qualitative PIGF results <12 pg/mL were set equal to 12 pg/mL for the purpose of statistical analysis. This approximation does not affect the reported test performance in terms of clinical sensitivity or specificity, but may slightly under report the significance of the difference between groups in the Kruskal-Wallis analysis.

Although the primary focus of this study was to assess the performance of PIGF in the discrimination between placental IUGR and constitutionally small fetuses (by estimating the sensitivity and the specificity of the test), analyses to account for the matched case-control study design were also carried out. Conditional logistic regression was used to obtain odds ratios with 95% CIs expressing the relationship between a positive test and pre-eclampsia for each assay as well as PIGF levels (log transformed given non-normal distribution and small study size).

Differences with $P$-values <0.05 were judged to be statistically significant. The STARD (Standards for Reporting of Diagnostic Accuracy) Initiative guidelines were consulted throughout the design and analysis of this study (Bossuyt et al., 2003).

4.3 Results

4.3.1 Clinical characteristics

Demographic characteristics and outcomes of women having fetuses with placental IUGR, women with constitutionally small fetuses and uncomplicated pregnancy controls are shown in Table 4.1. Maternal age and parity did not differ between the groups, although sampling in women with placental IUGR tended to occur earlier in gestation ($P$-value =0.04). All women were normotensive at the
time of sampling and had singleton pregnancies. None of the women were smokers at the time of sampling. One woman in the constitutionally small group reported smoking at the time of conception but had ceased smoking by 8 weeks gestation. Gestational age at delivery was earlier in the placental IUGR group. Pre-term delivery, lower infant birth weights, more SGA infants and intrauterine fetal demise were more common in the placental IUGR group as well.

In terms of ultrasound parameters, there were more cases with absent or reversed end diastolic flow at the time of sampling in fetuses with placental IUGR although this difference was not statistically significant. At the time of sampling, the median percentiles of fetal AC and femur length (FL) were lower in the placental IUGR group. Apart from AC percentile, there were no statistically significant differences in worst percentile measurements of head circumference, biparietal diameter, amniotic fluid index or FL between the groups although median percentiles were relatively lower in the placental IUGR group.

4.3.2 Plasma PlGF

All 9 placental IUGR cases had maternal PlGF levels below the detection limit of the assay (<12 pg/mL). One woman with a constitutionally small fetus and two uncomplicated pregnancy controls had PlGF concentrations <12 pg/mL. Women with placental IUGR had a median plasma PlGF concentration of 12.0 pg/mL [IQR: 12.0, 12.0]. Women with constitutionally small fetuses had a median concentration of 84.4 pg/mL [IQR: 16.4, 156.5] and normal pregnant women had a median concentration of 197 pg/mL [IQR 89.0, 449.0]. Differences between the three groups were statistically significant (P-value <0.001).

PlGF concentrations at the time of sampling by pregnancy outcome are shown in Figure 4.1. All women with placental IUGR (n=9) had PlGF concentrations below the 5th percentile cut-off for gestational age in uncomplicated pregnancy. All women with constitutionally small fetuses (n=6) had PlGF concentrations above the 5th percentile cut-off except for one woman who was sampled at 34+4 weeks gestation (corresponding to a false positive test result). Four uncomplicated pregnancy controls had PlGF concentrations below the 5th centile cut-off and were sampled at 26+5, 33+5, 36+1 and 36+4.
weeks gestation (corresponding to false positive test results). The remaining 75 uncomplicated controls had PlGF concentrations above the cut-offs. The three cases of syndromic IUGR all had PlGF concentrations within uncomplicated pregnancy ranges (Figure 4.1).

### 4.3.3 Identification of placental IUGR

The ability of a positive PlGF test to differentiate between placental IUGR, constitutionally small fetuses and uncomplicated pregnancy is shown in Table 4.2. All nine women with placental IUGR had a positive test result. One woman with a constitutionally small fetus and 4 women with uncomplicated pregnancies had positive PlGF test results. The differences between the groups of the 2x3 contingency table were statistically significant (P-value <0.0001) as expected and this was driven by the large group of uncomplicated pregnancy controls without a positive PlGF test.

The test performance of PlGF in differentiating between placental IUGR and constitutionally small fetuses is shown in Table 4.3. Sensitivity and specificity was 100% [95% CI: 66, 100] and 86% [95% CI: 42, 100], respectively. The difference between the two groups was statistically significant (P-value =0.0009).

In a sub-analysis, we combined the constitutionally small fetuses and the three syndromic IUGR cases to create a “non-placental” group and recalculated sensitivity and specificity. The three cases of syndromic IUGR were counted as true negative results as all had normal PlGF concentrations with a normal placental pathology. The sensitivity of PlGF to differentiate placental IUGR from non-placental IUGR/constitutionally small fetuses remained the same while specificity increased to 90% [95% CI: 56, 100] (Table 4.3).

Conditional logistic regression analyses assessing the relationship between a positive PlGF test and placental IUGR and constitutionally small fetuses yielded an undefined odds ratio. Among the constitutionally small fetuses and uncomplicated controls, there was no significant association between PlGF (continuous or log transformed) and the outcome. For placental IUGR, the regression model did not
converge due to complete separation between PIGF values for placental IUGR and matched controls. When all controls were considered in an unmatched analysis, a significant decline in the odds of placental IUGR was observed per one unit increase in PIGF (log transformed) among placental IUGR and uncomplicated controls (OR=0.101, 95% CI: 0.03, 0.335, P-value =0.0002). This association remained unchanged after adjustment for gestational age at sampling.

4.4 Discussion

In this study, we have shown that PIGF levels in maternal circulation have the potential to identify placental IUGR antenatally. A positive PIGF test result on the automated Triage® PIGF assay (concentration below the 5th percentile for gestational age in uncomplicated pregnancy) was more common in women with placental IUGR than among women with constitutionally small fetuses or uncomplicated pregnancies. We have also shown that a positive PIGF test identifies placental IUGR from constitutionally small fetuses with high sensitivity and specificity.

Results here agree with previous reports of lowered PIGF concentrations in the maternal circulation of women with growth-restricted fetuses (Levine et al., 2004; Poon et al., 2008; Romero et al., 2008; Taylor et al., 2003). Figure 4.1 shows the differences in PIGF levels between placental IUGR and uncomplicated pregnancies as well as pregnancies with constitutionally small fetuses. Previous studies have not investigated PIGF levels and placental IUGR nor looked at it in isolation from pre-eclampsia, another obstetric complication with placental implications. We believe that by grouping women with fetuses with placental IUGR and comparing them to women with constitutionally small fetuses, the true usefulness of PIGF, its ability to identify growth restriction due to a pathological placenta, is demonstrated. As it is challenging to diagnose placental IUGR prior to delivery, a readily available marker, such as one in the maternal circulation, could help to stratify these fetuses into high and low-risk groups. The relationship between placental dysfunction, IUGR and low PIGF concentrations may allow clinicians to utilize PIGF as a diagnostic tool, in conjunction with standard surveillance
technologies already used in clinical practice (Lausman and Kingdom, 2013). A diagnostic test, such as PI GF, to identify women with placental IUGR would modify clinical care (surveillance and timely interventions), reducing anxiety for patients not affected by placental disease and focus efforts on those who are affected.

Discriminating between growth restriction due to placental dysfunction and other causes such as infection, aneuploidy, and fetal syndromes is also clinically important. In the three women with syndromic IUGR, all PI GF concentrations were above the 5\textsuperscript{th} percentile for gestational age (i.e., a negative test result). When these cases were combined with constitutionally small fetuses in a secondary analysis (Table 4.3), specificity of PI GF increased slightly. Although preliminary, these results suggest that PI GF may have the ability to not only differentiate constitutionally small fetuses from those with placental IUGR but also syndromic IUGR from placental IUGR (i.e., placental versus non-placental). In this regard, the PI GF test may specifically identify placental compromise in placental IUGR.

Our uncomplicated pregnancy values confirm those previously reported for PI GF quantified on the Triage\textsuperscript{®} assay (Knudsen et al., 2012). Uncomplicated pregnancy concentrations follow the previously reported PI GF changes during pregnancy: a gradual increase up until about 33 weeks with a subsequent decrease until delivery (Levine et al., 2004; Levine et al., 2006; Makrydimas et al., 2008; Maynard et al., 2003). We have also, for the first time, observed this pattern of PI GF concentrations in women with constitutionally small fetuses. As these fetuses are small but healthy, we would assume the placenta is functioning properly as in uncomplicated pregnancy and this would explain the similar concentrations seen between their values and those of uncomplicated pregnancy. One of our women with a constitutionally small fetus had a PI GF concentration below the 5\textsuperscript{th} percentile (i.e., a subsequent false positive PI GF test result). There were also 4 false positive results in the uncomplicated pregnancy control group. The diagnostic performance of this assay begins to decrease after 35\textsuperscript{th} weeks gestation in pre-
eclampsia, as described in the product insert. The natural decline in PlGF at or near term may explain the positive results for these cases, as they clearly did not have placental IUGR.

Results from this study need to be confirmed in a larger prospective study of women with pregnancies with suspected IUGR. An investigation into this group of women would better elucidate the ability of PlGF to identify the pathological placenta. It is unlikely that PlGF could serve as a definitive diagnostic test for placental IUGR. In practice, PlGF would need to be used in conjunction with other laboratory and clinical investigations such as umbilical artery Doppler studies. While Doppler studies are inconsistent and not recommended for routine screening, Doppler testing could be used in conjunction with a positive PlGF test to confirm the diagnosis of placental IUGR, thereby improving the ability to correctly identify fetuses with pathological growth due to placental dysfunction (Crispi et al., 2006; Espinoza et al., 2007; Schlembach et al., 2007). Further investigations into the concurrent use of technologies such as umbilical artery Doppler and PlGF represent an exciting avenue for future research.

In summary, in this small study of 16 women with suspected IUGR, PlGF discriminated between those fetuses with abnormal placental pathology and those without pathology. These preliminary data provide compelling support for further investigation of PlGF in IUGR. The method for detecting PlGF in maternal circulation has advanced to point of care testing, where results can be available in a timely fashion and as such integration into routine clinical practice after validation appears feasible.

4.5 Prospective cohort study

As an extension of the pilot work described above, we are currently evaluating maternal PlGF to identify placenta IUGR in a large cohort of fetuses with AC or EFW <10\textsuperscript{th} percentile for gestational age. We postulate that PlGF will differentiate between pregnancies complicated by placental IUGR, confirmed by presence of placental lesions associated with placental dysfunction and those pregnancies without placental dysfunction in this cohort of fetuses (i.e., constitutionally small fetuses). In the
following sections, we present an interim analysis of PI GF and its association with placental IUGR in the women recruited into the study as of April 2014.

4.5.1 Study subjects

As of April 2014, a total of 106 pregnant women with suspected IUGR have been recruited into the ongoing PI GF in IUGR study (Appendix A). Women were consecutively recruited from tertiary care centres in Vancouver (BC Women’s Hospital) and Ottawa (Ottawa Hospital, General Campus). Ethics approval was granted by local Research Ethics Boards.

A pregnancy with suspected IUGR was defined as one with a fetus with an EFW or AC <10th percentile for gestational age on antenatal ultrasound. Inclusion was limited to singleton pregnancies. Women with documented hypertension or any signs or symptoms of pre-eclampsia based on the Society of Obstetricians and Gynaecologists of Canada guidelines (Magee et al., 2008) were ineligible for inclusion into the study. Women with fetuses with known chromosomal and/or congenital abnormalities at the time of enrolment were also excluded.

Detailed clinical information pertaining to the women as well as pregnancy and fetal/neonatal outcomes were collected by postpartum chart review. Pre-eclampsia was defined as hypertension (blood pressure $\geq 140/90$ mmHg, on at least two occasions $>4$ hours apart after 20 weeks gestation) and new onset proteinuria ($\geq 2+$ dipstick reading, $\geq 0.3$ g/day by 24 hour urine collection or $\geq 30$ mg/mmol by protein:creatinine ratio) (Magee et al., 2008). Gestational hypertension was defined as blood pressure $\geq 140/90$ mmHg in the absence of proteinuria. SGA was defined as a birthweight <3 percentile for gestational age and sex according to Canadian population birthweight charts (Kramer et al., 2001). Fetal biometry percentiles for gestational age on ultrasound were determined by local criteria at the time the ultrasound scan was performed. Umbilical artery Doppler studies were defined as abnormal if the systolic/diastolic (S/D) ratio was $>90$th percentile for gestational age (Acharya et al., 2005).
4.5.2 Blood sample collection and PlGF analysis

A maternal blood sample was collected at the time of the ultrasound identification of EFW or AC<10th percentile for gestational age. Venous blood was collected using standard EDTA plasma (purple top) tubes in the standard way. Plasma was isolated by centrifugation at 3000 rpm for 10 minutes and aliquots were stored at -80°C. Plasma samples were batch assayed for PlGF using the automated Triage® immunoassay (Alere, San Diego, CA, USA) according to the manufacturer’s instructions. This test device uses fluorescently labelled mouse monoclonal antibodies against human PlGF for quantification. In brief, plasma was thawed to 20°C and mixed by inversion. 250μL of thawed plasma was pipetted into the sample port of a new test cartridge. The cartridge was inserted into the Triage® meter and results were displayed on the meter in approximately 15min in pg/mL (PlGF). The detection range is 12-3000 pg/mL. Concentrations below the detection limit were recorded as "12 pg/mL". Laboratory staff was blinded to all information about the study subjects.

4.5.3 Evaluation of placental pathology

Placentas were collected at the time of delivery and sent to local Anatomical Pathology departments. Gross pathology examination was undertaken in the standard fashion. Trimmed placental weight, cord length, number of cord coils, cord insertion site, membrane appearance and maternal and fetal surface appearance were recorded by the reporting laboratory technician.

Tissue biopsies were excised from the placenta and membranes. Tissue was then fixed in 4% neutral buffered formalin and wax-embedded according to standard laboratory procedures. Tissue blocks were sectioned (5 microns) and stained with haematoxylin and eosin (H&E) using standard laboratory procedures. H&E slides were evaluated and graded by the Study Pathologist for lesions of maternal malperfusion, fetal villi maldevelopment, chronic villitis, perivillous fibrin deposition, fetal thrombotic vasculopathies, abruption, interplacental hematoma and chorioamnionitis (Redline et al., 2004a; Redline et al., 2004b). Each individual lesion was scored from 0 to 3 according to the predetermined criteria.
outlined in the Clinical Placental Pathology Evaluation Form (Appendix B). Our placental outcome of interest was the presence of lesion(s) reflecting placental dysfunction that would significantly contribute to reduced maternal-fetal exchange and subsequent compromised fetal growth. Therefore, the Study Pathologist assigned an integrated grade for each placenta from 0 to 3 based on the individual gradings of the following lesions: 1) maternal malperfusion, 2) fetal villi maldevelopment, 3) villitis of unknown aetiology (i.e., non-infectious), 4) perivillous fibrin deposition and 5) interplacental hematoma in conjunction with the placental weight (as a percentile for gestational age at delivery). An overall grade of 0 was an evaluation showing no significant pathology (i.e., grade 0 for each of the five lesions). An overall grade of 1 was defined as a grade of 1 for any of the five lesions or a grade of 2 for maternal malperfusion with appropriate placental weight for gestational age with grades of ≤1 for the remaining lesions. An overall grade of 2 was defined as a grade of 2 for maternal malperfusion with reduced placental weight (<10th percentile for gestational age) or a grade of 2 in any of the remaining lesions. An overall grade of 3 was defined as a grade of 3 for any of the lesions of interest or a grade of 2 for maternal malperfusion with severe reduction in placental weight (<3 percentile for gestational age) or with grade of 3 for any of the remaining lesions of interest.

The prevalence of the placental outcome of interest was evaluated according to maternal PlGF concentration at the time of sampling. Women with PlGF concentration <5th percentile for gestational age at sampling were grouped together. Women with PlGF concentration ≥5th percentile for gestational age formed the second group. The 5th percentile PlGF concentration cut-offs for gestational age were obtained from the product insert of the Triage® meter and were derived from uncomplicated pregnancies (Saffer et al., 2013).

4.5.4 Statistical analyses

Data were analysed using Prism 5.0 (GraphPad, San Diego, CA, USA). Normality of the data was tested using the D'Agostino and Pearson omnibus normality test. Descriptive data are expressed as
means with standard deviations for normally distributed data and medians with interquartile ranges for non-normally distributed data. Continuous variables were compared with the unpaired Student’s t-test (parametric) or Mann-Whitney U-test (non-parametric). Categorical variables were described using counts and proportions and compared using Chi-square test. P-values <0.05 were judged to be statistically significant.

For the interim analysis, a 2x2 contingency table was constructed to evaluate the association between PI GF concentration as a dichotomous exposure (i.e., PI GF <5\textsuperscript{th} percentile or ≥5\textsuperscript{th} percentile) and the placental outcome. Relative risk with 95% CIs was calculated using the contingency table. An interim sensitivity and specificity analysis with positive and negative predictive values and positive and negative likelihood ratios for a positive PI GF test to predict the placental outcome were calculated with 95% CIs. A positive PI GF test was defined as a PI GF concentration <5\textsuperscript{th} percentile for gestational age derived from uncomplicated pregnancies. These cut-offs were obtained from the product insert of the Triage meter.

4.5.5 Interim results

Of the 106 women recruited as of April 2014, 4 women withdrew their participation from the study, 9 women did not have their placentas collected at the time of delivery, 13 women were lost to follow up and 7 women had not delivered at time this thesis was written. In total, there were 73 women included in the analysis.

Of the 73 women with suspected IUGR, 29 (40\%) women had a PI GF below the 5\textsuperscript{th} percentile for gestational age. The remaining 44 women had PI GF concentrations above the 5\textsuperscript{th} percentile for gestational age. The demographic characteristics and outcomes of the groups are shown in Table 4.4. Maternal age, parity and smoking status did not differ significantly between the groups. Blood pressure at sampling was higher in women with PI GF <5\textsuperscript{th} percentile for gestational compared with women who had PI GF concentrations ≥5\textsuperscript{th} percentile for gestational age at sampling (118/72 mmHg vs 107/64 mmHg, P-value =0.002). Women with PI GF <5\textsuperscript{th} percentile at sampling were enrolled into the study.
approximately two weeks earlier than women with PIGF ≥5th percentile. The mean fetal AC percentile and mean EFW percentile at the time of sampling did not differ between the groups, however, there were more fetuses with abnormal umbilical artery Doppler at the time of sampling in the PIGF <5th percentile group (P-value =0.032).

Women with PIGF <5th percentile at sampling delivered earlier and consequently had babies with lower birthweight compared with the PIGF ≥5th percentile group. Fetuses in the PIGF <5th percentile group had lower AC and EFWs as well as more abnormal umbilical artery Doppler prior to delivery compared with the fetuses in the PIGF ≥5th percentile group. Three women (10%) in the PIGF <5th percentile group developed early-onset pre-eclampsia prior after enrolment into the study. Women in the PIGF <5th percentile group had higher rates of Caesarean section for fetal indication (P-value <0.0001) but the rate of induction for fetal indication was increased in the PIGF ≥5th percentile group. Trimmed placental weight was also decreased in the PIGF <5th percentile group.

Of the 29 women with PIGF <5th percentile at the time of sampling, 20 (67%) women had an overall placental grade ≥2 by our histological criteria (placental outcome) (Table 4.5). Of the 44 women with PIGF ≥5th percentile at sampling, 1 (2%) woman had the placental outcome. The difference in the prevalence of the placental outcome between the groups was statistically significant (P-value <0.0001). Women with PIGF <5th percentile at sampling had a 30% increased risk of having the placental outcome compared with women who had PIGF ≥5th percentile at sampling (Relative risk=30.4, 95% CI: 4.3, 214.0). Wide confidence intervals reflect the small sample size of this interim analysis.

Maternal PIGF at the time of sampling stratified by the placental outcome is shown visually in Figure 4.2. The performance of a positive PIGF test (i.e., <5th percentile for gestational age) to predict the placental outcome is shown in Table 4.6. A positive PIGF predicted the placental outcome with 95% [95% CI: 76, 100] sensitivity and 83% [95% CI: 70, 92] specificity. When analysis was limited to sampling
before 35 weeks gestation, sensitivity increased to 100% [95% CI: 82, 100] while specificity decreased to 78% [95% CI: 60, 91].

4.5.6 Discussion

We are conducting a multicentre study to evaluate the utility of PlGF to identify fetuses with IUGR of placental origin based on our pilot study (Benton et al., 2012). The ability to discriminate these fetuses from those without placental dysfunction would allow for improved clinical management of the truly at-risk fetus. Fundamental to developing novel tools to diagnose placental IUGR antenatally is adequately defining this outcome in a cohort of fetuses identified as being small on ultrasound. We have chosen to use clinical placental pathology to define the outcome of placental IUGR, systematically evaluating placentas for lesions associated with placental dysfunction that can result in compromised fetal growth (Appendix B) (Redline et al., 2004a; Redline et al., 2004b).

In the interim results of the first 73 women enrolled in the study, we found that women with PlGF <5th percentile for gestational age at the time of EFW or AC <10th percentile detection on ultrasound have an increased prevalence of the placental outcome (defined as an overall placenta pathology grade ≥2). Additionally, PlGF predicted this placental outcome with high sensitivity and specificity (95% [95% CI: 76, 100] sensitivity; 83% [95% CI: 70, 92] specificity).

While this study is going and definitive results are forthcoming, results support the growing body of evidence that PlGF may be an antenatal marker of placental dysfunction that is associated IUGR and pre-eclampsia. It is known that the most significant decreases in circulating PlGF occurs in cases of early-onset pre-eclampsia, a disease subtype of placental origin (Crispi et al., 2006; Kim et al., 2009; Levine et al., 2004; Maynard et al., 2003; Ohkuchi et al., 2007; Powers et al., 2012; Rana et al., 2007; Robinson et al., 2006; Taylor et al., 2003; Vatten et al., 2007). Interestingly, one study reported that in women with late-onset pre-eclampsia who had placental lesions associated with maternal malperfusion, circulating PlGF levels were lower compared with normotensive controls and women who had late-
onset pre-eclampsia without placental lesions (Soto et al., 2012). While SGA is a poor proxy for growth restriction, early studies investigating angiogenic factors in pregnancy found lower PlGF levels in the circulation of women who delivered SGA infants (Levine et al., 2005; Romero et al., 2008; Taylor et al., 2003), although the observed decreases were less profound than in pre-eclampsia. It is likely that these studies included both placental IUGR and constitutionally small fetuses in the SGA group, resulting in an artifactually weak correlation between PlGF and IUGR. A recent publication showed that in late-onset SGA fetuses, decreased maternal PlGF was associated with histological lesions of placental underperfusion (Triunfo et al., 2014) as our interim results suggest.

The strengths of this study are the well-characterised group of pregnancies with suspected IUGR and stratified outcomes based on placental phenotype, determined by histological evaluation of the placenta. To our knowledge, this is one of the first studies to take this approach when evaluating the clinical utility of PlGF. However, interim results are presented here and final conclusions and limitations will be addressed once our calculated sample size had been met (n=392) (Appendix A). If our results hold in the final analysis, PlGF could be used clinically as an additional tool to identify those fetuses with placental dysfunction. Future studies will be necessary to determine how best to integrate PlGF testing with surveillance tools already in use in clinical practice such as umbilical artery Doppler studies. We speculate that PlGF may have the most significant utility in cases of suspected IUGR with normal umbilical artery Doppler studies as a rule-out test for placental dysfunction as clinical uncertainty still remains in these cases (Parra-Saavedra et al., 2013; Triunfo et al., 2014). Correlations between PlGF and Doppler represent an exciting future direction of this work.

In summary, we present interim results of our ongoing PlGF in IUGR study using data from the first 73 women recruited into the study. We found that low maternal PlGF was associated with the placental outcome of placental dysfunction (defined as an overall placental grade ≥2 based on significant lesions of maternal malperfusion, fetal villi maldevelopment, villitis of unknown aetiology
(i.e., non-infectious), perivillous fibrin deposition and interplacental hemATOMA (P-value <0.0001). The sensitivity and specificity of a positive PlGF test (concentration <5th percentile for gestational age) to identity pregnancies that had the placental outcome was 95% [95% CI: 76, 100] and 83% [95 CI: 70, 92], respectively. Definitive results from the full study will be available following completion of study recruitment but PlGF appears to hold promise in identifying fetuses with placental dysfunction in SGA pregnancies.
Table 4.1 Demographic characteristics and outcomes of the study groups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placenta IUGR (n=9)</th>
<th>Constitutionally small fetuses (n=7)</th>
<th>Controls (n=79)</th>
<th>P-value (χ² or KW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>34 [28, 39]</td>
<td>32 [26, 36]</td>
<td>33 [31, 35]</td>
<td>0.79</td>
</tr>
<tr>
<td>GA at sampling (weeks)</td>
<td>24.6 [23.0, 33.3]</td>
<td>34.0 [24.6, 36.6]</td>
<td>33.0 [31.0, 35.0]</td>
<td>0.04</td>
</tr>
<tr>
<td>Nulliparous (%)</td>
<td>4 (44)</td>
<td>5 (71)</td>
<td>52 (66)</td>
<td>0.41</td>
</tr>
<tr>
<td>sBP at sampling (mmHg)</td>
<td>118 [108, 125]</td>
<td>110 [100, 120]</td>
<td>115 [108, 120]</td>
<td>0.68</td>
</tr>
<tr>
<td>dBP at sampling (mmHg)</td>
<td>70 [60, 77]</td>
<td>70 [70, 74]</td>
<td>70 [70, 80]</td>
<td>0.37</td>
</tr>
</tbody>
</table>

**Ultrasound**

- Absent or reversed EDF at sampling (%): 3 (33) vs 0 (0) n=6, 0.11*
- Uterine artery notching: 3 (60) n=5 vs 1 (25) n=4, 0.29*
- AC percentile at sampling (%): 1 [<1, 4] vs 5 [3, 9], 0.022*
- Worst AC percentile (%): 1 [<1, 3] vs 2 [<2, 4], 0.008*
- FL percentile at sampling (%): 1 [<1, 4] vs 12 [4, 34], 0.060*
- Worst FL percentile (%): 1 [<1, 4] vs 4 [2, 16], 0.077*
- BPD percentile at sampling (%): 4 [1, 15] vs 6 [4, 16], 0.48*
- Worst BPD percentile (%): 1 [<1, 7] vs 4 [1, 6], 0.47*
- HC percentile at sampling (%): 10 [1, 15] vs 20 [3, 38], 0.12*
- Worst HC percentile (%): 1 [<1, 6] vs 4 [1, 24], 0.17*
- AFI percentile at sampling (%): 18 [3, 41] n=8 vs 20 [4, 53] n=6, 0.38*
- Worst AFI percentile (%): 3 [<2.5, 28] vs 9 [3, 21], 0.69*

**Outcome**

- GA at delivery (weeks): 30.6 [24.9, 34.7] vs 37.6 [37.4, 38.5] vs 39.7 [38.3, 40.3], <0.0001
- Pre-term delivery (%)<37 weeks gestation): 8 (89) vs 2 (29) vs 1 (1), <0.0001
- Birthweight (g): 1050 [430, 1568] vs 2182 [2090, 2480] vs 3493 [3150, 3758], <0.0001
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placenta IUGR (n=9)</th>
<th>Constitutionally small fetuses (n=7)</th>
<th>Control (n=79)</th>
<th>P-value (X² or KW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGA &lt; 3rd percentile (%)</td>
<td>7 (78)</td>
<td>4 (50)</td>
<td>0 (0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Base deficit</td>
<td>-2.9 [-5.2, -0.7]</td>
<td>-4.5 [-6.3, -2.5]</td>
<td>-6.0 [-7.7, -4.5]</td>
<td>0.028</td>
</tr>
<tr>
<td>Intrauterine fetal demise (%)</td>
<td>4 (44)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Abnormal placental pathology (%)</td>
<td>9 (100)</td>
<td>0 (0)</td>
<td>0 (0) n=11</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Fisher’s exact test or Mann Whitney U-test.

Data are expressed as median [interquartile range] or n (%).

AC: abdominal circumference; AFI: amniotic fluid index; BPD: biparietal diameter; EDF: end diastolic flow; FL: femur length; GA: gestational age; HC: head circumference; KW: Kruskal-Wallis analysis of variance; sBP: systolic blood pressure; dBP: diastolic blood pressure; X²: Chi-square test.
Table 4.2 PLGF test results from women with placental IUGR, constitutionally small fetuses and uncomplicated pregnancies.

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Placental IUGR (n=9)</th>
<th>Constitutionally small fetuses (n=7)</th>
<th>Uncomplicated pregnancy (n=79)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive*</td>
<td>9</td>
<td>1</td>
<td>4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>6</td>
<td>75</td>
<td></td>
</tr>
</tbody>
</table>

* A positive PLGF result is defined as a PLGF concentration below the 5th percentile for gestational age at sampling derived from uncomplicated pregnancies, as described in the product insert. The P-value is from a Freeman-Halton test (extension of Fisher Exact) of the 2x3 table.
Table 4.3 Sensitivity and specificity of PI GF to identify placental IUGR from constitutionally small fetuses or from non-placental IUGR/constitutionally small fetuses.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity [95% CI]</th>
<th>Specificity [95% CI]</th>
<th>PPV [95% CI]</th>
<th>NPV [95% CI]</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental IUGR from constitutionally small fetuses* (n=16)</td>
<td>100 [66, 100]</td>
<td>86 [42, 100]</td>
<td>90 [56, 100]</td>
<td>100 [54, 100]</td>
<td>0.0009</td>
</tr>
<tr>
<td>Placental IUGR from non-placental IUGR/constitutionally small fetuses (n=19)</td>
<td>100 [66, 100]</td>
<td>90 [56, 100]</td>
<td>90 [56, 100]</td>
<td>100 [66, 100]</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* A positive PI GF result is defined as a PI GF concentration below the 5th percentile for gestational age at sampling derived from uncomplicated pregnancies, as described in the product insert. The P-value is from a Fisher Exact test of the 2x2 table.

CI: confidence interval; NPV: negative predictive value; PPV: positive predictive value.
### Table 4.4 Demographic characteristics and outcomes of the women by PI GF concentration at the time of sampling.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PI GF &lt;5th percentile (n=29)</th>
<th>PI GF ≥5th percentile (n=44)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SD, n (%) or median [IQR])</td>
<td>(Mean ± SD, n (%) or median [IQR])</td>
<td></td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>33 ± 4</td>
<td>32 ± 4</td>
<td>0.76</td>
</tr>
<tr>
<td>Nulliparity</td>
<td>19 (66)</td>
<td>26 (59)</td>
<td>0.58</td>
</tr>
<tr>
<td>Smoking</td>
<td>1 (3)</td>
<td>2 (5)</td>
<td>1.00</td>
</tr>
<tr>
<td>sBP at booking (mmHg)</td>
<td>118 ± 12</td>
<td>107 ± 10</td>
<td>0.002</td>
</tr>
<tr>
<td>dBP at booking (mmHg)</td>
<td>72 ± 12</td>
<td>64 ± 7</td>
<td>0.013</td>
</tr>
</tbody>
</table>

At sampling

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PI GF &lt;5th percentile (n=29)</th>
<th>PI GF ≥5th percentile (n=44)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SD, n (%) or median [IQR])</td>
<td>(Mean ± SD, n (%) or median [IQR])</td>
<td></td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>30.3 ± 4.3</td>
<td>32.6 ± 5</td>
<td>0.050</td>
</tr>
<tr>
<td>AC (mm)</td>
<td>224 ± 50</td>
<td>247 ± 51</td>
<td>0.15</td>
</tr>
<tr>
<td>AC percentile (%)</td>
<td>4 ± 2</td>
<td>5 ± 2</td>
<td>0.29</td>
</tr>
<tr>
<td>EFW (grams)</td>
<td>1241 ± 576</td>
<td>1637 ± 656</td>
<td>0.064</td>
</tr>
<tr>
<td>EFW percentile (%)</td>
<td>11 [10, 16]</td>
<td>19 [7, 31]</td>
<td>0.47</td>
</tr>
<tr>
<td>S/D ratio</td>
<td>3.2 [2.5, 4.8]</td>
<td>2.5 [2.5, 3.1]</td>
<td>0.04</td>
</tr>
<tr>
<td>S/D ratio &gt;90th percentile</td>
<td>7 (24)</td>
<td>3 (7)</td>
<td>0.032</td>
</tr>
<tr>
<td>Absent or reversed EDF</td>
<td>2 (7)</td>
<td>1 (2)</td>
<td>0.57</td>
</tr>
<tr>
<td>PI GF (pg/mL)</td>
<td>13.9 [12.0, 35.7]</td>
<td>225.0 [98.8, 409.0]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PI GF &lt;12 pg/mL</td>
<td>14 (48)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Outcome

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PI GF &lt;5th percentile (n=29)</th>
<th>PI GF ≥5th percentile (n=44)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SD, n (%) or median [IQR])</td>
<td>(Mean ± SD, n (%) or median [IQR])</td>
<td></td>
</tr>
<tr>
<td>Worst AC (mm)</td>
<td>250 ± 36</td>
<td>276 ± 26</td>
<td>0.009</td>
</tr>
<tr>
<td>Worst AC percentile</td>
<td>3 [1, 4]</td>
<td>4 [3, 5]</td>
<td>0.088</td>
</tr>
<tr>
<td>Worst EFW (grams)</td>
<td>1456 ± 547</td>
<td>2067 ± 459</td>
<td>0.0003</td>
</tr>
<tr>
<td>Worst EFW percentile</td>
<td>3 [6, 11]</td>
<td>9 [4, 21]</td>
<td>0.18</td>
</tr>
<tr>
<td>S/D ratio prior to delivery</td>
<td>3.6 [2.5, 5.5]</td>
<td>2.4 [2.2, 2.9]</td>
<td>0.002</td>
</tr>
<tr>
<td>Characteristic</td>
<td>PIGF &lt;5\textsuperscript{th} percentile (n=29)</td>
<td>PIGF ≥5\textsuperscript{th} percentile (n=44)</td>
<td>P-value (X\textsuperscript{2} or KW)</td>
</tr>
<tr>
<td>----------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td></td>
<td>(Mean ± SD, n (%)) or median [IQR]</td>
<td>(Mean ± SD, n (%)) or median [IQR]</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/D ratio &gt;90\textsuperscript{th} percentile prior to delivery</td>
<td>16 (55)</td>
<td>5 (11)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Absent or reversed EDF</td>
<td>7 (24)</td>
<td>2 (5)</td>
<td>0.025</td>
</tr>
<tr>
<td>GA at delivery (weeks)</td>
<td>34.7 ± 3.2</td>
<td>37.7 ± 1.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pre-term delivery (&lt;37 weeks gestation)</td>
<td>21 (72)</td>
<td>14 (32)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Male sex</td>
<td>17 (59)</td>
<td>19 (43)</td>
<td>0.24</td>
</tr>
<tr>
<td>Birthweight (grams)</td>
<td>1652 ± 710</td>
<td>2444 ± 420</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SGA &lt;3\textsuperscript{rd} percentile</td>
<td>13 (45)</td>
<td>16 (36)</td>
<td>0.63</td>
</tr>
<tr>
<td>Caesarean section for fetal indication</td>
<td>14 (48)</td>
<td>3 (7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Induction of labour for fetal indication</td>
<td>3 (10)</td>
<td>24 (55)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Early-onset pre-eclampsia</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>0.059</td>
</tr>
<tr>
<td>Stillbirth</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>0.40</td>
</tr>
<tr>
<td>Arterial cord pH</td>
<td>7.24 [7.19, 7.26]</td>
<td>7.25 [7.20, 7.30]</td>
<td>0.89</td>
</tr>
<tr>
<td>Trimmed placental weight (grams)</td>
<td>278 [210, 320]</td>
<td>366 [311, 464]</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

* Defined as onset before 34 weeks gestation.

AC: abdominal circumference; dBP: diastolic blood pressure; EDF: end diastolic flow; EFW: estimated fetal weight; GA: gestational age; IQR: interquartile range; KW: Kruskal-Wallis analysis of variance; S/D: systolic: diastolic; sBP: systolic blood pressure; SD: standard deviation; SGA: small for gestational age; X\textsuperscript{2}: Chi-square test.
Table 4.5 Rates of the placental outcome by PI GF concentration at the time of sampling.

<table>
<thead>
<tr>
<th>Placental outcome&lt;br&gt;◊ Defined as an overall placental grade ≥2.</th>
<th>PI GF &lt;5&lt;sup&gt;th&lt;/sup&gt; percentile (n=29)</th>
<th>PI GF ≥5&lt;sup&gt;th&lt;/sup&gt; percentile (n=44)</th>
<th>Fisher’s exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>20</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>No</td>
<td>9</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6 Sensitivity and specificity of a positive PI GF test to predict the placental outcome in pregnancies with suspected IUGR.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity [95% CI]</th>
<th>Specificity [95% CI]</th>
<th>PPV [95% CI]</th>
<th>NPV [95% CI]</th>
<th>+LR [95% CI]</th>
<th>-LR [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling at any GA*</td>
<td>95 [76, 100]</td>
<td>83 [70, 92]</td>
<td>69 [49, 85]</td>
<td>98 [88, 100]</td>
<td>5.5 [3.0, 10.0]</td>
<td>0.06 [0.009, 0.4]</td>
</tr>
<tr>
<td>(n=73)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling &lt;35 weeks</td>
<td>100 [82, 100]</td>
<td>78 [60, 91]</td>
<td>73 [52, 88]</td>
<td>100 [86, 100]</td>
<td>4.6 [2.4, 8.8]</td>
<td>--</td>
</tr>
<tr>
<td>(n=51)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

* A positive PI GF result was defined as a PI GF concentration < 5th percentile for gestational age at sampling as described in the product insert of the Triage® assay.

+LR: positive likelihood ratio; -LR: negative likelihood ratio; CI: confidence interval; GA: gestational age.

NPV: negative predictive value; PPV: positive predictive value.
Figure 4.1 PIGF concentrations from women with placental IUGR fetuses, syndromic IUGR fetuses, constitutionally small fetuses and uncomplicated pregnancies at the time of sampling. Constitutionally small fetuses (red triangles) and uncomplicated pregnancy controls (black squares) had increased PIGF levels compared with placental IUGR cases (blue triangles). Case of syndromic IUGR (green circles) had PIGF levels within uncomplicated pregnancy ranges. The black dashed black line represents the 5th percentile PIGF concentration cut-off according to the product insert. The y-axis is log transformed. Two blue triangles overlap at 33^{+2} weeks gestation due to the sampling of these women occurring at the same gestational age.
Figure 4.2 Maternal PI GF concentrations at the time of sampling stratified by overall placental pathology grade. Women with no pathology (Grade 0, black squares) or mild pathology (Grade 1, grey circles) had increased levels of circulating PI GF compared with cases of moderate (Grade 2, blue triangles) and severe malperfusion pathology (Grade 3, red diamonds). The black arrows indicated the three women who developed early-onset pre-eclampsia after sampling. The black dashed black line represents the 5th percentile PI GF concentration cut-off according to the product insert. The y-axis is log transformed.
Chapter 5. Maternal PlGF and placental morphology in pregnancies with suspected intrauterine growth restriction

5.1 Introduction

In fetuses with suspected IUGR, a proportion will have histological evidence of placental dysfunction following delivery (Kingdom et al., 2012; Parra-Saavedra; Redline, 2008; Triunfo et al., 2014). These lesions may reflect downstream effects of abnormal placentation in early gestation that is believed to precipitate placental dysfunction later in pregnancy when fetal demands exceed the exchange capacity of the placenta, leading to compromised fetal growth (Kingdom et al., 2012). Conversely, a proportion of fetuses with suspected growth restriction will have no histopathological evidence of significant placental dysfunction and may represent the group of constitutionally small fetuses with the population of fetuses (RCOG, 2013). The ability to identify placental dysfunction antenatally would improve clinical management of to the truly at-risk fetus (i.e., the one with disrupted growth due to placental dysfunction) within the population of fetuses antenatally identified as being small (Bamberg and Kalache, 2004).

Novel biomarkers such as maternal PlGF may provide additional information for discriminating between fetuses with and without placental dysfunction in pregnancies with suspected IUGR. This line of inquiry is currently being investigated by our research group (Chapter 4) and others (Triunfo et al., 2014). To date, there are no studies that have attempted to correlate maternal PlGF concentration and quantitative measures of placenta structure in pregnancies with suspected IUGR. As such, we performed quantitative analyses of placental morphology in a subset of placentas collected from the women enrolled as part of the ongoing study described in Chapter 4 (Appendix A). Our hypothesis is that low maternal PlGF concentration is associated with altered placental structure in this group of fetuses. A better understanding of the association between maternal PlGF and abnormal placental morphology may help to further elucidate the pathological processes PlGF reflects in women with pregnancy...
complications.

5.2 Methods

5.2.1 Study subjects

We collected additional placenta tissue for quantitative analyses from 36 women with suspected IUGR recruited into the ongoing PIGF in IUGR study (Chapter 4; Appendix A). These women were consecutively recruited from tertiary care centres in Vancouver (BC Women’s Hospital) and Ottawa (Ottawa Hospital, General Campus). Ethics approval was granted by local Research Ethics Boards.

A pregnancy with suspected IUGR was defined as one with a fetus with an EFW or AC <10th percentile for gestational age on antenatal ultrasound. Inclusion was limited to singleton pregnancies. Women with documented hypertension or any signs or symptoms of pre-eclampsia based on the Society of Obstetricians and Gynaecologists of Canada guidelines (Magee et al., 2008) were ineligible for inclusion into the study. Women with fetuses with known chromosomal and/or congenital abnormalities were also excluded.

Detailed clinical information pertaining to the women as well as pregnancy and fetal/neonatal outcomes were collected by postpartum chart review. Pre-eclampsia was defined as hypertension (blood pressure ≥140/90 mmHg, on at least two occasions >4 hours apart after 20 weeks gestation) and new onset proteinuria (≥2+ dipstick reading, ≥0.3 g/day by 24 hour urine collection or ≥30 mg/mmol by protein:creatinine ratio) (Magee et al., 2008). Gestational hypertension was defined as blood pressure ≥140/90 mmHg in the absence of proteinuria. SGA was defined as a birthweight <3 percentile for gestational age and sex according to Canadian population birthweight charts (Kramer et al., 2001). Fetal biometry percentiles for gestational age on ultrasound were determined by local criteria at the time the ultrasound scan was performed. Umbilical artery Doppler studies were defined as abnormal if the systolic:diastolic (S/D) ratio was >90th percentile for gestational age (Acharya et al., 2005).
5.2.2 Blood sample collection and PI GF analysis

A maternal blood sample was collected at the time of the ultrasound identification of EFW or AC <10th percentile for gestational age. Venous blood was collected using standard EDTA plasma (purple top) tubes in the standard way. Plasma was isolated by centrifugation at 3000 rpm for 10 minutes and aliquots were stored at -80°C. Plasma samples were batch assayed for PI GF using the automated Triage® immunoassay (Alere, San Diego, CA, USA) according to the manufacturer’s instructions. This test device uses fluorescently labelled mouse monoclonal antibodies against human PI GF for quantification. In brief, plasma was thawed to 20°C and mixed by inversion. 250μL of thawed plasma was pipetted into the sample port of a new test cartridge. The cartridge was inserted into the Triage® meter and results were displayed on the meter in approximately 15min in pg/mL (PI GF). The detection range is 12-3000 pg/mL. Concentrations below the detection limit were recorded as "12 pg/mL". Laboratory staff was blinded to all information about the women.

5.2.3 Quantitative assessment of placental morphology

Placenta tissue sections were quantitatively assessed for the following morphological parameters: syncytial knots, trophoblast area, proliferation and vascularity as previously described (Warrander et al., 2012).

Placentas were collected at the time of delivery and sent to local Anatomical Pathology departments. Following examination for gross pathology, four random biopsies of the fetal villous tissue (~1cm³ per block, two excised from the centre and two excised from the edge) were dissected and fixed in 4% neutral buffered formalin for 24 hours. After fixing, tissue blocks were wax embedded. Blocks were cut into 5 micron sections for H&E staining and immunohistochemistry (IHC). Following staining, slides were scanned using the Aperio ScanScope® CS System (Aperio, Vista, CA, USA) and ten random images per tissue section were taken using ImageScope™ software (Aperio, Vista, CA, USA) giving a total of 40 images per placenta for each parameter evaluated.
H&E stained slides were used to quantify syncytial knots. Tissue sections were dewaxed in xylene and rehydrated in a series of graded ethanol concentrations. Sections were stained with modified Harris’s haematoxylin solution (Sigma-Aldrich, St. Louis MI, USA) for 10 minutes then dipped in acid-alcohol for differentiation. Slides were stained with eosin (Sigma-Aldrich) for 2 minutes, rinsed in cold tap water then dehydrated and mounted with DPX Mountant (Sigma-Aldrich). The number of syncytial knots were counted manually and expressed as the number of knots per mm² of villous tissue as previously described (Warrander et al., 2012). A syncytial knots was defined as an area of 10 or more aggregated nuclei protruding from the villus surface that was not in contact with adjacent villi. Villous area was quantified using ImageJ software (NIH, Bethesda, MD, USA) (Rasband, 2012).

IHC was used to assess trophoblast area (pan-cytokeratin; Dako, Carpinteria, CA, USA), proliferation (Ki67; Novus Biologicals, Littleton, CO, USA) and vascularity (CD31; Novus Biologicals). Tissue sections were dewaxed in xylene and rehydrated in a series of graded ethanol concentrations. Endogenous peroxidases were quenched using 3% H₂O₂ in methanol for 10 minutes. Antigen retrieval was performed using the Decloaking Chamber™ Pro (Biocare Medical, Concord, CA, USA) pressure cooker at a maximum temperature of 125°C for 4 minutes in 0.01 M sodium citrate buffer. Serum-free protein block (Dako) was applied to sections for 30 minutes at room temperature in humidity chambers. Sections were incubated with primary antibodies for cytokeratin (1:500 dilution), Ki67 (1:150 dilution) and CD31 (1:500 dilution) diluted in 1X PBS and negative controls (non-immune mouse immunoglobulins) overnight at 4°C in humidity chambers. Sections were then incubated with biotinylated goat anti-mouse and goat anti-rabbit IgGs (LSAB2-HRP Kit, Dako) for 1 hour, followed by incubation with peroxidase-labelled streptavidin-horseradish peroxidase for 30 minutes (LSAB2-HRP Kit, Dako). Staining was completed by exposing sections to 3-3’-diaminobenzidine (DAB) substrate-chromogen (Dako) at room temperature. Sections were counterstained with haematoxylin and mounted with coverslips using Immu-Mount™ (Thermo Scientific, Pittsburgh, PA, USA).
Trophoblast area was expressed as the proportion of villous area positive for cytokeratin staining to total villous area quantified using ImageJ (Rasband, 2012). Proliferative index was the number of Ki67positive nuclei (manually counted) as a proportion of the total villous area (mm²). Villous area was quantified using ImageJ software (Rasband, 2012). Vascularity was expressed as the number of capillaries per villus as well as the percentage of avascular villi per total villi.

5.2.4 Statistical analyses

Data were analysed using Prism 5.0 (GraphPad, San Diego, CA, USA). Normality of the data was tested using the D’Agostino and Pearson omnibus normality test. Descriptive data are expressed as means with standard deviations for normally distributed data and medians and interquartile ranges for non-normally distributed data. Continuous variables were compared with unpaired Student’s t-tests (parametric) or Mann-Whitney U-tests (non-parametric). Categorical variables were described using counts and proportions and compared using the Chi-square test. P-values <0.05 were judged to be statistically significant.

The quantitative parameters of placental morphology were compared between women with PlGF <12pg/mL at sampling, women with PlGF between 12pg/mL and the < 5th centile for gestational age at sampling and those women with normal PlGF levels (>5th percentile for gestational age at sampling) to determine if low PlGF was associated with abnormal placental morphology. The 5th percentile PlGF concentration for gestational age derived from uncomplicated pregnancies and cut-offs were obtained from the product insert of the Triage® meter.

Additionally, we collected placentas from ten women with no pregnancy complications and normal PlGF levels (PlGF >5th percentile for gestational age at sampling) during the same time period to serve as a reference group. These women had no documented concerns or diagnoses of hypertension, pre-eclampsia, growth restriction or fetal anomalies at the time of enrolment or at any time during their pregnancy. An uncomplicated pregnancy was confirmed by chart review following delivery.
5.3 Results

5.3.1 Clinical characteristics

In total, 36 placentas from women with suspected IUGR and 10 placentas from women with uncomplicated pregnancies were included in the analyses. At the time of blood sampling, there were 7 women with PlGF <12 pg/mL, 9 women with PlGF ≥12 pg/mL but below the 5th percentile for gestational and 20 women with PlGF ≥5th percentile for gestational age (Normal PlGF group). All women with uncomplicated pregnancies had PlGF ≥5th percentile for gestational age at the time of sampling (Uncomplicated group).

Demographics characteristics and outcomes of the four groups are shown in Table 5.1. Maternal age, parity, smoking status and gestational age at sampling did not differ between the groups. At the time of sampling, fetal AC and EFW percentiles did not differ between the SGA groups (PlGF <12 pg/mL, PlGF <5th percentile and normal PlGF) but were significantly lower than the uncomplicated pregnancy controls as was expected (evaluated with Tukey’s multiple comparison test). Women with PlGF <12 pg/mL had increased S/D ratio and S/D ratio >90th percentile for gestational age at the time of sampling compared with women in the other groups. Women with PlGF <12 pg/mL delivered earlier and had more SGA infants compared with the other groups. Abnormal umbilical artery Doppler was more common in this group prior to delivery and more women in this group were delivered by Caesarean section for fetal indication than in the other groups.

5.3.2 Placental structure

Placentas from women with low PlGF had increased syncytial knots per villous area (mm²) (Figure 5.1). The greatest increase in syncytial knot density was seen in the placentas from women PlGF <12 pg/mL group (15.3 ±3.2 knots/mm²). This increase was significantly different when this group was compared with the normal PlGF group (7.1 ±3.3, P-value <0.0001) and the uncomplicated pregnancy group (7.1 ±2.7, P-value <0.0001) (Figure 5.1). Interestingly, the density of syncytial knots did not differ
between placentas pregnancies with suspected IUGR with normal PI GF and placentas from uncomplicated pregnancies (7.1 ±3.3 vs 7.1 ±2.7, P-value >0.05).

There was a reduction in syncytiotrophoblast area in placentas from women with PlGF <12 pg/mL (Figure 5.2). Syncytiotrophoblast area was significantly reduced in this group compared with placentas from women suspected IUGR with normal PlGF (17.8 ±3.6% vs 29.0 ±6.6%, P-value <0.01) as well as placentas from uncomplicated pregnancies (17.8 ±3.6% vs 29.1 ±6.7%, P-value <0.01). There was no difference in syncytiotrophoblast area between placentas from women with PlGF <5th percentile (but ≥12 pg/mL), the normal PlGF group and the uncomplicated pregnancy group (P-value >0.05).

Proliferation as indicated by an increase in the number of Ki67-positive nuclei per villous area was increased in placentas from women with PlGF <12 pg/mL (768 ±206 nuclei/mm²) (Figure 5.3). This increase was significantly different compared with placentas from women with suspect IUGR but normal PlGF concentrations (222 ±83, P-value <0.001) as well as placenta from uncomplicated pregnancies (170 ±56, P-value <0.001).

Placentas with low PI GF had reduced vascularity (Figure 5.4). Placentas from women with PlGF <12 pg/mL had an average of 3.5 ±0.3 capillaries per villus as did placentas from women with PI GF between the <5th percentile and ≥12 pg/mL (3.5 ± 0.3 capillaries/villus). The number of capillaries per villus in these groups were both significantly decreased compared with the normal PI GF group (4.2 ±0.4 capillaries/villus, both P-values <0.05) and the uncomplicated pregnancy group (4.2 ± 0.4, both P-values <0.01). There was no difference in the number of capillaries per villus between the normal PI GF group and the uncomplicated pregnancy group. Also, placentas from women with PI GF <12 pg/mL had more avascular villi (8.3 ± 4.5%) compared with placentas from the normal PI GF group (1.6 ±1.2 %, P-value <0.001) and the placentas from uncomplicated pregnancies (2.7 ± 1.5%, P-value <0.001).
5.4 Discussion

In this study, we assessed placental structure in placentas collected from pregnancies with suspected IUGR and compared these quantitative parameters according to maternal PI GF levels at the time growth restriction was diagnosed (i.e., at the time of a fetal EFW or AC <10th percentile for gestational age on ultrasound). The parameters included syncytial knot density, trophoblast area, proliferation and vascularity. We found that there were significant differences in placental structure in placentas from women with PI GF <5th percentile at the time of sampling and those with PI GF in normal ranges within this cohort of pregnancies with suspected IUGR. Compared to placentas from women with normal PI GF at the time of sampling (PI GF >5th percentile for gestational age), placentas from the PI GF <5th percentile group had increased syncytial knots density, decreased syncytiotrophoblast area and increased proliferative index. Villus vascularity was also reduced and the proportion of avascular villi was increased. Interestingly, when placentas from women normal PI GF were compared with placentas from uncomplicated pregnancies (and also normal PI GF levels), the parameters of placental structure was not significantly different between the groups.

These findings suggest that low PI GF in the maternal circulation may reflect altered placental structure in pregnancies with suspected IUGR supporting the hypothesis that it is an antenatal marker of placental well-being. In addition to altered placental structure in the PI GF <12 pg/mL group, the pregnancy outcomes in this group were also more adverse than the other groups in terms of gestational age at delivery, infant birth weight, SGA status and Caesarean section for fetal indication (Table 1). Despite being antenatally suspected of having IUGR, the group with normal PI GF levels had placental structure as well as pregnancy outcome that were similar to those of the uncomplicated pregnancy group. Considering both the clinical outcomes and the quantitative parameters of placental structure, it would appear that low PI GF identifies pregnancies with placental compromise with a cohort of pregnancies with suspected IUGR.
Changes in these placental structural features have been previously reported in placentas from pregnancies complicated by pre-eclampsia, SGA and reduced fetal movements with adverse pregnancy outcomes (Cali et al., 2013; Heazell et al., 2007; Longtine et al., 2012; Krebs et al., 1996; Prusac et al., 2011; Sankaret et al., 2012; Torry et al., 2004; Warrander et al., 2012). To our knowledge, this is the first study to assess features of placental structure in placentas stratified by maternal PI GF concentration. Assessing the correlation between PI GF concentration and placental structure highlights the differences in placental compromise with a cohort of pregnancies with suspected IUGR. Placentas from women with normal PI GF concentrations had similar structure to placentas collected from uncomplicated pregnancies, suggesting that while fetuses in this group were antenatally identified as being SGA on ultrasound, they may represent constitutionally small fetuses with this group. It is clinically important to differentiate these two groups of fetuses and novel biomarkers that reflect placental and/or compromise would provide an additional tool for this differentiation.

Abnormalities in placental morphology (both in a clinical and quantitative context) in placentas from women with low PI GF levels support the hypothesis that PI GF reflects placental dysfunction and/or compromise in these pregnancies (Benton et al., 2012; Chapter 4). The degree to which placentation and placenta function must be disrupted for complications to arise is unknown and future research in this area should aim to uncover this association. From our work, it appears that PI GF <12 pg/mL reflects severe compromise to the placenta as these fetuses had more severe outcomes compared to the other groups. Understanding the aspects of placental structure that are disrupted in placental IUGR and reflected by low PI GF will help to elucidate the mechanisms that lead to this changes and altered fetal growth. It is still not know what alters circulating PI GF and the other angiogenic factor concentration in pre-eclampsia and IUGR and uncovering the significance of the relationship between PI GF and placental structure and function may allow for targeted therapies and intervention in the future.
In summary, using quantitative assessment of placental structure, we have found that placentas from women with PIGF <12 pg/mL had increased syncytial knot density, decreased trophoblast area, increased proliferation and reduced vascularity compared with women with normal PIGF concentrations and uncomplicated pregnancies. This study provides insight into the relationship between circulating maternal PIGF and abnormal placental structure in pregnancies with suspected IUGR and provides support for future investigations into the utility of PIGF to detect placental dysfunction antenatally.
Table 5.1 Demographic characteristics and outcomes of the women according to PlGF concentration at the time of sampling.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PIGF &lt;12 pg/mL (n=7) (Mean ± SD, n (%)) or median [IQR]</th>
<th>PIGF &lt;5th percentile° (n=9) (Mean ± SD, n (%)) or median [IQR]</th>
<th>Normal PIGF° (n=20) (Mean ± SD, n (%)) or median [IQR]</th>
<th>Uncomplicated (n=10) (Mean ± SD, n (%)) or median [IQR]</th>
<th>P-value (Student’s t-test, MW or (X^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At sampling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>30 ± 5</td>
<td>33 ± 5</td>
<td>32 ± 4</td>
<td>31 ± 6</td>
<td>0.71</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>5 (71)</td>
<td>5 (56)</td>
<td>11 (55)</td>
<td>7 (70)</td>
<td>0.78</td>
</tr>
<tr>
<td>Smoking status</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>1 (10)</td>
<td>0.68</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>30.4 ± 3.5</td>
<td>30.5 ± 5.2</td>
<td>31.7 ± 5.0</td>
<td>32.9 ± 32.28</td>
<td>0.61</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
<td>121 ± 11</td>
<td>113 ± 13</td>
<td>105 ± 12</td>
<td>107 ± 8</td>
<td>0.017</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
<td>71 ± 12</td>
<td>70 ± 7</td>
<td>65 ± 8</td>
<td>60 ± 7</td>
<td>0.017</td>
</tr>
<tr>
<td>AC percentile (%)</td>
<td>3.6 ± 2.8</td>
<td>5.1 ± 2.4</td>
<td>5.3 ± 2.4</td>
<td>33.4 ± 20.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EFW percentile (%)</td>
<td>12.3 ± 9.9</td>
<td>14.4 ± 5.4</td>
<td>22.5 ± 11.0</td>
<td>50.0 ± 17.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>S/D ratio</td>
<td>4.8 ± 2.5</td>
<td>3.2 ± 1.0</td>
<td>2.7 ± 1.0</td>
<td>--</td>
<td>0.005</td>
</tr>
<tr>
<td>S/D &gt;90th percentile</td>
<td>4 (57)</td>
<td>2 (22)</td>
<td>1 (5)</td>
<td>--</td>
<td>0.01</td>
</tr>
<tr>
<td>Absent or reversed EDF</td>
<td>1 (14)</td>
<td>0 (0)</td>
<td>0 (20)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/D ratio delivery*</td>
<td>5.0 ± 2.2</td>
<td>3.4 ± 1.5</td>
<td>2.7 ± 1.0</td>
<td>--</td>
<td>0.001</td>
</tr>
<tr>
<td>S/D &gt;90th percentile delivery*</td>
<td>5 (71)</td>
<td>4 (44)</td>
<td>2 (10)</td>
<td>--</td>
<td>0.006</td>
</tr>
<tr>
<td>Characteristic</td>
<td>PIGF &lt;12 pg/mL (n=7)</td>
<td>PIGF &lt;5th percentile* (n=9)</td>
<td>Normal PIGF◊ (n=20)</td>
<td>Uncomplicated (n=10)</td>
<td>P-value (Student’s t-test, MW or X²)</td>
</tr>
<tr>
<td>--------------------------------------------</td>
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</tr>
<tr>
<td><strong>Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent or reversed EDF delivery*</td>
<td>3 (43)</td>
<td>1 (11)</td>
<td>0 (0)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GA at delivery (weeks)</td>
<td>32.6 ± 3.3</td>
<td>36.1 ± 2.9</td>
<td>38.0 ± 1.7</td>
<td>39.1 ± 1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pre-term delivery (&lt;37 weeks gestation)</td>
<td>6 (86)</td>
<td>5 (55)</td>
<td>6 (30)</td>
<td>0 (0)</td>
<td>0.002</td>
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<tr>
<td>Male sex</td>
<td>5 (71)</td>
<td>4 (44)</td>
<td>11 (55)</td>
<td>3 (30)</td>
<td>0.36</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>1288 ± 530</td>
<td>2031 ± 694</td>
<td>2414 ± 457</td>
<td>3082 ± 472</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SGA &lt;3rd percentile</td>
<td>5 (71)</td>
<td>2 (22)</td>
<td>5 (25)</td>
<td>0 (0)</td>
<td>0.02</td>
</tr>
<tr>
<td>Caesarean section for fetal indication</td>
<td>5 (71)</td>
<td>4 (44)</td>
<td>2 (10)</td>
<td>1 (10)</td>
<td>0.007</td>
</tr>
<tr>
<td>Induction of labour for fetal indication</td>
<td>1 (14)</td>
<td>1 (11)</td>
<td>11 (55)</td>
<td>0 (10)</td>
<td>0.005</td>
</tr>
<tr>
<td>Arterial cord pH</td>
<td>7.20 ± 0.05</td>
<td>7.26 ± 0.06</td>
<td>7.21 ± 0.11</td>
<td>7.24 ± 0.07</td>
<td>0.49</td>
</tr>
<tr>
<td>Trimmed placental weight</td>
<td>201 ± 53</td>
<td>328 ± 115</td>
<td>369 ± 97</td>
<td>472 ± 112</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* PIGF <5th percentile for gestational age but ≥12 pg/mL. Shortened for brevity.

◊ Defined as a PIGF concentration >5th percentile for gestational age in uncomplicated pregnancy.

* Doppler study immediately prior to delivery.

AC: abdominal circumference; dBP: diastolic blood pressure; EDF: end diastolic flow; EFW: estimated fetal weight; GA: gestational age; sBP: systolic blood pressure; S/D: systolic to diastolic; SGA: small for gestational age.
Figure 5.1 Number of syncytial knots per villous area by PIGF group. (A) Placentas from women with PIGF <12pl/mL had an increased density of syncytial knots compared with placentas from pregnancies with suspected growth restriction and normal PIGF levels (P-value <0.0001) and placentas from uncomplicated pregnancies (P-value <0.0001). (B) Representative images of haematoxylin and eosin stained tissue sections from pregnancies with suspected IUGR and PIGF <12 pg/mL group or normal PIGF. Arrows indicate syncytial knots.
Trophoblast area was significantly reduced in the placentas from women with PlGF <12 pg/mL compared with placentas from pregnancies with suspected growth restriction and normal PlGF levels ($P$-value <0.01) and placentas from uncomplicated pregnancies ($P$-value <0.01).

(B) Representative images of tissue sections stained with cytokeratin from pregnancies with suspected IUGR and PlGF <12 pg/mL group or normal PlGF.
Figure 5.3 Proliferative index by PlGF group. (A) The number of Ki67-positive nuclei per villous area is significantly increased in the placentas from women with PlGF <12 pg/mL compared to placentas from women with normal PlGF concentration and uncomplicated pregnancies (P-value <0.0001). (B) Representative images from tissue sections stained with Ki67 immunohistochemistry from pregnancies with suspected IUGR and PlGF <12 pg/mL group or normal PlGF. Arrows indicated Ki67-positive nuclei.
Figure 5.4 Placental villi vascularity by PIGF group. (A) The number of capillaries per villus in placentas from women with PIGF<12pg/mL and from women with PIGF ≥12pg/mL– <5\textsuperscript{th} percentile was significantly reduced compared with placentas in the normal PIGF group (P-value <0.05) and placentas from uncomplicated pregnancies (P-value <0.01). (B) Representative images of tissue sections stained with CD31 from pregnancies with suspected IUGR and PIGF <12 pg/mL or normal PIGF.
Chapter 6. Summary and conclusions

In this thesis, I investigated circulating angiogenic factors in placentally-mediated complications such as pre-eclampsia and IUGR. In particular, I focused on PlGF and its association with placental IUGR as this relationship has not been thoroughly investigated to date. This section summarises the main findings and their significance, discusses the strengths and limitations of my studies and proposes future directions for this area of research.

6.1 General discussion

Pre-eclampsia and placental IUGR are pregnancy complications that have significant consequences on the well-being of mothers and their babies. A priority in obstetrics is the early and accurate identification of women who will or who are developing these complications so that they may be managed appropriately. Improved prediction and diagnosis could improve care for these high-risk mothers and babies with the intent of reducing the associated adverse outcomes as well as identifying women who will benefit the most from intervention and treatment.

6.1.1 Pre-eclampsia

For over a decade, angiogenic factors have been proposed as diagnostic markers for pre-eclampsia. Recently, technology for the quantification of PlGF and sFlt-1 in plasma or serum samples has reached point of care testing. Point of care testing allows for rapid, automated testing for biomarkers at the bedside or in a clinic, supporting their use in routine prenatal care. In Chapter 2, we compared the performance of two commercially available angiogenic factor tests. We found that the single analyte assay (Triage® PlGF) was better able to diagnose pre-eclampsia with onset before 35 weeks gestation compared with the ratio assay (Elecsys® sFlt-1/PlGF Ratio) using product insert cut-offs to define a positive test result. Additionally, as sensitivity and specificity of these markers are influenced by the gestational age at sampling due to the physiological changes in circulating levels of these markers with
advancing gestation, we found that gestational-age dependent cut-offs performed better than fixed cut-offs in our study.

The results of Chapter 2 contribute to the growing body of evidence supporting the use of angiogenic factors, particularly PlGF, in the diagnosis of pre-eclampsia. While our study used cases of pre-eclampsia with no clinical uncertainty surrounding the diagnosis (i.e., conventional criteria of hypertension and proteinuria were present), we postulate that these markers will have the greatest utility in cases of clinical uncertainty surrounding the diagnosis of pre-eclampsia. For example, it is now widely accepted that pre-eclampsia can manifest with or without hypertension or proteinuria. The British Eclampsia Survey Team (BEST) found that only 57% of women with eclampsia had documented hypertension with proteinuria within the week prior to the first seizure (Douglas and Redman, 1994). Women with non-proteinuric, pre-existing or gestational hypertension can evolve into pre-eclampsia and have been found to have worse outcomes than their classically-defined pre-eclampsia counterparts (Magee et al., 2007). Information from the Pre-eclampsia Integrated Estimate of Risk (PIERS) Study dataset showed that 254 of 2024 women with pre-eclampsia did not meet the classical definition of pre-eclampsia (i.e., hypertension with proteinuria) on the day of their hospital admission (von Dadelszen et al., 2011). Moreover, of the 106 women who had an adverse maternal outcome within 48 hours of hospital admission, 21 (20%) had isolated HELLP (haemolysis, elevated liver enzymes, low platelets) syndrome and 6 (5%) had hyperuricemic hypertension (unpublished data). It would be useful to have additional tests to confirm the diagnosis of pre-eclampsia in these cases. Although we did not evaluate these markers in women with suspected pre-eclampsia or with non-classical manifestations of the syndrome in our study, other research groups are currently investigating this question (Chappell et al., 2013). In a recent publication of women presenting before 35 weeks gestation with suspected pre-eclampsia, PlGF concentrations <5th percentile for gestational age predicted pre-eclampsia onset requiring delivery within 14 days with 96% [95% CI: 89, 99] sensitivity (Chappell et al., 2013). Similar
results have been reported for sFlt-1, sEng and the sFlt-1/PIGF ratio to identify women requiring
delivery within two weeks or those would progress into severe disease after initial presentation with
suspected pre-eclampsia (Chaiworapongsa et al., 2011; Rana et al., 2012a, Rana et al., 2012b).

Additionally, the relationship between these markers and adverse maternal and neonatal outcomes
would also be clinically useful in addition to the identification of the pre-eclampsia syndrome itself
(Sibai, 2007). One study reported high predictive power (AUC 0.96) of PIGF to predict adverse outcomes
in women with suspected pre-eclampsia or IUGR. Adverse outcomes included HELLP, stillbirth and
elective delivery before 34 weeks gestation (Su et al., 2001). PIGF is thought to have value in predicting
adverse outcomes such as preterm delivery in women with pre-eclampsia (Gullai et al., 2013; Molvarec
et al., 2013). The ability to predict adverse outcomes may have more clinical importance as it is the
devastating events of pre-eclampsia that are of clinical concern as opposed to the disease itself.
Continued work in this area is ongoing and foreshadows exciting advances in obstetrics which will lead
to improved maternal and fetal outcomes.

In Chapter 3, we found that angiogenic factors do not predict the onset of pre-eclampsia or other
placentally-mediated pregnancy complications such as SGA delivery, stillbirth and placental abruption
with adequate sensitivity and specificity. In our high-risk pregnancy cohort, all markers performed
poorly in the prediction of a composite outcome defined as early-onset pre-eclampsia, stillbirth,
placental abruption and/or SGA live birth. Using multivariable logistic regression, we did find that an
increase in PIGF concentration was associated with a significant decrease in odds of the composite
outcome, independent of other covariates (maternal age, gestational age, uterine artery notching
between 20–24 weeks and unexplained abnormal serum screen result). A PIGF concentration <5th
percentile for gestational age was also associated with 4-fold higher odds of the composite outcome
(adjusted OR=3.78, 95% CI: 1.39, 10.23). sFlt-1 and sEng were not significantly associated with the
composite outcome (adjusted OR=2.09, 95% CI: 0.49, 8.91 and adjusted OR=0.92, 95% CI: 0.26, 3.28, respectively).

A definitive test to predict pre-eclampsia and other pregnancy complications is currently elusive. It is likely that prediction models will need to include several predictor variables in order to achieve adequate clinical performance. The results of Chapter 3 suggest that PI GF may be a suitable candidate for inclusion in such models. A multivariable prediction modelling approach that combines other predictive factors such as maternal characteristics, uterine artery Doppler and parameters of fetal well-being is likely to prove useful. Work in this area is ongoing.

In Chapter 3, we also performed an exploratory analysis to investigate the relationship between angiogenic factor concentrations and lesions of placental dysfunction in this high-risk pregnancy cohort. We found decreased PI GF and increased sEng in the second trimester was significantly associated with placental lesions of malperfusion and fetal stromal-vascular maldevelopment (Redline et al., 2004a; Redline et al., 2004b). The relationship between angiogenic factors in the maternal circulation and placental lesions associated with placental dysfunction has yet to be established. As placental dysfunction can lead to the development of complications such as pre-eclampsia and IUGR, biomarkers that reflect placental functional status would be particularly useful in clinical practice. In this preliminary study, it appears that PI GF and sEng may reflect abnormal placental development in early pregnancy that will lead to placental dysfunction in later gestation. Future work in this area would include further investigation into the relationship between angiogenic factors and placental lesions and more importantly, ascertainment of the relationship between changes in these angiogenic factors and adverse maternal and fetal outcomes associated with these lesions. We were unable to evaluate these relationships in our study due to the small sample size. As histological findings are downstream manifestations of abnormal placentation and placental dysfunction, these markers may reflect these
abnormal processes early in pregnancy and provide new tools by which to identify placental abnormalities during pregnancy.

6.1.2 Intrauterine growth restriction

In Chapter 4, we conducted a pilot study of maternal PlGF in pregnancies with IUGR to evaluate the ability of PlGF to identify fetuses with abnormal placental pathology as a cause of growth restriction within this group. Our findings suggest that low PlGF differentiates placental IUGR from constitutionally small fetuses (fetuses detected to be small on ultrasound but with no placental pathology at the time of delivery) with high sensitivity and specificity when placental pathology is used to define these outcomes. This was a novel approach to evaluate PlGF as a diagnostic marker in IUGR and to our knowledge, this was the first study to show this association. Due to the small sample size, our results require confirmation in a large cohort of fetuses with suspected IUGR and we are currently conducting a multicentre study to this effect (Appendix A). Interim results of this ongoing study presented in Chapter 4 using data from women recruited up until April 2014 show that low maternal PlGF identifies fetuses who will have severe placental pathology at the time of delivery. While we require the study to reach its calculated sample size of 396 women to have an adequate precision around the sensitivity estimate (important in the clinical setting), these interim results appear promising and support our hypothesis.

Follow up studies to this ongoing study include investigating the incremental value of PlGF testing in addition to information already gained from current clinical assessments for identifying placental IUGR using multivariable predictive modelling. Multivariable logistic regression can be used to assess the incremental value of PlGF testing, in addition to information from assessment tools already used in routine clinical practice, to determine the probability of placental IUGR. Based on contributory medical history and current surveillance tests, we propose that the following predictor variables could be included in the regression model: history of an IUGR/SGA pregnancy, maternal age, maternal height, uterine artery Doppler notching between 20–24 weeks gestation, umbilical artery Doppler S/D ratio
and/or fetal assessments such as umbilical artery absent-reversed end diastolic flow, decreased middle cerebral artery resistance, notched ductus venosus and amniotic fluid index. Predictive modelling will represent an advance in the diagnosis of placental IUGR if results are successful.

An additional objective of the ongoing study will be to determine the relationship between maternal PlGF, umbilical artery Doppler and placental IUGR in pregnancies with SGA fetuses. At present, umbilical artery Doppler studies are used in clinical practice to identify fetuses at increased risk of compromise due to increased placental vascular resistance (RCOG, 2013). However, fetuses with normal umbilical artery Doppler studies still have evidence of placental dysfunction at delivery (Parra-Saavedra et al., 2013; Triunfo et al., 2014). It would be particularly interesting to see if PlGF discriminates fetuses with placental dysfunction in the group of fetuses with normal Doppler studies (Triunfo et al., 2014).

In Chapter 5, we quantitatively assessed placental morphology from pregnancies with suspected IUGR, sub-dividing this group by PlGF concentration. The results of this study show that low PlGF, particularly PlGF <12 pg/mL, is associated with abnormal placental structure. Placentas from women with PlGF <12 pg/mL at the time of SGA identification on ultrasound had increased numbers of syncytial knots, decreased trophoblast area, increased proliferative and reduced vascularity. These parameters in placentas from women with normal PlGF level at the time of SGA identification on ultrasound were similar to placentas from uncomplicated pregnancies. These findings suggest that PlGF may identify a subset of SGA fetuses with placental compromise and supports the hypothesis that PlGF acts as an antenatal marker of placental dysfunction.

6.2 Conclusion

Pre-eclampsia and IUGR are pregnancy complications that threaten the lives and well-being of mothers and babies worldwide. The ability to accurately identify women with these complications would improve outcomes for both mother and child by allowing for enhanced surveillance and appropriate management in order to reduce the adverse outcomes associated with these complications. Work is this
thesis supports the use of angiogenic factors in the detection of pre-eclampsia and placental IUGR. This work also helps to elucidate the associations between angiogenic factors and placental lesions, providing support for future work to better understand the mechanisms by which placental function and angiogenic factors are altered in pre-eclampsia and IUGR. Studies remain to be done to determine how best to implement the use of PI GF in clinical practice but this area holds promise for improving the care of pregnant women and their babies.
References


presenting to the obstetrical triage area with the suspicion of preeclampsia. *J Matern Fetal Neonatal Med*, 24(10), 1187-207.


Appendix

A. PlGF in IUGR Study: Working Protocol

Research Study, February 2012

Preamble:

Intrauterine growth restriction (IUGR) is a pathological process that reduces the growth trajectory of a fetus. IUGR can result from placental dysfunction (placental IUGR) or be caused by fetal syndromes, congenital infection and/or aneuploidy (syndromic IUGR) (Dugoff, 2010; Goetzl, 2010; Lakhoo, 2007; Maruyama et al., 2007; Oepkes and van Scheltema, 2007; Ralston et al., 2001). Fetuses with IUGR are at increased risk for preterm delivery, stillbirth, serious neonatal complications and sequelae later in child and adulthood (Breeze and Lees, 2007; Eber, 2007; Garite et al., 2004).

Management of IUGR requires increased fetal surveillance with subsequent intervention to balance the risks of prematurity with the risks of continued exposure to placental dysfunction (Halliday, 2009; Marsal, 2009; Maruyama et al., 2007). This surveillance and subsequent neonatal course carries significant burdens to the healthcare system (e.g., resources utilisation, costs) and increased parental and clinician anxiety about the pregnancy and infant outcomes.

Clinical suspicion/diagnosis of IUGR is precipitated by any indication that the fetus is small for gestational age (e.g., decreased symphysis-fundal height, estimated fetal weight (EFW) <10th percentile for gestational age or fetal abdominal circumference (AC) <10th percentile for gestational age on ultrasound) (Harkness and Mari, 2004; Maruyama et al., 2007). However, in this population of fetuses, only a proportion will have placental IUGR (an additional proportion will have syndromic IUGR) (RCOG, 2013). Most fetuses in this group are small and otherwise healthy (RCOG, 2013). These constitutionally small fetuses achieve their individual growth potential and are at low or no risk for adverse outcomes.

It is important for clinicians to make an accurate diagnosis of IUGR for timely and appropriate antenatal management. Advances in fetal surveillance and genetic screening have improved antenatal diagnosis of syndromic IUGR. However, it remains a clinical challenge to identify placental IUGR prior to delivery despite advances in antenatal screening and surveillance (Maulik, 2006; Soothill et al., 1999; Zhang et al., 2010). As such, IUGR is over-diagnosed in small for gestational age fetuses. Many misdiagnoses of IUGR, particularly placental IUGR, can be attributed to surveillance techniques providing inadequate information about the functional status of the placenta. Biomarkers found in maternal circulation that reflect the functional status of the placenta may provide clinicians with an additional tool to identify placental dysfunction antenatally. Based on our previous research (Benton et al., 2012), placental growth factor (PIGF) in the maternal circulation during pregnancy may identify placental dysfunction antenatally and hence, may be useful in the diagnosis of IUGR.
Study Objectives:

1) To determine if low PIGF at the time of ultrasound identification of a small for gestational age fetus (EFW or AC <10th percentile) is associated with an outcome of placental IUGR, defined by abnormal placental pathology at delivery.

2) To assess the ability of PIGF to diagnose placental IUGR antenatally using the STARD criteria for diagnostic testing (Bossuyt et al., 2003).

3) Investigate the incremental value of PIGF testing in addition to information from current clinical assessments for identifying placental IUGR antenatally with multivariable modelling.

4) To collect and store samples from these pregnancies for future biomarker research related to IUGR.

Research Methods:

This is a multi-centre, prospective cohort study involving pregnant women who have a fetus with suspected or diagnosed IUGR (i.e., a fetus identified as being small for gestational age). This study is funded by the Canadian Institute for Health Research and involves collaboration between research sites at the University of British Columbia and the University of Ottawa (Ottawa, Ontario).

i. Recruitment

Women will be recruited from the Diagnostic Ambulatory Program (DAP) at BC Women’s Hospital and from the OB Ultrasound Department at the Ottawa Hospital, General and Civic Campuses. Women will be identified by the study’s Research Assistants according to referral information and clinic lists. Eligible women may also be made known to the Research Assistants by physicians and/or ultrasound technicians; however, only the study staff will approach women with the invitation to participate in the study. Eligible women will be approached by the Research Assistants to discuss the study and consent form in a private room. The subject will have as much time as she needs to read through the study material and consent forms in this quiet room.

ii. Inclusion criteria

Women meeting the following inclusion criteria are eligible to participate in the study provided written, informed consent is obtained:

1. Clinical suspicion or diagnosis of IUGR as indicated by estimated fetal weight (EFW) <10th percentile for gestational age on ultrasound and/or fetal abdominal circumference (AC) <10th percentile for gestational age on ultrasound.


4. Gestational age 18-45+ weeks.
Women will be excluded from participating for any of the following:

1. Any fetal anomaly or syndrome known to exist at the time of enrolment.
2. Confirmed premature rupture of membranes due to infection prior to enrolment.
3. Documented hypertension or any signs/symptoms of pre-eclampsia at the time of enrolment.

iii. Sample size

A sample size of 392 women has been calculated based on preliminary data. We want to ensure that the specificity of identifying placental IUGR can be estimated to within 5 percentage points (i.e., the upper and lower limits of the 95% confidence interval for our estimate of specificity will be within 5 percentage points of our point estimate). A specificity of 85% will ensure that the lower limit of the confidence interval in our study will be no lower than 80%, a value considered to be the minimum clinically acceptable level of specificity for a diagnostic test. The sample size is calculated as follows:

Point estimate for specificity, \( p = 0.85 \)

Desired lower of 95% Confidence Interval (CI) = 0.80

Standard Error (SE) of a proportion = \( p\sqrt{\frac{1-p}{n}} \) where \( n \) = sample size

Formula: \[ 95\% \text{ CI} = \text{Point Estimate} \pm 1.96 \times \text{SE} \]

\[ 0.80 = 0.85 - (1.96 \times \sqrt{0.85 \times 0.15}) \]

Solving for \( n = 196 \)

Solving for placental IUGR (50%) = 196*2

\[ n = 392 \]

In other words, we anticipate that ~50% of women in our population will have placental IUGR based on our pilot data. Therefore, the calculated sample size of 196 women represents 50% of the total sample size.

iv. Confidentiality

After written informed consent is obtained, all women will be de-identified with a unique subject study number. All biological samples and other information relating to each woman will be collected and stored using only this number. No identifying information will be contained in the number nor will any identifying information be stored or published.

v. Data collection

We will perform a thorough postpartum chart review of all enrolled women. Hospital charts will be reviewed for patient medical and obstetrical history, pregnancy outcomes, perinatal outcomes, intervention and surveillance, placental pathology reports as well as other pertinent information using a standard data collection form. The study staff will perform these chart reviews in consultation with the
Principal Investigators. Data will then be inputted into a secure study database. This study database is password protected. No identifying information will be inputted into this database and subject information will be de-identified using only the study number. After information is entered into the database, the paper copy of the data collection form will be stored in a locked cabinet in the Principal Investigators’ offices and stored there for 7 years after the final results related to this study are published.

vi. Sample collection and processing

Enrolled women will be asked to give a blood sample (~30mL) at the time of enrolment. Venous blood will be collected using two standard 10 mL EDTA plasma (purple top) tubes and two standard 4 mL serum tubes (red top). Venous blood will be collected in the standard way by Research Staff trained in phlebotomy. Immediately after collection, whole blood will be centrifuged at 3000 rpm for 20min. Plasma and serum will be immediately collected into aliquots of 250μL in 1.5mL Eppendorf tubes and frozen at -80°C. Aliquot tubes will be labelled with barcodes that are linked to the subject’s study number and date of collection. No identifying information will be written on the tubes or tube labels. Our laboratory staff will be blinded to all information about all study subjects. Plasma and serum samples will be batch assayed for our candidate markers according to the length of freezing time to minimise intra-assay variability.

Plasma and serum samples will be stored at the von Dadselszen and Gruslin laboratories for future biomarker testing. After the testing of our candidate markers is complete, remaining aliquots will be divided and stored between the two laboratories. This distribution will also protect the loss of samples should there be any sort of damage or malfunction to our freezers.

vii. Biomarker analysis

Plasma will be analysed for PlGF using the automated Triage immunoassay (Alere, San Diego, CA, USA) according to the manufacturer’s instructions and our previous work. The Triage meter and test cartridges will be provided as an in-kind donation from Alere International.

The test device uses fluorescently labelled mouse monoclonal antibodies against human PlGF or sEng for quantification. In brief, plasma is thawed to 20°C and mixed by inversion. 250μL of thawed plasma is pipetted into the sample port of a new test cartridge. The cartridge is inserted into the Triage meter and results are displayed on the meter in approximately 15min in pg/mL. The detection range for the Triage PlGF assay range from 12-3000 pg/mL. Thus far, our samples have had the range of 12-2854 pg/mL. Should concentrations exceed the upper limit of detection, we will dilute plasma to ensure detection is within the linear portion of the standard curve.

Biomarker analyses will occur at the von Dadselszen and Gruslin laboratories. Testing of samples will occur only after delivery of the index pregnancy of each enrolled woman. Biomarker results will not be recorded in patient charts nor be used in any way to guide clinical care of these women. Women will
be made aware that participating in this study will have no impact on their clinical care and they will continue to receive the standard of care at each site. Physicians and Research Assistants will be blinded to the biomarker results. Data will be merged and then unblinded after the delivery of the final woman enrolled in the study. Data will be kept confidential from Alere at all times until all study analyses have been completed.

viii. Placental pathology

The outcome of placental IUGR will be determined by rigorous histopathological assessment of the placenta, using a hierarchical and systematic approach, following delivery (Redline et al., 2003; Redline et al., 2004a; Redline et al., 2004b).

Placentas from enrolled women will be sent to local Anatomical Pathology departments following delivery. Gross pathology examination was undertaken in the standard fashion and placental weight, cord length, number of cord coils, cord insertion site, membrane appearance and maternal and fetal surface appearance will be recorded by the reporting technician. Biopsies of placental tissue will be collected from random areas of the villous parenchyma (4 sections), fetal membranes (1 section) and umbilical cord (1 section). Tissue will be fixed in 4% neutral buffered formalin and then wax-embedded using standard laboratory procedures. Tissue sections will be stained with haematoxylin and eosin in the standard way. Digital images of the stained sections will be taken using the Aperio® ScanScope and stored on a portable, external hard drive. All tissue blocks, slides and images will be labelled only with the patient’s study number.

Tissue sections will be review by the Study Pathologist according to the “Clinical Placental Pathology Evaluation Form” developed in accordance with the scheme endorsed by the Society for Paediatric Pathology (Appendix B) (Redline et al., 2003; Redline et al., 2004a; Redline et al., 2004b). The Study Pathologist will be blinded all aspects of the woman’s history, pregnancy course, pregnancy outcomes and neonatal outcomes apart from gestational age at delivery. Each individual lesion will be graded from 0 (not present) to 3 (severe). Lastly, a final overall grade from 0 to 3 for the placenta will be assessed based on the individual grading for each lesion and the placental weight percentile for gestational age.

In addition to routine clinical pathology, additional placental villous tissue will also be collected for quantitative basic science investigations using immunohistochemistry and image analysis software (not routinely done in clinical pathology). Placental sections will be quantitatively assessed for the following morphological parameters: syncytial knots, proliferation, trophoblast area and vascularity (Warrander et al., 2012). An additional 4 blocks (1cm³) of villous tissue will be dissected from each and fixed in formalin for 18-24 hours. After fixing, tissue will be processed in the standard method and embedded in paraffin. Paraffin blocks will be sectioned and stained using immunohistochemistry procedures. Digital images will be taken using Aperio® ScanScope. All tissue blocks, slides and images will be labelled only with the patient’s study number.
Analyses:

1. Association of low PlGF and placental underperfusion

In this cohort study, women will be grouped based on their PlGF concentration (the exposure) at the time of enrolment into the study (i.e., at the time of an EFW or AC <10th percentile identification). Women with PlGF concentrations <5th percentile for gestational age at sampling will be grouped into the “Low PlGF group”. Women with PlGF concentrations ≥5th percentile for gestational age at sampling will be grouped together to form the “Normal PlGF group”. The 5th percentile cut-offs for gestational age are provided in the product insert of the Triage® meter and are derived from uncomplicated pregnancies (Saffer et al., 2013).

The primary outcome of interest in these two groups is the abnormal placental pathology related to placental underperfusion at the time of delivery. Abnormal placental pathology related to placental underperfusion is defined as an overall grade of ≥2 on the Placental Evaluation Form. We will compare the prevalence of this placental outcome between the groups using a 2x2 contingency table and Fisher’s exact test as follows:

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Outcome</th>
<th>Overall Placenta Grade ≥2</th>
<th>Overall Placenta Grade ≤1</th>
<th>Fisher’s exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low PlGF at sampling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal PlGF at sampling</td>
<td></td>
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</tr>
</tbody>
</table>

The association of PlGF and the placental outcome will be assessed using logistic regression to calculate the odds ratio with 95% confidence intervals (crude and adjusted for confounders). Confounders include maternal age, maternal height, parity, past history of an SGA infant, and smoking status during pregnancy.

2. Evaluation of diagnostic accuracy:

We will evaluate the ability of PlGF to identify placental pathology in pregnancies with small for gestational age fetuses using sensitivity and specificity, positive and negative predictive values and likelihood ratios (all with 95% confidence intervals) calculated from a 2x2 contingency table (below).

<table>
<thead>
<tr>
<th>Test</th>
<th>Disease</th>
<th>Overall Placenta Grade ≥2</th>
<th>Overall Placenta Grade ≤1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (PlGF &lt;5th percentile)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (PlGF ≥5th percentile)</td>
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<td></td>
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</tr>
</tbody>
</table>
A positive PI GF test is defined as a PI GF level < 5th percentile for gestational age at sampling. A negative PI GF test is defined as a PI GF concentration ≥ 5th percentile for gestational age at sampling. Again, the 5th percentile cut-offs for gestational age are provided in the product insert of the Triage® meter and are derived from uncomplicated pregnancies (Saffer et al., 2013). We will use the product insert cut-offs for a positive PI GF test but can identify fixed cut-off from receiver operator curves (ROC), if needed.

3. Multivariable modelling:

This objective will investigate the incremental value of PI GF testing, in addition to information from assessment tools already used in routine clinical practice, to determine the probability of placental IUGR. We will develop a multivariable logistic regression model that will provide the diagnostic probability of placental IUGR based on PI GF and other predictors. The following variables will be included in our regression model:

1) IUGR in previous pregnancy
2) Maternal age
3) Uterine artery Doppler notching between 20-24 weeks gestation
4) Umbilical artery Doppler (S/D ratio or absent-reversed end diastolic flow,)
5) Other fetal assessment (decreased middle cerebral artery resistance, notched ductus venosus, or < 50 mm amniotic fluid index)

Appropriate transformations (e.g., log) will be applied to variables that are not normally distributed. β-coefficients and standard errors from logistic regression will be exponentiated to obtain odds ratios and 95% CIs to evaluate the independent contribution of PI GF in identifying placental IUGR. For each variable included in the regression model, a minimum of 10 women with placental IUGR will be needed to estimate a robust regression model. We believe it is feasible to assemble this large cohort. This model should be able to discriminate between fetuses with placental IUGR and those that are constitutionally small, and thereby address a critical need in current obstetrics practice.
# B. PIgf in IUGR Study: Clinical Placental Pathology Evaluation Form

**STUDY NUMBER:** ____________________

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Parameter</th>
</tr>
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<tbody>
<tr>
<td><strong>Macroscopic</strong></td>
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<tr>
<td>Placental weight (grams)</td>
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</tr>
<tr>
<td>* Record <em>after</em> microscopic grading is completed</td>
<td></td>
</tr>
<tr>
<td>Fetal:placental weight ratio</td>
<td></td>
</tr>
<tr>
<td>* Record <em>after</em> microscopic grading is completed</td>
<td></td>
</tr>
<tr>
<td>Peripheral cord insertion (&lt;3cm from margin)? (Y/N)</td>
<td></td>
</tr>
<tr>
<td>Velamentous cord insertion? (Y/N)</td>
<td></td>
</tr>
<tr>
<td>Extrachorialis? (Y/N)</td>
<td></td>
</tr>
<tr>
<td>Accessary lobes? (Y/N)</td>
<td></td>
</tr>
<tr>
<td>Two vessel cord? (Y/N)</td>
<td></td>
</tr>
<tr>
<td>Length of umbilical cord (cm)</td>
<td></td>
</tr>
<tr>
<td>Excessive coiling of cord for GA? (Y/N)</td>
<td></td>
</tr>
<tr>
<td>True knot(s) in cord? (Y/N)</td>
<td></td>
</tr>
<tr>
<td><strong>Microscopic</strong></td>
<td></td>
</tr>
<tr>
<td>1. Maternal malperfusion (MMP)</td>
<td></td>
</tr>
<tr>
<td>▪ 0: None present</td>
<td></td>
</tr>
<tr>
<td>▪ 1: Mild DVH* and/or 1-3 peripheral infarcts (focal marginal infarcts excluded in term placentas)</td>
<td></td>
</tr>
<tr>
<td>▪ 2: Moderate DVH and/or &gt;3 total infarcts</td>
<td></td>
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<tr>
<td>▪ 3: Severe DVH, ≥5 total infarcts or &gt;3 central infarcts or &gt;3cm in size</td>
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<tr>
<td>*Based on the proportion/size of intermediate villi surrounded by sparse clusters of small mature distal villi and volume of intervillous space</td>
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<tr>
<td>1- Mild reduction in size of intermediate villi with slightly dispersed distal villi that appear more uniformly small than normal</td>
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<tr>
<td>2- Moderate reduction, moderate dispersal</td>
<td></td>
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<tr>
<td>3- Very thin intermediate villi with very sparse clusters with abundant intervillous space</td>
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</tr>
<tr>
<td>Include grade for accelerated villous maturation:</td>
<td></td>
</tr>
<tr>
<td>▪ 0: focal and infrequent</td>
<td></td>
</tr>
<tr>
<td>▪ 1: focal scattered knots (sparse and patchy)</td>
<td></td>
</tr>
<tr>
<td>▪ 2: multi-focal knots</td>
<td></td>
</tr>
<tr>
<td>▪ 3: diffuse abundant knots</td>
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</tr>
</tbody>
</table>
2. Fetal villi maldevelopment  
- 0: No dysmorphology  
- 1: Mild  
- 2: Moderate  
- 3: Severe  

Includes:  
Villous capillary proliferative lesion – chorangiosis (>10 terminal villi with ≥10 capillaries in several sections) and chorangiomatosis  
Dysmorphic villi (irregular villous contour, abnormal vascular pattern) and immature distal villi

3. Villitis  
- 0: No villitis  
- Infectious origin  
- Non-infectious origin, unknown origin  
  - 1: Focal  
  - 2: Multifocal  
  - 3: Diffuse/massive

4. Perivillous fibrin deposition  
- 0: None present  
- 1: Focal (<10% of volume)  
- 2: Patchy (10-50% of volume)  
- 3: Diffuse (>50% of volume)

5. Fetal thrombotic vasculopathy (vascular obstruction)  
- 0: None  
- 1: Patchy (1-5% of vessels affected)  
- 2: Multifocal (>5-20%)  
- 3: Diffuse (>20%)  

Features of obstruction in delivering fetal vessels including obliteratorive or hemorrhagic changes due to occlusion and areas showing avascular fibrotic distal villi

6. Evidence of abruption  
- 0: No evidence  
- Acute abruption: Retroplacental blood clot (decidual basalis) with compression of overlying parenchyma  
- Chronic marginal separation: Marginal clot with hemosiderin within peripheral chorionic plate and/or membranes

7. Interplacental hematoma/thrombi  
- 0: None present  
- 1: 1-5 focal hematomas  
- 2: 5-10 focal or any hematoma 2-5cm in diameter  
- 3: >10 hematomas or any hematoma >5cm in diameter

Thrombus/hematoma within the intervillous space
8. Chorioamnionitis
   - 0: None present
   - If present, is it acute? Stage?

9. Miscellaneous findings
   - Note as needed

OVERALL GRADE:
   - Grade 0: No significant pathology (Grade 0 in all categories)
   - Grade 1: Grade 1 in MMP, chorangiosis, villitis, fibrin, interplacental hematoma OR Grade 2 in MMP with good placental weight and good fetal to placental ratio and other categories Grade ≤1
   - Grade 2: Grade 2 in MMP with reduction in placental weight (<10\textsuperscript{th} percentile) with no grades of 3 in other categories OR Grade 2 in chorangiosis, villitis, fibrin, interplacental hematoma
   - Grade 3: Grade 3 in MMP, chorangiosis, villitis, fibrin, interplacental hematoma OR Grade 2 in MMP with significant reduction in placental weight (<3rd percentile) OR Grade 2 in MMP with Grade 3 in chorangiosis, villitis, fibrin, interplacental hematoma grade