NOVEL FACTORS IN PREECLAMPSIA

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DECLARATION

I declare that this thesis has been composed by myself and that the work described has been performed by myself unless clearly stated in the text. All information from sources outside of this study has been acknowledged. This work has not been submitted for any other degree or professional qualification.

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ABSTRACT

Preeclampsia (PE) is a hypertensive disorder of human pregnancy that is characterised by widespread vascular endothelial cell activation, inflammation, and oxidative stress. The regulation of chemokines and adhesion molecules in these cells is important in inflammatory responses. This thesis explores the hypothesis that the levels of soluble Fractalkine (sFkn), a marker of inflammation, are increased in PE, and that over-expression of the protective enzyme, heme oxygenase-1 (HO-1), reduces sFkn. We found that sFkn release was not increased in plasma and placenta lysates in patients with PE compared to normal pregnancy. However, it was induced by the pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), in human umbilical endothelial cells (HUVECs). Honokiol, an antioxidant agent, significantly reduced sFkn release. Collectively, these studies indicate that cytoprotective HO-1 pathway and Honokiol may offer partial protection against PE, by down-regulating sFkn and reducing the impact of pro-inflammatory factors in PE.

Affymetrix Gene array profile of human placenta identified high expression of two new factors involved in the pathogenesis of PE, Noggin, and Leucine rich and immunoglobulin like domains protein 1 (Lrig1). This led us to investigate whether the expression level of Noggin and Lrig1 changes in preeclamptic placentas. Quantitative polymerase chain reaction (qPCR) revealed no significant differences in expression of Noggin at the messenger RNA (mRNA) level between normal and

preeclamptic placenta. Western blot (WB) analysis of Noggin demonstrated an increase in the expression levels throughout gestation. In contrast, the expression of Lrig1, both at mRNA and at the protein level, was significantly higher in the PE placenta compared to the normal placenta. Immunohistochemical staining showed that Noggin and Lrig1 are expressed and localised in the cytotrophoblast and syncytiotrophoblast. These data suggest that Noggin and Lrig1 are found in human placenta and their expression is altered in PE. Further studies are needed to validate the significance of these early studies.

Key Words: ■ Preeclampsia ■ Chemokine ■ Fractalkine ■ Honokiol ■ heme oxygenase ■ Lrig1 ■ Noggin ■ Endothelial cells ■ placenta

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ABBREVIATIONS

Ad	Adenovirus
ADAM	Metallopeptidase domain
ADMA	Asymmetrical dimethylarginine
Akt	Protein kinase B
Alk	Activin-like kinases
APS	Ammonium persulphate
BBE	Bovine brain extract
BMPR2	Bone morphogenetic protein receptor type II
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
BVR	Biliverdin reductase
CD31	Cell adhesion molecule
cDNA	Complementary DNA
CECs	circulating endothelial cells
CGMP	Cyclic guanosine monophosphate
CMV	Cytomegalovirus
CO	Carbon monoxide
CORM	Carbon monoxide-releasing molecule
CrPP	Chromium protoporphyrin
CsCl	Caesium chloride
Cth	cystathionine γ-lyase
CX3CR1	Fractalkine receptor 1
Cyt7	Cytokeratin 7
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco’s modified eagle medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNase	Deoxyribonuclease
DTT	Dithiothreitol
EBM	Endothelium basal medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immuno sorbant assay
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
Fe	Free iron
Fkn	Fractalkine
Flt-1	Fms-like tyrosine kinase 1
GDF	Growth differentiation factor
GM-CSF	Granulocyte macrophage colony stimulating factor
H_2S	Hydrogen sulphide
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
hEGF	Human epidermal growth factor
HELLP	Haemolysis, elevated liver enzymes, low platelet count
HET	Heterozygous
HIF-α	Hypoxia inducible factor-alpha
Hmox1	Heme oxygenase (decycling) 1 gene

HO	Heme oxygenase
HRP	Horseradish peroxidase
HUVECs	Human umbilical endothelial cells
ICAM	Intercellular adhesion molecule
iCORM	inactivated carbon monoxide depleted molecule
IFN-γ	Interferon gamma
IFU	Infective units
Ig	immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IUGR	Intrauterine growth restriction
JNK	Jun N-terminal kinases
KO	homozygous knockout
Lef1	factor-1
Lrig1	Leucine-rich repeats and immunoglobulin-Like domains 1
LRRs	Leucine-rich repeats
M199	Medium 199
MAdCAM	Mucosal addressin cell adhesion molecule
MAPK	Mitogen-activated protein kinases
ME	Mercaptoethanol
mRNA	Messenger RNA
m-TOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-κB
NGS	Non-immune goat serum
NHBPEP	National High Blood Pressure Education Program

NK	Natural killer
NO	Nitric oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-tween-20
PCR	Real-time polymerase chain reaction
PE	Preeclampsia
PFU	Plaque forming units
PlGF	Placental growth factor
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
sFkn	Soluble Fractalkine
sFlt1	Soluble Fms-like tyrosine kinase-1
siRNA	Small interfering RNA
SnPP	Tin protoporphyrin IX
STOX1	Storkhead box 1
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-tween-20
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-β
Th	T helper

TNF-α	Tumour necrosis factor-α
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand factor
WB	Western blot
WHO	World health organization
WT	wild-type
β-ME	β-Mercaptoethanol

CHAPTER ONE: INTRODUCTION

1.1 The placenta

The placenta is a feto-maternal organ, which develops inside the lining of the uterus during pregnancy. The placenta is composed of both fetal and maternal components. The fetal part, develops from the chorion frondosum and the maternal part is derived from the endometrium. Endometrial cells differentiate into enlarged secretory decidua cells, accompanied by an infiltration of uterine natural killer cells that aid remodelling of the spiral arteries (Aplin et al., 2008; Moffett-King, 2002).

The fetus is connected by the umbilical cord to the placenta. The umbilical cord inserts into the chorionic plate, from which the vessels branch out over the surface of the placenta. These vessels further divide to form a highly vascularised network, which is covered by a thin layer of cells. A healthy placenta plays a crucial role for normal pregnancy, and it is important to understand the factors that control function of the placenta (Figure 1.1).

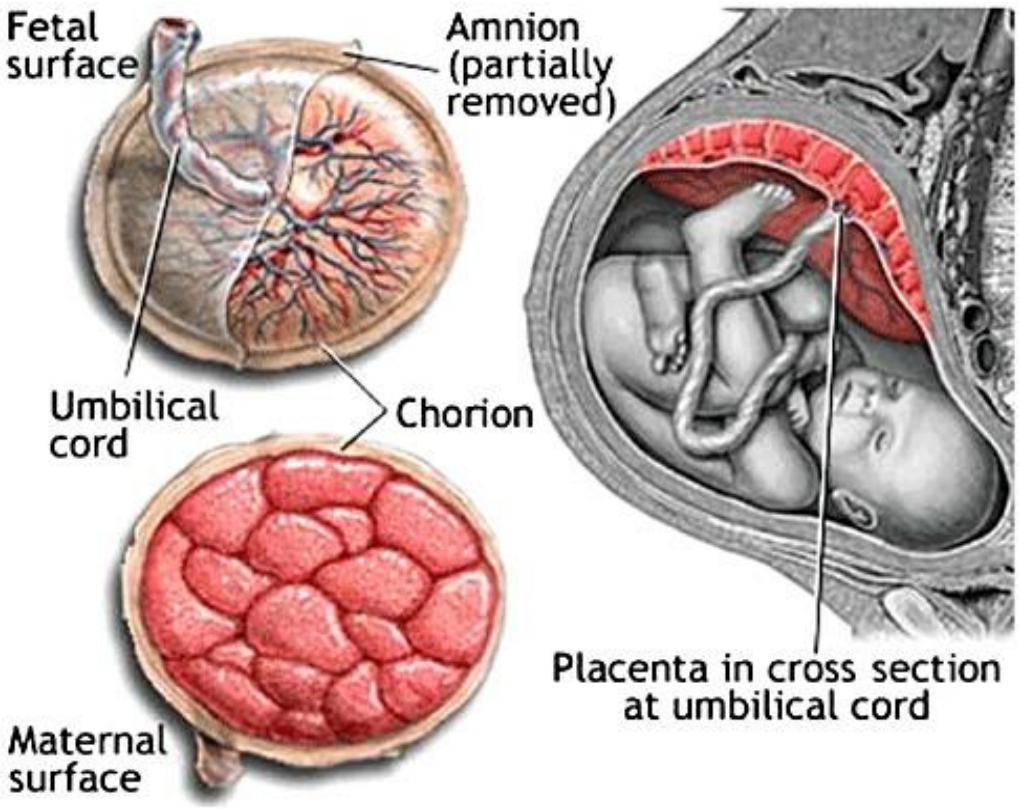

Figure 1.1 The human placenta anatomy diagram. The human placenta including mother's blood vessels, umbilical cord and placental membrane.The inner cell mass will later form the embryo and other placental compartments (Adapted from ((Bárcena et al., 2011)).

1.1.1 Placental development

The placenta begins to develop upon implantation of the blastocyst into the endometrium. The morula, consisting of a ball of cells derived from the division of the zygote undergoes differentiation and compaction to form the blastocyst. The outer trophectoderm layer of the blastocyst differentiates into the trophoblast, which forms the outer layer of the placenta, and is composed of two cell layers, the cytotrophoblast and the overlying syncytiotrophoblast. The inner cell mass forms the embryo, amnion and the umbilical cord (Benirschke et al., 2000). The extraembryonic mesoderm layer, also derived from the inner cell mass, gives rise to the placental stroma and vessels (Figure 1.2). The syncytiotrophoblast (or syncytium) is a continuous and unicytoplasmic layer, which covers the placenta lining the intervillous space. Cytotrophoblast cells, which lie beneath this layer, proliferate and differentiate fusing with the overlying syncytiotrophoblast. In spite of cytotrophoblast cell numbers increasing throughout gestation (Simpson et al., 1992), this layer later becomes discontinuous because of the increase in the villous surface area by the third trimester (Jones et al., 2008).

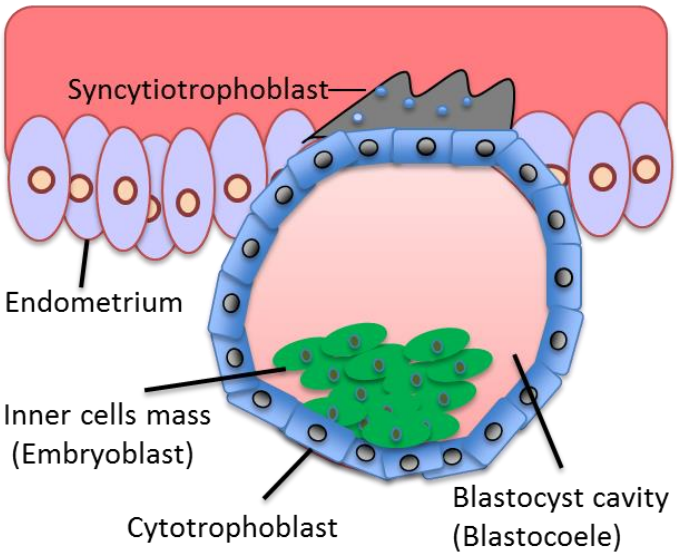

Figure 1.2 The implanting human blastocyst. The blastocyst contains a fluid-filled cavity called the blastocoele. The outer cells are the trophectoderm which form the trophoblast cells of the placenta. The inner cell mass will later form the embryo and other placental compartments.

Cytotrophoblasts also differentiate to form columns of extravilloust trophoblasts, which break through the syncytiotrophoblast and either invade interstitially into the uterine wall, or endovascularly into uterine blood vessels (Pijnenborg et al., 1998). The endovascular trophoblasts facilitate the transformation of the maternal spiral arteries in the decidua and myometrium. Vascular smooth muscle and endothelial cells are lost from the arterial walls, a process which is dependent upon pro-apoptotic and anti-apoptotic signals, and are replaced by these trophoblast cells. This process of vascular remodeling alters the arteries so their diameter is increased and they become less convoluted (Bulmer et al., 2012). This maximises blood flow to the placenta whilst also preventing shear stress by reducing resistance (Harris et al., 2006). In normal placental

development, the myometrium and extravillous cytotrophoblasts invade the uterine spiral arteries of the decidua. These fetal-derived cells replace the endothelial layer of the uterine vessels, transforming them from high resistance vessels to flaccid, low resistance cells. This allows the increase in uterine blood flow needed to sustain the fetus during pregnancy (Meekins et al., 1994). Important factors such as uterine size, shape, and trophoblast invasion might influence uterine vascularity during pregnancy (Pollheimer and Knofler, 2012; Crespi and Semeniuk, 2004).

1.1.2 Placental vascular remodelling

Vascular remodeling plays a significant role in successful pregnancy outcome, and influences the well-being of the mother as well as fetal development for newborn and future life (Song et al., 2010). However, defects in any changes in the placental vasculature system may lead to complications during pregnancy (Wang et al., 2013). Pregnancy also involves intensive placental uterine vasculature remodeling of large and small arteries and veins, as well as functional and structural invasion of the myometrial and decidual layers. In pregnant women, the plasma volume will rise by approximately 45% of the average non-pregnant volume for white European women, by about 1250 ml to about 2600 ml (Hytten, 1985).

1.1.3 Placental function

The placenta plays an important role in maternal health and fetal development (Haas, 2014; Ji et al., 2013; Pollheimer and Knofler, 2012; Hunkapiller and Fisher, 2008). The placenta and the umbilical cord are a transport system for the exchange of substances between the mother and fetus. In the placenta there are extravillous trophoblasts facilitating the exchange of substances. These include the transient “transport” of gases, macromolecules (proteins), micronutrients, metabolic waste products (glucose, amino acids) and signalling molecules between the maternal and fetal circulations; the synthesis of glycogen and fatty acids to nourish the fetus in early pregnancy; the production of hormones such as human chorionic gonadotrophin, progesterone, oestrogen and insulin-like growth factors; the protection of the fetus from rejection by the maternal immune system and the protection of the fetus from maternal toxins and pathogens.

1.2 Preeclampsia

Preeclampsia (PE) is a maternal pregnancy disorder that has an unknown cause and develops into a specific, multisystem disorder of pregnancy. PE is considered to be a two stage disorder; the first stage is associated with abnormal placental formation and release of placental factors into the maternal circulation. The second stage is characterised by maternal systemic pathophysiological changes, such as proteinurea and hypertension, which occur in the second trimester after 20 weeks gestation (Roberts and Hubel, 2009). Untreated PE may develop into a disorder known as eclampsia, which can lead to acute renal failure, seizures, pulmonary oedema, acute liver injury, haemolysis, and/or thrombocytopenia (Schaap et al., 2014; Munjuluri et al., 2005). The last three features of the disease occur together as part of the haemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome, which is a variant of PE (Haram et al., 2009).

1.2.1 Epidemiology of preeclampsia

In Africa and Asia, nearly one-tenth of all maternal deaths are associated with hypertensive disorders of pregnancy, whereas one-quarter of maternal deaths in Latin America have been associated with these complications (Streatfield et al., 2014). In America, PE affects 5% to 8% of pregnancies (Association, 2012). PE can cause morbidity (2% to 8%) and mortality (10% to 15%) worldwide, and neonatal mortality is significantly elevated (Duley, 2009; Saigal and Doyle, 2008). The 2016

World health organization (WHO) statistics indicate that pregnancy and childbirth complications are the second cause of death among 15 to 19 year olds globally, and an estimated 50,000-76,000 maternal and 500,000 fetal deaths occur as a result of this condition every year (Raghupathy, 2013). In addition, a study conducted by Lisonkova et al. (2014) found that late-onset PE was significantly associated with a high risk of fetal and neonatal death, but early-onset PE was not. Furthermore, HELLP syndrome occurs in approximately 10% to 20% of women with severe PE (Karumanchi et al., 2005).

1.2.2 Risk factors for preeclampsia

There are a number of theories about the initiating cause of PE and the descriptions in research articles can be difficult to understand. The aetiology of PE is currently unknown, and it is defined as “the disease of theories” (Aris et al., 2009). Many theories focus on placental and maternal factors contributing to the pathogenesis of PE (Kanasaki and Kalluri, 2009). Researchers have found many significant factors that contribute to the risk of PE including body mass, lifestyle and diet, age (under 20 years and over 40 years), nulliparity (first pregnancy), multiple pregnancies, pre-existing medical conditions such as diabetes, family history of PE, stroke, and the anti-phospholipid antibody syndrome (Brown et al., 2006; Duckitt and Harrington, 2005). Moreover, a recent study in China showed that population and regional ethnicity contributed to the prevalence of PE, where Chinese women had a low risk of developing PE as compared to Caucasians (Xiao et al., 2014). However,

the degree of scrutiny, method and definition of PE may have contributed to the low incidence decribed in the study.

1.2.3 Clinical characteristics of preeclampsia

Nowadays, the criteria for clinical diagnosis of PE syndrome is defined by the onset of an elevated blood pressure (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥90 mmHg), and proteinuria (defined as the urinary excretion of ≥300 mg of protein in 24 hours). PE can occur around >20 weeks to term (Nankali et al., 2013; Liu et al., 2008). In addition to the symptoms of proteinuria and hypertension, PE also results in hyperreflexia, loss of vision and headache (Preeclampsia, 2016; Vogell et al., 2014). Cohen et al. (2014) found that expression of angiogenic factors could be used as surrogate markers to diagnose PE. However, the level of sensitivity and specificity of these assays needs to be improved if they are to rule in PE.

PE can be divided into two subgroups, early-onset (<34 weeks) and late-onset (>34 weeks), and the management of the two subgroups differs. Early-onset PE has been significantly associated with a high risk of fetal death, whereas late-onset PE has no such association (Lisonkova et al., 2014). In early-onset PE, the symptoms are clinically very severe and commonly result in the delivery of a very immature, growth-retarded fetus. Cases with late-onset PE frequently do not exhibit placental changes, such as unmodified spiral arteries (Hahn et al., 2006; Sebire et al., 2005).

1.2.4 Complications of preeclampsia

PE is associated with short-term and long-term consequences for maternal and fetal health (Adams et al., 2014). Complications may lead to maternal organ damage, vascular and connective tissue disease in later life (Bilhartz et al., 2011), chronic hypertension (Ghossein-Doha et al., 2014; McDonald et al., 2013; Lykke et al., 2009) and insulin resistance (Manten et al., 2007).

Furthermore, there is evidence that PE complications influence fetal health both in the short term and long term. Complications can lead to growth restriction, low birth weight, respiratory distress syndrome (Li et al., 2014B; Duckitt and Harrington, 2005) insulin resistance, cardiovascular disease, inflammatory response and metabolic diseases (Liu et al., 2011A). In severe, particularly early-onset (prior to completion of 34 gestational weeks) PE, the fetus often suffers from increasing nutritional and respiratory insufficiency as a result of reduced blood flow to the placenta, which can lead to impaired growth, asphyxia, and even death (Redman and Sargent, 2005).

1.2.5 Prevention and treatment of preeclampsia

Due to the lack of success in the pharmacological management of PE, the induction of labour still remains the only known manging for this disorder. The current mangment, which is to induce delivery before 35 weeks, involves taking into account the maternal risks of continuing the pregnancy as well as fetal development (Chanprapaph, 2004). Several studies have demonstrated that daily low-dose (60 mg aspirin) aspirin reduces the risk of PE (Bujold et al., 2010: Wallenburg et al., 1986). However, there are no symptoms to warn a woman having PE after 20 weeks of gestation, and the effectiveness of aspirin is debatable (Bujold et al., 2010). There is a need for a drug which prevents PE, reverses the symptoms and does not have any dangerous side effects. The WHO has made recommendations to health care providers in the management of PE and the possible treatment for PE (Table 1.1). For each recommendation, the quality of the supporting evidence was graded as moderate, high, very low, and low.

Table 1.1 Prevention/ treatment of preeclampsia (WHO, 2014).

Recommendation	Quality of Evidence	Strength
Low-dose acetylsalicylic acid (aspirin, 75 mg) for the prevention of PE and its related complications should be initiated before 20 (+0) weeks of pregnancy.	Low	Weak
Women with severe hypertension during pregnancy should receive treatment with anti-hypertensive drugs.	Very low	Strong
The choice and route of administration of an anti-hypertensive drug for severe hypertension during pregnancy, in preference to others, should be based primarily on the prescribing clinician's experience with that particular drug, its cost and local availability.	Very low	Weak
Induction of labour is recommended for women with severe PE at a gestational age when the fetal is not viable or unlikely to achieve viability within one or two weeks.	Very low	Strong
In women with severe PE, a viable fetal and before 34 weeks of gestation, a policy of expectant management is recommended, provided that uncontrolled maternal hypertension, increasing maternal organ dysfunction or fetal distress are absent and can be monitored.	Very low	Weak
In women with severe PE, a viable fetal and between 34 and 36 (plus 6 days) weeks of gestation, a policy of expectant management may be recommended, provided that uncontrolled maternal hypertension, increasing maternal organ dysfunction or fetal distress are absent and can be monitored.	Very low	Weak

In women treated with anti-hypertensive drugs antenatally, continued anti-hypertensive treatment post-partum is recommended.	Very low	Strong
Treatment with antihypertensive drugs is recommended for severe postpartum hypertension.	Very low	Strong
In areas where dietary calcium intake is low, calcium supplementation during pregnancy (at doses of 1.5 to 2.0 g elemental calcium/day) is recommended for the prevention of PE in all women, but especially those at high risk of developing PE.	Moderate	Strong
Low-dose acetylsalicylic acid (aspirin, 75 mg) is recommended for the prevention of PE in women at high risk of developing the condition.	Moderate	Strong
Magnesium sulphate is recommended for the prevention of eclampsia in women with severe PE in preference to other anticonvulsants.	High	Strong

1.3 Causes of preeclampsia

The real origin of PE is still a mystery, but it is generally accepted that PE originates in the placenta rather than the fetus. Several dogmas have been established such as abnormal spiral artery remodelling, aberrant maternal systemic inflammation, angiogenic imbalance, and failed trophoblast invasion (Burton et al., 2009; Redman and Sargent, 2009; Caniggia et al., 1999).

The insufficient invasion of the maternal spiral arteries by extra villas cytotrophoblastic cells can lead to placental endothelial dysfunction in PE (Young et al., 2010). A number of factors have been found to play a pathogenic role in PE including imbalances of anti-angiogenic factors, oxidative stress, cytokines involved in hypoxic placenta response and inflammation, changes in genes such as the STOX1 gene (Haram et al., 2014), reduced heme oxygenase-1 (HO-1) and its gaseous product, carbon monoxide (CO). The pathophysiological mechanisms of PE are highlighted in Figure 1.3.

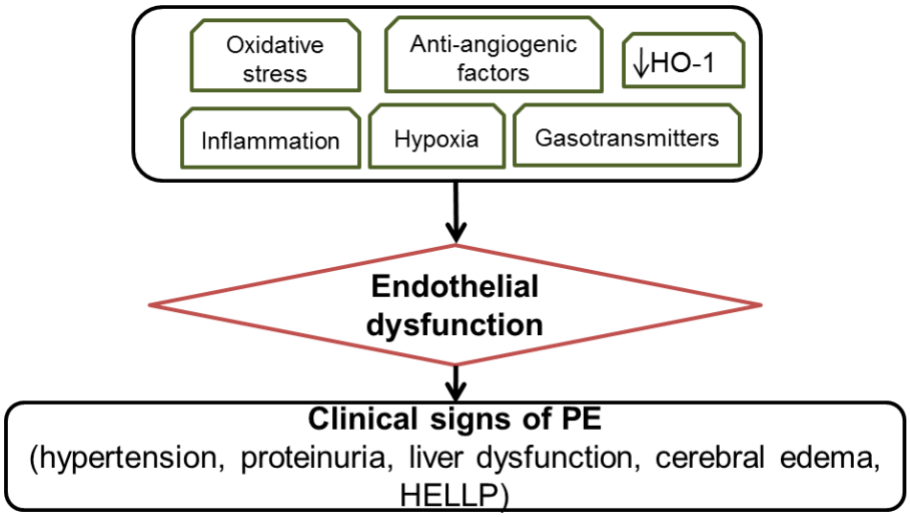

Figure 1.3 Pathophysiology of preeclampsia. Various factors have been found to contribute to the pathophysiology of PE. All factors contribute to the endothelial dysfunction seen in PE, leading to its clinical presentation.

1.3.1 Placental changes associated with preeclampsia

In PE, remodeling of the maternal spiral arterial walls is incomplete. The extra villas cytotrophoblast invasion of the spiral arteries is limited to the superficial decidua and the myometrial segments remain narrow and undilated (Meekins et al., 1994). The cytotrophoblasts fail to adopt an endothelial cell surface adhesion phenotype and to down-regulate the epithelial type adhesion molecules (Zhou et al., 2002), and hence inadequately invade the maternal spiral arteries (Figure 1.4). In addition to shallow invasion of the decidua (Kadyrov et al., 2003; Khong et al., 1986) there is increased apoptosis of extravillous trophoblast cells (Difederico et al., 1999; Genbacev et al., 1999), which is associated with reduced transformation of the spiral arteries (Sheppard, 1981). Spiral arteries remain convoluted with a small lumenal diameter, decreasing blood flow to the placenta and/or increasing turbulence. These two effects could result in increased hypoxia and increased shear stress respectively, both potentially resulting in irreparable damage to the placenta. Furthermore, the reduction in perfusion in a preeclamptic placenta is associated with maternal endothelial cell dysfunction (Ducat et al., 2016; Scioscia et al., 2015).

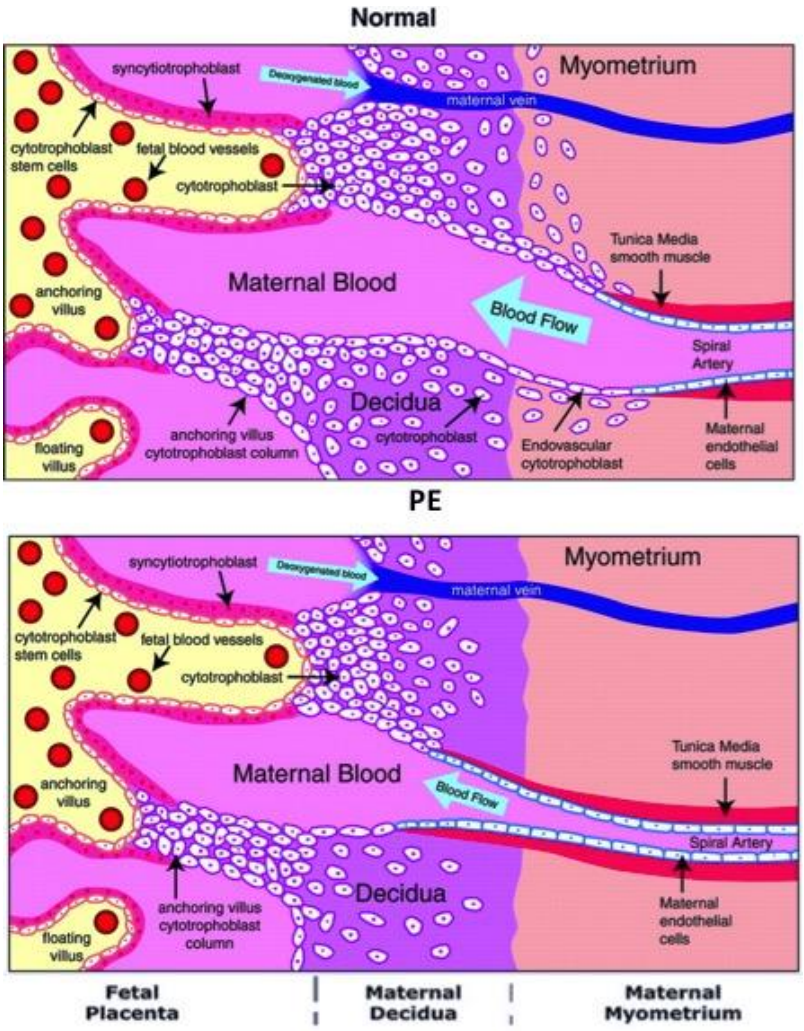

Figure 1.4 Vascular characterization in normal and abnormal placentation. During the process of vascular invasion, the cytotrophoblasts penetrate the arterial walls, replacing the maternal endothelium. The spiral arteries become dilated and lose smooth muscle, leading to the appearance of large vessel cytotrophoblasts (purple). In normal placental development, spiral arterioles transforming them from small-caliber resistance vessels to high-caliber capacitance vessels capable of providing placental perfusion adequate to sustain the growing fetus. In PE, cytotrophoblasts fail to adopt an invasive endothelial phenotype. Instead, invasion of the spiral arterioles are narrow, have high resistance and low blood flow (Adapted from (Lam et al., 2005)).

1.3.2 Endothelial cell dysfunction and preeclampsia

1.3.2.1 Endothelium

The role of the endothelium in regulation of local and basal control of vessel tone was first described in 1980 by Furchgott and Zawadzki. The vascular endothelium is a metabolically active organ weighing approximately 1 kg with an estimated total surface area of ~350 m^2 in humans (Pries et al., 2000). The endothelium lines the walls of blood vessels and is a continuous single-layer of cells. In large vessels, such as arteries and veins, the endothelium forms the intima. The endothelium in large vessels is surrounded by media, which contains smooth muscle cells, and an outer layer, the adventitia. In contrast, capillaries contain only the endothelium with no muscular or adventitial layers (Wilson and Hunt, 2002).

The endothelium of capillaries can be continuous, but can also be discontinuous or fenestrated, depending upon the requirements of the tissue. Typically, fenestrated endothelium is found in organs that are involved in filtration or secretion- examples include the kidney glomerulus and intestine/endocrine glands (Aird, 2003). The endothelium acts as an anatomical barrier, which prevents influx of circulating blood inside the vessel wall, controls vascular tone through secretion of vasoactive factors and regulates local cellular growth, as well as the deposition of the

extracellular matrix. Indeed, the endothelium protects the vessel from the harmful influence of toxic substances and cells that circulate in the blood by mediating hemostatic, inflammatory, and reparative responses to local injury. Additionally, the endothelium controls leukocyte trafficking from blood to tissues during an inflammatory response, and maintains the balance between the pro- and anti-coagulation systems in blood flow (Pries et al., 2000). Endothelial dysfunction contributes directly to the morbidity and mortality of sepsis and other severe systemic infections.

1.3.2.2 Endothelial cell activation

Endothelial cell excessive or over-sustained activation and injury is recognized as playing an important role in both drug-induced and inflammatory mediated injury to the endothelium. Both endothelial cell activation and endothelial cell damage are two dissimilar phenomena, yet the two are likely to overlap through the activation process (Pober and Cotran, 1990). Endothelial cell activation is a reversible process, which involves alteration of the morphological structure of the endothelial cells (increases in cell size and cytoplasmic organelles), without loss of endothelial cell integrity. Endothelial cells can be activated by cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) and withdrawal of such cytokines restablishes the dormant, non-activated state of endothelial cells (Blann, 2000; Brenner et al., 1989; Pober, 1988). Uncontrolled endothelial cell activation can lead to endothelial apoptosis and injury, which causes endothelial fragmentation and detachment of

endothelial cells from the intima (Bach et al., 1997).

The terms “endothelial cell activation,” “endothelial cell injury/endothelial cell damage,” and “endothelial cell dysfunction” are not interchangeable and should be used with a clear definition of each (Blann, 2000). “Endothelial cell activation” is distinct from sub-lethal injury with consequent endothelial cell dysfunction. Endothelial cell activation may also lead to endothelial cell dysfunction without evidence of vascular injury, as seen in the vascular leak syndrome induced by interleukin (IL)-2 (Pober, 1988).

The process of endothelial cell activation resulting in endothelial cell dysfunction and endothelial cell injury involves a series of immediate and delayed events. Immediate immunological activation of the endothelial cells (type I endothelial cell activation) involves the release of stored proteins independent of protein synthesis. This is followed by an early delayed activation (type II endothelial cell activation) that involves *de novo* protein synthesis, which also results in protein secretion. Endothelial cell dysfunction resulting from uncontrolled and/or persistent endothelial cell activation can result in critical levels of endothelial adhesion molecules, pro-coagulant molecules, vasodilators, cytokines, chemokines, and endothelial cell necrosis (Zhang et al., 2010).

The expression of adhesion molecules on the surface of the endothelial cell, as well as soluble factors released by the endothelium are good markers of endothelial cell activation. Adhesion molecules play an

important role in recruiting leukocytes in many types of vascular injury. There are numerous endothelial cell adhesion molecules, belonging to the immunoglobulin (Ig) superfamily, which facilitate interaction between the endothelial cell and leukocytes. One example is intercellular adhesion molecule (ICAM). The ICAM family is divided into ICAM-1, ICAM-2, and recently, ICAM-3, a third ICAM family member. Other adhesion molecules include vascular cell adhesion molecule-1 (VCAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). A recent study found that ICAM-1 and VCAM-1 are key adhesion molecules in identifying endothelial activation (Farzadnia et al., 2013).

Additional biomarkers that are commonly used for endothelial cell activation include E-selectin, P-selectin and endothelin-1. Amongst these biomarkers only E-selectin, von Willebrand factor (vWF), MAdCAM-1, Asymmetric dimethylarginine (ADMA), and circulating endothelial cells (CECs) that are detached from the endothelium, are considered to be endothelial-specific markers of activated endothelial cells (Lintermans et al., 2014; Zhang et al., 2010).

Other markers, although reliable and sensitive for vascular inflammation and injury, are not endothelial-specific and can come from multiple types of activated cells, such as neutrophils, platelets, mast cells, macrophages, antigen presenting cells or T lymphocytes (Lintermans et al., 2014; Galkina and Ley, 2009; Juhn et al., 2008). Activation of neutrophils and endothelial cells is an early critical event in drug-induced

vascular injury and in major pathologies (Mikaelian et al., 2014; Louden et al., 2006). Increased expression of ICAM-1 has been reported in vasculitic lesions involving nerve and muscle (Panegyres et al., 1992). The levels of soluble ICAM-1 have been shown to be higher in individuals who develop atherosclerosis, thereby suggesting that soluble ICAM-1 may serve as a biomarker for such vascular lesions (Lu et al., 2010). In addition, E-selectin, expressed on endothelial cells, binds to the carbohydrate ligands on leukocytes to aid in diapedesis. In patients with diffuse vasculitis, skin vessels that have increased expression of both E-selectin and ICAM-1 can be related to an inflammatory response (Johnson et al., 2006).

1.3.2.3 Endothelial cell dysfunction

Endothelial cell dysfunction has been referred to as the disruption of any of the processes that are required to maintain healthy endothelial cells or all their functions (Schwartz et al., 2009), and it can result in a variety of pathophysiological complications of vascular relaxation (vasodilation). Several studies have shown that endothelial dysfunction is provoked by chronic systemic infection, obesity, decreased prostaglandin and elevated C-reactive protein (Bivalacqua et al., 2003; Prasad et al., 2002; Thogersen et al., 1998).

Endothelial cell dysfunction can arise due to the impairment of the nitric oxide (NO) - cyclic guanosine monophosphate (cGMP) pathway ultimately leading to increased vasoconstriction of the vessels. Patients

with hypertension, diabetes, hypercholesterolemia, congestive heart failure or atherosclerosis have a deficiency of normal endothelium, hence the vasodilatory impact of acetylcholine is directly reduced or abolished due to endothelial damage (Schwartz et al., 2009). Endothelial cell dysfunction can occur when there is an imbalance between relaxing and contracting factors, such as NO and endothelin, or if there are imbalances between anticoagulant mediators and procoagulants, or between growth-inhibiting and growth-promoting substances (De Meyer et al., 1997). Studies *in vitro* have reported that soluble fms-like tyrosine kinase-1 (sFlt-1), in concert with the pro-inflammatory cytokine TNF-α, causes endothelial dysfunction, but sFlt-1 does not induce endothelial dysfunction alone *in vivo* (Cindrova-Davies et al., 2011; Bergmann et al., 2010).

1.3.2.4 Endothelial cell dysfunction in preeclampsia

Preeclamptic women show widespread endothelilal cell dysfunction, which is the cause of the hypertensive phenotype and clinical characteristics of PE (Gathiram and Moodley, 2016; Gilbert et al., 2008). Indeed, there are numerous reports that markers of endothelial cell activation are increased in the plasma or serum of preeclamptic women. It has futher been reported that CECs (Echeverri et al., 2015; Tuzcu et al., 2015; Canbaken et al., 2007) and other markers associated with endothelil cell damage, such as soluble ICAM-1, VCAM-1 (Farzadnia et al., 2013; Cross et al., 1994), and E-selectin are elevated in preeclamptic pregnancies compared to normal pregnancies (Austgulen et al., 1997).

In addition, vWF and endothelin are also elevated in preeclamptic women (Velxing-Arts et al., 2002; Clark et al., 1992). Furthermore, endothelial cell dysfunction in PE correlates with a reduction in NO production (Echeverri et al., 2015). Moreover, angiogenic imbalance is thought to induce endothelial dysfunction in PE by increasing the level of sFlt-1 in maternal plasma. sFlt-1 acts as an antagonist by binding to the vascular endothelial growth factor (VEGF) receptor, or by binding to the binding domains of placental growth factor (PLGF) and VEGF, thus preventing both PLGF and VEGF from interacting with the growth factor receptors on the cell surface (Gilbert et al., 2012; Levine et al., 2004). Being born small for gestational age with low birthweight may accelerate the endothelial dysfunction and development atherosclerosis (Visentin et al., 2014; Andraweera et al., 2012).

1.3.3 Angiogenesis and preeclampsia

1.3.3.1 Angiogenesis

The formation of new blood vessels occurs through two methods, namely, vasculogenesis and angiogenesis. Vasculogenesis is the formation of tube-like structures from endothelial precursor cells and occurs during early embryogenesis, whilst angiogenesis is the formation of new blood vessels from pre-existing vessels to make new connections.

There are two forms of angiogenesis: sprouting and

intussusceptive angiogenesis. Both occur in virtually all tissues and organs as well as *in utero* and in adults during the later stages of embryogenesis, during wound healing, menstruation, pregnancy adaptation underlying increasing blood flow in uterine, and most notably cancer (Adair and Montani, 2011; Flamme et al., 1997; Pardanaud et al., 1989). Whereas sprouting angiogenesis can generate new blood vessels, intussusceptive angiogenesis forms blood vessels by splitting of a pre-existing vessel (Adair and Montani, 2011). The endothelial cell lattice created by vasculogenesis then serves as a scaffold for angiogenesis (Papetti and Herman, 2001).

Insufficient angiogenesis occurs in diseases such as coronary artery disease, peripheral arterial disease, stroke, retinopathy of prematurity and delayed wound healing (Burton, 2009). Some studies indicate that dysregulation of angiogenesis is involved in the pathogenesis of PE (Venkatesha et al., 2006; Ahmad and Ahmed, 2004; Maynard et al., 2003). Over 20 endogenous proteins can stimulate angiogenesis (Cao et al., 1996) including growth factors, matrix metalloproteinases, cytokines, and integrins (Pardanaud et al., 1989, Ushio-Fukai, 2006).

During this process, the extracellular matrix surrounding the endothelial cells is degraded and the cell-cell interaction is disrupted, leading to the migration, proliferation and finally the creation of new vessels by endothelial cells (Ushio-Fukai, 2006).

Angiogenic factors are crucial for stimulating endothelial cell migration and proliferation. Important examples of these factors are VEGF, as well as PlGF, and their receptors, VEGFR-1/Flt-1 and VEGFR-2 (Flk-1/KDR). VEGF binds to both receptors, whereas PlGF is specific for VEGFR-1 (Widmer et al., 2007; Ahmad and Ahmed, 2004). Normal placentation involves angiogenesis within the placental villi of fetal origin, as well as on the maternal side (Verlohren et al., 2012).

1.3.3.2 Vascular Endothelial Growth Factor

VEGF was first described as a protein able to induce vascular permeability in tumours (Senger et al., 1983), and belongs to the platelet derived growth factor (PDGF)/VEGF family of growth factors which also includes PlGF (Maglione et al., 1991), VEGF-A, VEGF-B (Olofsson et al., 1996), VEGF-C (Joukov et al., 1996), VEGF-D (Achen et al., 1998) and VEGF-E (Ogawa et al., 1998). It is widely documented that VEGF is one of the most important growth factors for vasculogenesis and for the stimulation of endothelial proliferation and migration during angiogenesis. VEGF mediated cell proliferation and migration are mediated by VEGFR-2, whilst VEGFR-1/Flt-1 plays a role in endothelial cell differentiation and release of HO (Bussolati et al., 2004). A study by Ahmad et al. (2011) found that when endothelial cells were incubated with VEGF-A there was increased expression and secretion of sFlt-1 mRNA. Moreover, adenoviral over-expression of VEGF-A in mice led to 8-fold increase in circulating levels of sFlt-1) (Figure 1.5).

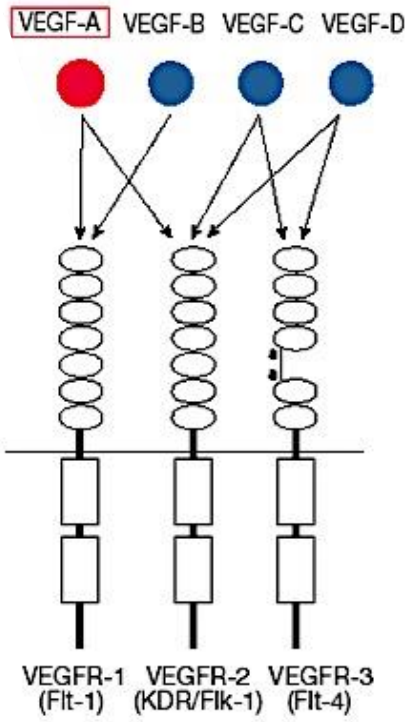

Figure 1.5 The VEGF and VEGFR system. All VEGF-A isoforms interact with VEGFR-1 and VEGFR-2; VEGF-B, PlGF-1 and -2 bind only VEGFR-1; VEGF-C and VEGF-D bind both VEGFR-2 and VEGFR-3 (Adapted from (Pandey et al., 2017) modified by me).

1.3.3.3 Placental Growth Factor

In 1991, PlGF was discovered in the human placenta (Ribatti, 2008; Maglione et al., 1991). PlGF is expressed in placenta, endometrium and trophoblast cells, and is involved in maturation of uterine natural killer (NK) cells (Nejabati et al., 2017). Human PlGF has 4 isoforms: PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$) and PlGF-4 ($PlGF_{224}$). PlGF-1 and PlGF-3 bind exclusively to VEGFR-1/Flt-1, which is highly expressed in vascular endothelial cells. Unlike VEGF, binding of PlGF to VEGFR-1/Flt-1 causes phosphorylation of alternative tyrosine residues and gene expression in endothelium (Autiero et al., 2003B). In mouse models, PlGF has been shown to have a major role in healing and

angiogenic and inflammatory cell recruitment (Maes, 2006). A recent study has shown that PlGF causes tissue damage, which could lead to early pregnancy loss through induction of inflammatory reactions, and may be accompanied by reduced angiogenesis (Nejabati et al., 2017).

Moreover, PlGF is not required for vascular development and its level is low in healthy adult tissues (Carmeliet et al., 2001). However, in normal endothelial cells, PlGF stimulates angiogenesis by amplifying the effect of VEGF, and it also promotes monocyte migration (Clauss et al., 1996), stimulates NO release (Bussolati et al., 2001) and prolongs survival and stability of capillary-networks (Cai et al., 2003). Furthermore, PlGF affects endothelial cells directly by binding to VEGFR-1/Flt-1 and inducing its own signaling, as well as amplifying VEGF-mediated angiogenesis (Carmeliet et al., 2001). Angiogenesis can occur due to the intermolecular crosstalk between VEGFR-1/Flt-1 and VEGFR-2 as a result of activation of VEGFR-1/Flt-1 by PlGF (Autiero et al., 2003A; Autiero et al., 2003B).

1.3.3.4 Fms-like tyrosine kinase-1

Flt-1, also known as VEGFR-1, was identified as a VEGF receptor on human endothelial cells. sFlt-1 is a splice variant of the Flt-1 receptor, which binds to the proangiogenic factors VEGF and PlGF (Shibuya, 2006). sFlt-1 contains the extracellular ligand-binding domain of VEGFR-1, but lacks the transmembrane and intracellular signalling domain (Claesson-Welsh, 2012; Luttun and Carmeliet., 2003). Circulating sFlt-1

acts as a high-affinity antagonist of VEGF- and PlGF-mediated biological activities by binding these ligands and by preventing ligand-receptor dimerisation with full- length VEGF receptors (Luttun and Carmeliet., 2003).

In pregnancy, sFlt-1 mRNA is highly expressed in villous and extravillous trophoblasts (Huang et al., 2013). The sFlt-1 protein is present in the supernatant from villous cultures, indicating that vascular growth in the placenta may be locally regulated by this soluble factor produced by the placenta (Clark et al., 1998A). The sFlt-1 protein may regulate the bioavailability of VEGF through heterodimerisation with VEGF receptors, which abolishes VEGF- mediated signal transduction (Pavlakovic et al., 2010).

1.3.3.5 Angiogenic factors in preeclampsia

Angiogenesis plays a key role in the pathogenesis of PE (Venkatesha et al., 2006; Maynard et al., 2003; Ahmad and Ahmed, 2004; Clark et al., 1998B) and plasma levels of angiogenic factors can be used as a marker of PE (Ushio-Fukai, 2006). Loss of VEGF activity (Ahmed, 1997) and elevation of sFlt-1 in the blood circulation in pregnant rats was shown to be associated with PE (Maynard et al., 2003). This led researchers to investigate angiogenic factors, such as sFlt-1 and PlGF as possible PE markers (Noori, 2011; Cudmore et al., 2007; Levine et al., 2006; Levine et al., 2004; Maynard et al., 2003). To be more precise, the imbalance in PE is described as an increase in sFlt-1 (Zhou et al., 2002; Ahmad and

Ahmed, 2004; Levine et al., 2006) and a decrease in PlGF (Levine et al., 2006) (Figure 1.6). In PE, placental expression of sFlt-1 is significantly elevated, leading to increased circulating sFlt-1 (Maynard et al., 2003). Numerous studies in rodents have attributed PE symptoms to be a direct consequence of increased sFlt-1 expression or circulating levels (Bytautiene et al., 2010; Costantine et al., 2010; Levine et al., 2004). A study showed that sFlt-1 could only induce hypertension and proteinuria in mice above a certain, critical threshold (Bergmann et al., 2010). Indeed, Bergmann et al. (2010) showed that reduction of sFlt- 1 below a certain threshold no longer induced preeclamptic symptoms. Moreover, Ahmad and Ahmed (2004) showed that sFlt-1 is responsible for inhibiting angiogenesis in PE.

This occurred by binding of sFlt-1 to PlGF and VEGF, which prevented endothelial stimulation. It has also been reported that PlGF is reduced in maternal circulation in women with PE (Wortelboer et al., 2010; Gaiser, 2005). PlGF levels were shown to be reduced *in vitro* tissue culture experiments from preeclamptic placental explants (Ramma and Ahmed, 2011). In addition, Wang and colleagues (2013) showed that reduction in the level of PlGF in PE was associated with loss of hydrogen sulfide (H_2S) and the H_2S producing enzyme cystathionine γ-lyase (Cth).

1.3.4 Inflammation and preeclampsia

Figure 1.6 Vascular dysfunction in preeclampsia. In PE there is an excess of sFlt-1 and a reduction in PlGF in the maternal circulation, which leads to vascular dysfunction (Adapted from (Ahmed et al., 1997) modified by me).

1.3.4.1 Inflammation

During acute and chronic inflammation, a variety of soluble factors are involved in recruiting leukocytes through the expression of cellular adhesion molecules and chemo-attractants. These soluble mediators modulate the activation of resident cells (such as fibroblasts, endothelial cells, tissue macrophages, and mast cells) and newly recruited

inflammatory cells (such as monocytes, lymphocytes, neutrophils, and eosinophils) (Feghali and Wright, 1997). Soluble mediators include inflammatory lipid metabolites such as platelet activating factor and derivatives of arachidonic acid (prostaglandins, leukotrienes, and lipoxins) which are generated from cellular phospholipids, the

endogeneous vasodilator NO, and cytokines which orchestrate the inflammatory response by regulating the state of cellular activation and the systemic responses to inflammation (Schleimer, 1988).

1.3.4.2 Inflammatory cytokine expression

Cytokines are central to the inflammatory process and are soluble, extracellular proteins that regulate immunological inflammatory responses, cell growth, differentiation, development and repair processes (Franzen et al., 2004). In the systemic circulation, TNF-α, IL-1β and IL-6 are important inducers of the acute defence responses to trauma and infection (Wang and Shuaib, 2002; Dinarello et al., 1986) (Figure 1.7). These cytokines are part of a large group termed the pro-inflammatory cytokines, which also includes IFN-γ, IL-12 and granulocyte macrophage colony stimulating factor (GM-CSF) (Flohe et al., 2008). Increased levels of pro-inflammatory cytokines can cause detrimental effects, since they can bind to common receptors on the surface of target cells and increase the expression of several downstream targets. TNF-α is a potent inducer of other pro-inflammatory cytokines, and increased levels of TNF-α further increases the levels of other inflammatory factors. One consequence of elevated levels of pro-inflammatory factors is higher production of NO (Leonard and Myint., 2006), which may induce cell death.

IFN-γ is a type II IFN that is essential for the control of viral infection, intracellular bacteria and tumor malignancies. IFN-γ is mainly produced

by NK cells, and NKT cells, and it directly promotes antiviral mechanisms by induction of antiviral enzymes. However, its main function is in immunomodulation.

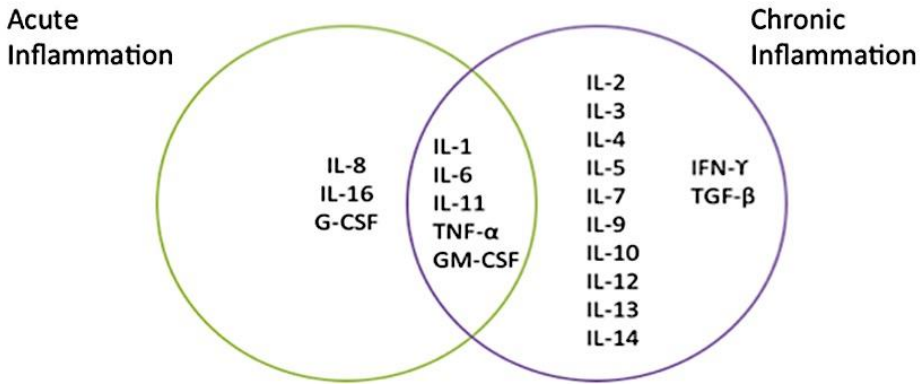

Figure 1.7 Summary of the cytokines involved during acute and chronic inflammation.

1.3.4.3 Chemokines and inflammation

Chemokines are small, chemo-attractant cytokines (8-12 kDa) that are secreted by immune and stromal cells. Chemokines are involved in regulating directional cell migration *in vivo* (Rot and von Andrian., 2004). Chemokines are characterized by a conserved cysteine, and can be divided into four subfamilies, C, CC, CXC and CX3C (Duchesnes et al., 2006; Murphy et al., 2002; Murphy, 1997). The ligands are named according to subclass followed by L for ligand and a number. Chemokines induce chemotaxis in responsive cells such as neutrophils, monocytes, lymphocytes, and eosinophils. Chemokines play a critical role in cellular recruitment, retention, and resolution of

inflammation (Speyer and Ward, 2011). As such chemokines and their receptors are considered to be therapeutic targets in chronic inflammatory disorders (Jones et al., 2010; Haringman, 2003).

Chemokines can be broadly divided into two categories: inflammatory chemokines, which recruit leukocytes in response to physiological stress and homeostatic chemokines, which are responsible for coordinating basal leukocyte trafficking, and also the formation of the architecture of secondary lymphoid organs. Their role is primarily to regulate cell trafficking of different leukocytes via interactions with seven transmembrane G protein coupled receptors (Bryant and Slade, 2015; Deshmane et al., 2009).

Two variations have also been identified: lymphotactin, which is specific for lymphocytes, and is missing a cysteine at its amnino terminus; and Fractalkine (Fkn), which is known to induce both adhesion and migration of leukocytes. Inflammatory chemokines are generally not expressed in resting tissues, but are expressed upon leucocyte and stromal cell activation in response to pro-inflammatory mediators such as TNF-α and IFN-γ (Sica et al., 2008; Herlaar and Brown, 1999). Although most inflammatory chemokines are regulated at the transcriptional level by pro-inflammatory mediators, some (such as CXCL1, CXCL4, CXCL8 and CCL5) are produced by platelets and stored as preformed proteins in α-granules which are then released upon platelet activation (Golledge, 2013; Nesmelova et al., 2008). There is a high degree of promiscuity in

the chemokine/receptor interactions, with most receptors capable of binding a range of chemokines and most chemokines interacting with more than one receptor. Inflammatory chemokine receptors bind only to one or two constitutive chemokines (Townson and Nibbs, 2002; Gosling et al., 2000).

1.3.4.4 Fractalkine

Fractalkine (Fkn), also known as neurotactin, is the only known member of the CX3C chemokine family. Fkn is a large protein of 373 amino acids that is expressed as a membrane-bound molecule. Fkn consistis of an extracellular N-terminal domain (residues 1–76), a mucin-like stalk (residues 77–317), a transmembrane α helix (residues 318–336), and a short cytoplasmic tail (residues 337–373).

The extracellular chemokine domain is attached to the cell surface via a mucin-like stalk, and the extracellular domain contains the novel cysteine arrangement, Cys XXX Cys, in which two cysteine molecules are separated by three other amino acids (Sasaki et al., 2014). The main source of Fkn is endothelial cells. Fkn specifically binds to Fkn receptor 1 (CX3CR1) or G-protein coupled receptor 13 and functions as an adhesion molecule, a chemotactic agent, and can mediate immune injury (Hoffmann et al., 2010; Koziolek et al., 2009). Fkn can be cleaved and shed from the cell surface through the activity of matrix metalloproteases such as ADAM (a-disintergin and metalloproteinase) 10 and 17 (Jones et al., 2010), which produces the soluble form of Fkn (sFkn) (Allen et al.,

2007; Shimoya et al., 2003; Bacon et al., 2002; Garton et al., 2001; Muehlhoefer et al., 2000; Papadopoulos et al., 1999; Harrison et al., 1998). sFkn is a chemoattractant for T cells, monocytes, and NK cells, and can be induced in primary endothelial cells by inflammatory cytokines such as IFN-γ and TNF-α (Zhang et al., 2001). sFkn can function as a pro-inflammatory agent that activates receptive inflammatory cells (Figure 1.8).

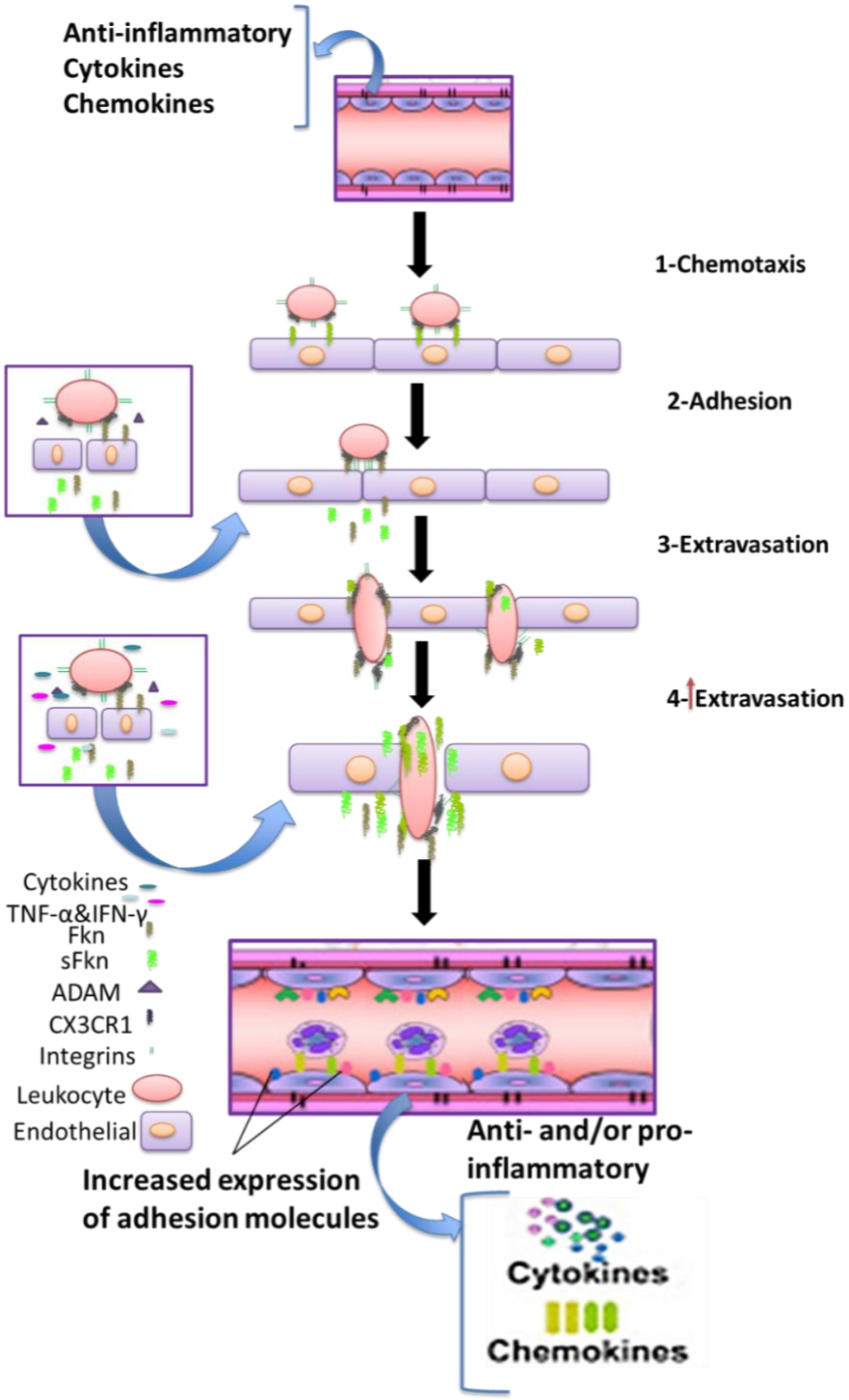

Figure 1.8 Mechanism of action of Fractalkine and soluble fraktalkine in endothelial cells. When Fkn is present in endothelial cells, the chemokine domain is presented at the top of the membrane bound mucin-like stalk, where it acts as an adhesion molecule. Immobilised Fkn exhibits rapid and high affinity binding to CX3CR1. Interaction among Fkn and CX3CR1 improves the integrin affinity, which results in firmer adhesion. The cleavage of membrane-bound Fkn by the metalloproteinase ADAM10/ADAM17 produces the soluble form. sFkn binds to CX3CR1, assisting leukocyte extravasation through the vascular wall into the tissue. Cytokines (TNF-α and IFN-γ) induce the expression of Fkn/sFkn.

1.3.4.5 Inflammation in preeclampsia

Generalised systemic inflammation is common to all pregnancies (Romero et al., 2007; Redman and Sargent, 2003). Redman and colleagues (1999) have proposed that PE arises as a result of an excessive maternal intravascular inflammatory response to pregnancy. They went on to specify that PE is not intrinsically different from normal pregnancy, but it is the extreme end of a continuous spectrum of inflammatory responses that are a feature of pregnancy itself (Redman and Sargent, 2010; Redman et al., 2005). Furthermore, it has also been proposed that as a consequence of poor placentation, reduction in uterine blood flow and increase in oxidative and endoplasmic reticulum stress, potent pro-inflammatory mediators may be the cause of PE (Redman and Sargent, 2010; Burton, 2009; Redman et al., 2005).

Studies have stipulated that the contribution of endothelial dysfunction in PE can be viewed in a larger context as part of the inflammatory network, which involves the release of pro-inflammatory cytokines, increases in chemokines adherence to the vascular endothelium and consequent vascular dysfunction (Sprague and Khalil, 2009; Zhang et al., 2007; Redman et al., 1999).

Fkn is expressed at the apical microvillous plasma membrane of the syncytiotrophoblast in human placenta, from where it is released into the maternal circulation by constitutive metalloprotease dependent shedding (Siwetz et al., 2014). According to previous studies on placental explants,

the release of placental Fkn significantly increases from the first trimester toward the term of pregnancy (Siwetz et al., 2014). The CX3CR1 signalling pathway induces angiogenesis through two sequential steps; the induction of VEGF and hypoxia inducible factor alpha (HIF-α) (Liu et al., 2011).

Secretion of inflammatory cytokines, especially TNF-α and Fkn, may act locally as a key angiogenic factors (Szukiewicz et al., 2013). Shimoya and colleagues (2003) found that in amniotic fluid Fkn may contribute to the immunodefence mechanism during pregnancy. TNF-α may up-regulate the expression and release of placental Fkn, which may contribute to PE (Siwetz et al., 2015). Also, Siwetz and colleagues (2015) reported that sFkn may also contribute to PE. Further studies are required on sFkn and CX3CR1 signalling in PE.

1.3.5 Heme oxygenases and preeclampsia

The HO enzyme was identified in 1968 by Tenhunen and colleagues. HO is a microsomal enzyme responsible for the rate-limiting breakdown of heme to produce equimolar amounts of CO, free iron (Fe) and biliverdin, which is converted to bilirubin by biliverdin reductase (BVR) (Figure 1.7) (Wang et al., 2016; Zhao et al., 2014; George and Granger, 2013; Willis et al., 1996).

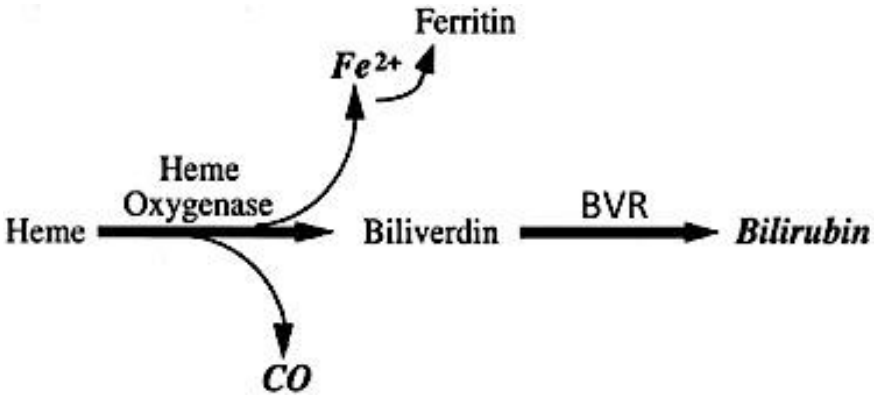

Figure 1.7 Reaction of enzymatic heme oxygenases. Iron, CO, and biliverdin are released during the reaction. Biliverdin is then converted to bilirubin by BVR, (Adapted from (Abraham and Kappas, 2008) modified by me).

There are three major isoforms of HO, which are products of different genes (Cruse and Maines, 1988). HO-1 is a 32 kDa protein, which is the inducible form. HO-1 is present at very low levels in most quiescent tissues. The spleen and the liver are the only exception, where HO-1 is highly expressed most likely due to its role in the recycling of erythrocyte and heme degradation (Braggins et al., 1986).

HO-2 is approximately 36 kDa, and shares similar substrate specificity with HO-1 (Trakshel et al., 1986). It is constitutively expressed in the brain, endothelium and testis, supporting a role for this enzyme in both the nervous and male reproductive systems (Bainbridge and Smith, 2005; Elbirt et al., 1999). However, HO-2 is present at lower levels in most tissues including the liver, kidney, spleen the cardiovascular system and the vasculature comprising the endothelial and smooth muscle cell lining of blood vessels (Maines, 1988). Both HO-2 and HO-1 share less than 50% homology in amino acid and nucleotide sequences with one

another (Rotenberg and Maines, 1990). HO-3 is a 33 kDa protein and is a poor catalyst of heme. The HO-3 transcript can be found in a variety of organs including the spleen, liver, thymus, prostate, heart, kidney, brain and testes (McCoubrey et al., 1997). Free heme is produced from hemoglobin upon lysis of red blood cells. Heme is a pro-oxidant and pro-inflammatory agent, which can be toxic to several cellular components including lipid bilayers, mitochondria, the cytoskeleton and the components of the nucleus (Maines, 1997). As a result, the elimination of heme from the body is essential to prevent excessive oxidative stress, inflammation and also to maintain cell survival (Elbirt et al., 1999). HO and its catalytic products have important roles in ischemia/reperfusion injury, inflammation, immune dysfunction, and the control of vascular tone and apoptosis (Bainbridge and Smith, 2005; Elbirt et al., 1999).

Bilirubin is an antioxidant and it can scavenge free radicals and prevent lipid peroxidation. CO also plays a role in the control of vascular tone by inhibiting the vasoconstrictors endothelin-1 (Morita et al., 1995). CO has vasodilatory effects as well as anti-apoptotic functions, and can protect cells under “stress” conditions (Li Volti et al., 2002; Agarwal and Nick, 2000; Morita et al., 1995). Brouard et al. (2000) reported that the anti-apoptotic effect of HO-1 was due to the production of CO in endothelial cells expressing HO-1, and that CO may also act as an intercellular signalling molecule to protect those endothelial cells which do not express HO-1.

1.3.5.1 Heme oxygenase-1 and inflammation

Induction of HO-1 is associated with reduced inflammation, whilst its inhibition is pro-inflammatory (Willoughby et al., 2000; Willis et al., 1996). The anti-inflammatory property of HO-1 is supported by the fact that the only reported case of a human who lacked the HO-1 enzyme died from an inflammatory condition (Yachie et al., 1999). Furthermore, HO-1 deficient mice exhibited increased expression of pro-inflammatory cytokines, including IL-1β, IFN-γ, TNF-α, and lL-6 and developed a chronic inflammatory state characterized by splenomegaly, lymphadenopathy, leukocytosis, as well as hepatic and renal inflammation that progressed with age (Poss and Tonegawa, 1997).

Studies have shown that the expression of HO-1 in inflammatory conditions, including pulmonary inflammation, cardiac ischemia and reperfusion injury and hypertension, is potently cytoprotective (Fujita et al, 2001; Yet et al., 2001; Otterbein et al., 1999). Furthermore, elevated levels of HO-1 mRNA and protein were observed in human atherosclerotic plaques (Wang et al., 1998), and in vascular endothelial and smooth muscle cells exposed to oxidised low-density lipoprotein (Ishikawa et al., 1997).

In addition, an increase in HO-1 gene expression has also been shown to inhibit vascular smooth muscle cell proliferation, hence preventing atherosclerotic lesions (Duckers et al., 2001). Many studies have suggested that CO is largely responsible for the anti-inflammatory nature

of HO-1 (Lee and Chau, 2002; Otterbein et al., 2000). CO is an efficient anti-inflammatory mediator and inhibits the production of pro-inflammatory cytokines TNF-α, IL-1β or IL-6 from lipopolysaccharide-stimulated macrophages (Sawle et al., 2005; Otterbein et al., 2000).

However, ferritin, biliverdin and bilirubin may also mediate this effect (Gray et al., 2002; Vile et al., 1994; Nakagami et al., 1993). For example, they inhibit nuclear factor-κB (NF-κB) by altering the expression of various adhesion molecules such as E-selectin, ICAM-1, and VCAM-1, in order to minimize its interaction with the vascular endothelium (Barreiro et al., 2002).

1.3.5.2 Heme oxygenase-1 in preecampsia

The correlation between HO-1 expression and placental development suggests that HO-1 might be essential for normal embryonic development (Watanabe et al., 2004). The deletion of the HO-1 gene, Hmox1, in mice has pathological consequences for pregnancy, namely suboptimal placentation followed by intrauterine growth restriction (IUGR) and fetal lethality (Zenclussen et al., 2011). Production of CO via heme catabolism by HO-1 positively influences placenta formation, which leads to proper oxygen and nutrient supply and results in adequate fetal growth. Maternal hypoxia during the early stages of placentation activates the invasive endovascular trophoblast cell lineage and promotes uterine vascularization, positively influencing placentation (Zenclussen et al., 2011). The increased expression of HO-1 in

development, the association of impaired growth with HO-1 disruption, and the effect of HO on placental vasodilation strongly suggest a role for HO-1 in fetal growth. In PE, HO-1 levels are reduced (Zenclussen et al., 2011). It has been found that women with PE have reduced HO-1 mRNA in the circulation (Nakamura et al., 2009). Furthermore, the placenta of women with PE was found to have decreased protein levels of HO-1 (Ahmed et al., 2000).

Endothelial cell dysfunction in PE has also been correlated with decreased HO expression, and HO deficiency has been shown to cause severe endothelial damage as indicated by increased levels of vWF (Yachie et al., 1999). Furthermore, placental HO-1 is believed to be a negative regulator of sFlt-1. Indeed, adenoviral overexpression of HO-1 led to a reduction in the release of sFlt-1 from endothelial cells, whilst small interfering RNA (siRNA) mediated knockdown of HO-1 led to increased sFlt-1 release (Cudmore et al., 2007). As such, HO-1 has been identified and proposed as an opportunity for therapeutic Intervention in PE.

1.3.6 Genetic factors and preeclampsia

Researchers have started analysing the genes involved in the risk of developing PE. Certain genetic polymorphisms have been found to contribute to PE. These include genes implicated in cytokines and angiogenesis (Chelbi and Vaiman, 2008). For instance polymorphisms in VEGF, specifically VEGF-A, have been associated with an increased risk

of PE (Verlohren et al., 2012; Zhou et al., 2002).

In addition, one study looked at TNF-α-238 polymorphism and found there to be an increased frequency of the AA genotype in women with PE compared to normal pregnancy, which suggested that the AA genotype could increase the risk of PE (Naderi et al., 2014). Furthermore, IFN-γ and lL-6 gene polymorphisms have been shown to influence the expression of these genes in PE compared to normal pregnancy (Pinheiro et al., 2015).

This thesis will investigate the relative contribution of Noggin and leucine rich repeats immunoglobulin-like domains 1 (Lrig1) to PE. Gene expression profiling of placental tissue from women with and without PE using Affymetrix U95A microarray chips found Noggin and Lrig1 mRNA (GenBank accession number U01134) to be up-regulated in preeclamptic placenta (data not shown). Noggin is involved in regulation of vasculogenesis and angiogenesis (Nimmagadda et al., 2005) and is not widely detected in adult tissues. Lrig1 plays an important role in morphogenesis.

1.4. Honokiol

Honokiol, an active component of the herb ‘Houpo’ a biphenolic compound present in the cones, bark, and leaves of the Magnolia tree, has been used in traditional Chinese medicine to treat a number of vascular diseases (Chang et al., 2013; Zhang et al., 2008; Chen et al.,

2007). Honokiol is a small molecule with the molecular formula $C_{18}H_{18}O_2$.

Honokiol has multiple biological activities including anti-inflammatory (Liou et al., 2003), anti-oxidative (Zhao and Liu, 2011), anti-angiogenic (Bai et al., 2003), anti-arrhythmic (Tsai et al., 1999), anti-thrombocytic (HU et al., 2005), anti-tumour (Yang et al., 2002), anxiolytic (Kuribara et al., 1999), anti-microbial (Park et al., 2004), and anti-fungal (Ho et al., 2001) activities.

Furthermore, Honokiol has been shown to have pro-apoptotic effects in a number of cancer cell lines (Singh and Katiyar, 2013). Honokiol was shown to provide therapeutic benefit not only through its effects on tumour cells and angiogenesis, but also through inhibiting lymphangiogenesis and metastasis via the VEGFR-3 pathway (Wen et al., 2015). Additionally, Chen et al. (2014) found that Honokiol may be a potential therapeutic choice in the treatment of osteoarthritis patients. Honokiol is also promising for the prevention and treatment of chronic asthma (Lin et al., 2012).

Pretreatment with Honokiol has been shown to block TNF-α-induced protein expression of matrix metalloproteinases (Zhu et al., 2014). In 2004, Park and colleagues suggested that there might be a way to inhibit the mechanisms of pro-inflammatory cytokines. Honokiol has been reported to down-regulate TNF-α and IFN-γ expression through VEGFR-2 (Wen et al., 2015). Research carried out on the effects of Honokiol on endothelial cells has shown that Honokiol inhibits vascular vessel

formation of stem cell-derived endothelial cells (Kim et al., 2012). However, no previous studies have been carried out regarding the potential role of Honokiol inhibiting Fkn.

Since endothelial dysfunction is associated with PE, we chose to investigate whether it was possible to reduce sFkn release from Human Umbilical Vein Endothelial Cells (HUVEC). We explored the effect of Honokiol on sFkn, as Honokiol is a natural anti-inflammatory product and anti-inflammatory therapies have been proposed to play a protective role in PE (Ramma and Ahmed, 2015; Afzal, 2011).

CHAPTER TWO: HYPOTHESIS AND AIMS

2.1 Hypothesis and aims

This thesis tested the hypothesis that sFkn release is up-regulated in PE through pro-inflammatory cytokines, which induce release of sFkn in endothelial cells, and that over-expression of HO-1 reduces sFkn release. Furthermore, we hypothesised that variation in the gene and protein expression of Noggin and Lrig1 in human placenta is associated with normal and abnormal placentation. The aims of this thesis were to:

(I) To determine whether TNF-α and IFN-γ stimulate the release of sFkn in cultured endothelial cells and whether Honoikol can reduce sFkn release. As well as, investigate the levels of sFkn in women with normal pregnancy, HELLP syndrome and women with PE.

(II) To assess the release of sFkn in cell cultured lysate explants from heart and lung tissue from Hmox1 homozygous knockout mouse models.

(III) Determine the presence and localisation of immunoreactive Noggin and Lrig1 in human placenta from normal and PE pregnancy, and to investigate the effects of Noggin and Lrig1 on angiogenesis in endothelial cells.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Materials and methods

3.1.1 Reagents

Cell culture and tissue culture media were obtained from Sigma Chemical Co Ltd (Poole, UK). The ELISA kits were procured from R&D systems (Abington, UK) and the protein assay reagents from Bio-Rad (Hercules, CA). Distilled water and Dulbecco's phosphate-buffered saline (PBS) were autoclaved before use. Sterile tissue culture treated plastics were purchased (Sigma-Aldrich) and glassware was washed and autoclaved before use. Media was warmed to 37°C before use and was stored at -4°C. All media was used within 3 months. The sources of all purchased materials and gifts are outlined in Appendix I. Apparatus and equipment utilised during the studies are detailed in Appendix II together with corresponding suppliers. Formulations of all solutions and buffers are listed in Appendix III.

3.1.2 Human Umbilical Vascular Endothelial Cells

The fetus connects to the mother by the umbilical cord, which contains two arteries and one vein (Li et al., 2014A; Zhao et al., 2011). Human umbilical cords were obtained from term delivery of healthy pregnant women. HUVECs were isolated with ethical permission from Edinburgh's Queen Mary Hospital. The Maruyama (1963) protocol has been modified to pioneer a fresh approach to the isolation of HUVECs from umbilical cords. HUVECs were isolated from the veins of umbilical cords through incubation with collagenase 24 hours after birth. Detached endothelial

cells were collected by flushing the collagenase solution through the umbilical vein with Earle's salts and supplemented with Medium 199 (M199) (containing 2.5 ng/ml basic Fibroblast Growth Factor, 2 mM L-Glutamine, 100 U/ml penicillin, 20 ng/ml Epidermal Growth Factor, 10 mg/ml ml penicillin-streptomycin, and 20% fetal bovine serum (FBS)) into a sterile 50 ml tube. The suspension was centrifuged at 1200 rpm for 5 minutes, and the resulting cell pellet re-suspended in 10 ml M199 media supplemented with 20% FBS. Cells were seeded in 1% gelatin-coated tissue culture flasks, and allowed to attach overnight at 37°C in an incubator with an atmosphere of 95% air, 5% CO_2. The medium was replaced the following day to remove erythrocytes and the cells were grown to confluence.

3.1.3 Cell culture

Cell cultures were maintained in a humidified incubator with an atmosphere of 95% air, 5% CO_2 at 37°C. Cells were seeded in 75 cm^2 tissue culture flasks in 20% FBS; appropriate ratio is listed in table 3.1. Cells were cultured in endothelal basal medium (EBM) containing human epidermal growth factor (hEGF), hydrocortisone, bovine brain extract (BBE), FBS plus 2 mM L-glutamine and 0.1 µg/ml penicillin-streptomycin. The medium was changed every 2 to 3 days. Confluent cells were sub-cultured by aspiration of the medium from the flask, and the cells were washed two times with 5 ml of PBS to remove the serum. Cells were detached by incubation with 2.5 ml of 0.25% trypsin/ ImM ethylenediaminetetraacetic acid (EDTA) for 2-5 minutes. Where

preservation of extracellular proteins was required, cells were incubated for 5-10 minutes with 5 ml 0.2% EDTA. After cells were detached, 9 ml of the respective growth medium was added to inactivate the digestion of the extracellular matrix.

The cell suspension was transferred to a sterile 15 ml tube, and the cells were centrifuged at 1200 rpm for 5 minutes to pellet the cells. The resultant supernatant was aspirated completely before the cell pellet was resuspended in 1 ml of the respective growth medium. Finally, the cells were either split between new culture flasks, or counted by haemocytometer in preparation for experiments requiring a certain number of cells per well prior to seeding in T25 cm^2 flasks, or tissue culture plates with 10% culture medium for 24 hours. The cells were then seeded onto gelatin coated culture plates in the following manner:

Table 3.1 Cell density according to different plates.

Tissue Culture Plate Type (No. Wells/Plate)	Seeding density (No) Cells/Well)	Volume/Well (ml)
6	250,000	1
12	150,000	0.5
24	100,000	0.4
96	10,000	0.1

3.1.4 Cell cryopreservation

A reserve stock of each cell type was maintained through cryopreservation. Confluent cells were trypsinised and pelleted before resuspension in 1 ml of freshly made precooled culture medium

containing 10% dimethyl sulfoxide (DMSO) and 10% FBS. The cell suspension was then transferred to sterile cryovials labelled with cell type, passage number, and date of storage and name of the owner. The cryovials were cooled in a freezing container (Sigma-Aldrich) at a rate of -1°C per minutes and placed in a -80°C freezer. When cryopreserved cells were required, cells were thawed at 37°C in a water bath for 10 minutes and immediately transferred to tissue culture flasks containing the respective serum-containing growth medium. Cells were incubated for 16 hours to allow cells to adhere, followed by two washes with PBS to remove all traces of DMSO. Fresh growth medium was then added.

3.1.5 Effect of stimulators and inhibitors on cells

Several agents were utilised in the treatment of HUVECs during the study. Table 3.2 shows working concentrations of all agents used. HUVECs were incubated with 1, 10, and 100 of ng/ml TNF-α or IFN-γ at 37^{o}C for 24 hours. Additionally, HUVECs were incubated with 10 ng/ml TNF-α and IFN-γ at 37^{o}C for 0, 25, 75, and 100 hours. HUVECs were also incubated with 10 ng/ml IL-1β at 37^{o}C for 24 hours.

HUVECs were incubated with different concentrations of PlGF, which was used at 10 ng/ml and 50 ng/ml; VEGF-A, which was used at 10 ng/ml and 50 ng/ml, and VEGF-E, which was used at 10 ng/ml and 50 ng/ml, at 37^{o}C for 24 hours.

Where appropriate, confluent HUVECs were treated with 2 µg/ml and 20

µg/ml Honokiol. Honokiol was dissolved in DMSO for 24 hours and was then added to cell culture plates (Sigma-Aldrich). Furthermore, HUVECs were incubated at 37ºC with various inhibitors or stimulators. HUVECs were pre-incubated with 1 µg/ml actinomycin D and 5 µg/ml cycloheximide (Sigma-Aldrich). Chromium protoporphyrin (CrPP) (40 µM), tin-protoporphyrin (SnPP) (50 µg/ml) were dissolved in 0.2 N NaOH, adjusted to physiological pH 7.4 with 1 N HCl, (Sigma-Aldrich). In other experiments, HUVECs were pre-treated with 10 ng/ml bilirubin, 10 µM inactivated carbon monoxide depleted molecule (iCORM-2), and 5 µm/ml hemin for 1 hour at 37ºC. The preparation of hemin, SnPP, and bilirubin was performed in the dark room since they are sensitive to light. Aliquots were taken in dark brown tubes and frozen at -80°C. The cell supernatants were collected until required for the experiments regarding sFkn release.

HUVECs were used in this study because they are easy to grow and it has been shown previously that they do not express Noggin (Kang et al., 2009). Confluent HUVECs in 6 or 12 well plates were treated with altered concentrations of 5 µg/ml Noggin and 4 µg/ml of Bone morphogenetic protein 4 (BMP4). All treatments were prepared in bovine serum albumin (BSA). HUVECs were incubated for 24 hours with the treatment and the cells and supernatants were subsequently collected and stored at -80°C until required.

Table 3.2 Stimulators and inhibitors employed in the study.

Agent	Working Concentration
IL-1β	10 ng/ml
TNF-α	10 ng/ml
IFN-γ	10 ng/ml
PLGF	10, 50 ng/ml
VEGF-A	10, 50 ng/ml
VEGF-E	10, 50 ng/ml
Honokiol	2, 20 µg/ml
Actinomycin D	1 µg/ml
Cycloheximide	5 µg/ml
CrPP	40 µM
SnPP	50 µg/ml
Bilirubin	10 ng/ml
CORM	10 µM
iCORM-2	10 µM
Hemin	5 µm/ml
Noggin	5 µg/ml
BMP4	4 µg/ml

3.1.6 Clinical study

Gestational age refers to the length of pregnancy based on the last menstrual period and/or early ultrasound evaluations (less than 20 weeks of gestation) (Jehan et al., 2010; Kramer, 1988). Preterm is a premature labour in other words, before 37 weeks of pregnancy. According to the American College of Obstetricians and Gynecologists, PE occurs after 20 weeks of gestation and is characterised by a blood pressure of 160 mm Hg systolic or higher, or 110 mmHg diastolic or higher. Other

indications of PE are proteinuria of more than 300 mg in a 24 hour period (two separate urine samples collected at least 4 hours apart both with proteinuria more than 300 mg) (Preeclampsia., 2016, Harrington et al., 1996). High blood pressure can lead to IUGR or elements of HELLP syndrome.

Human placental tissue and blood samples were collected from women recruited from Hospital de Maternitat, Barcelona Spain, and umbilical vein samples were collected from patients at the Royal Infirmary of Edinburgh. Patients with diabetes, infections, kidney disease, congenital malformations and chromosomal anomalies (number and/or structure) were excluded. Human placental tissues were obtained from term pregnancies by elective caesarean section, and from first and second trimester terminated pregnancies. The patient consent form can be found in Appendix IV.

3.1.6.1 Tissue and blood samples preparation of placental homogenates

The villous tissue was dissected into small pieces of 2 mm. Twelve pieces of the tissue were incubated for 24 hours in phenol red-free Dulbecco's modified eagle medium (DMEM) containing 0.1% FBS supplemented with 5,000U per ml Penicillin/ 2 mM L-glutamine, and 5 mg/ml penicillin-streptomycin and 0.1% BSA and transferred to the laboratory on ice in preparation for explants or cell culture. The villous tissue pieces were separated from the medium (supernatant) by centrifugation at 13,000

rpm for 5 minutes and were stored at 4°C for 20 minutes, after which the supernatants containing extracted protein were transferred into clean 1.5 ml Eppendorf tubes and stored at -80°C until analysis. Tissue was collected and sent to the laboratory for processing. Paraffin blocks of tissue were also generated for histology. Blood samples were obtained after delivery. The collection tubes were stored at 4°C for 30 minutes, and the blood samples were centrifuged at 2000 rpm for 15 minutes at 4°C. The plasma was separated and stored at −70°C until analysis.

3.1.7 Mouse studies

The study protocols were consistent with good animal practices under approval from our local animal care and use committee. Animal experiments were approved by the local ethics committee of Aston University, according to the Home Office Animals (Scientific Procedures) Act 1986 (United Kingdom). Homozygous knockout (KO) Hmox1$^{-/-}$ mice were supplied by Anupam Agarwal (The University of Alabama at Birmingham, USA). Mice were rederived at Edinburugh University. Re-deriving and breeding was conducted in house. Briefly, *In vitro* fertilisation with sperm from Hmox1$^{-/-}$ mice and eggs from heterozygous (HET) Hmox1$^{+/-}$ mice was performed as described (Ahmad et al., 2006; Poss and Tonegawa., 1997; Hogan et al., 1986). Pseudopregnant (C57BL/6 × DBA/2) F1 or Swiss Webster females were used as two-cell embryo recipients (Poss and Tonegawa, 1997), rederived and bred by Hmox1$^{+/-}$ to Hmox1$^{+/-}$ mating in accordance with local regulations. Hmox1$^{+/-}$ mice were obtained by mating male chimeras

with C57BL/6 females, these Hmox1$^{+/-}$ animals were intercrossed to produce Hmox1$^{-/-}$ mice. Genotypes of mice were determined by real-time polymerase chain reaction (PCR) of ear punch tissue samples and tail biopsies. Mice were purchased from 5-6 weeks of age and weighed 20-25 g. Mice were provided food and water *ad libitum*. Three groups of C57BL/6 mice (two mice per group) wild-type (WT) (Hmox1$^{+/+}$) (control), HET (Hmox1$^{+/-}$) and KO (Hmox1$^{-/-}$) mice was used for the experiments. Mice were used after at least three days of acclimatisation. Once the mice had been sacrificed the chest cavity was cut to reveal organs, such as the heart and lungs, which were stored at -80°C for further studies. This was carried out by the lab technician.

The heart and lung were immediately flushed with 500 µl PBS, through the right ventricle, to remove blood and were kept on ice until all tissues were collected. Tissues were excised and cut into 1 mm^2 pieces. Five to eight pieces of heart and lung were kept in phenol red-free DMEM containing 5% FBS. These steps were repeated with all the samples before centrifuging at 1000 rpm for 5 minutes at 18°C. Tissues were equilibrated in 24 well plates for 24 hours. The medium was changed to fresh phenol red-free DMEM containing 5% FBS after 24 hours, and heart and lung explants were collected and stored at –80°C prior to assay for sFkn. Other cut pieces of the heart and lung were placed into a 24 well plate, at 6 to 8 pieces per well, and cultured in the absence or presence of TNF-α, IFN-γ (20 ng/ml) for up to 48 hours. sFkn levels were measured in conditioned media.

3.1.8 Quantitative real time polymerase chain reaction

Cells were harvested as follows: the media was aspirated from the flask and discarded. The cells were washed in PBS three times to remove surplus media. The cells were then trypsinised and incubated in a cell culture incubator. The trypsin was deactivated with media. Gentle agitation by pipetting achieved cell separation. Cells were transferred to a 50 ml falcon tube and centrifuged at 13000 rpm for 10 minutes. The supernatant was removed and the cells resuspended in buffer 200 µl buffer RLT with beta-mercaptoethanol (β-ME) (10 µl β-me per 1 µl buffer RLT), with gentle agitation to re-suspend the cells. Cells were then freeze/thawed on dry ice 3 times to rupture the cell wall and release cell contents. Samples were incubated on ice and centrifuged at 4ºC, 13000 rpm for 5 minutes. The supernatant was transferred to a new eppendorf tube and incubated on ice. The pellet was discarded.

Human tissue was cut on ice and transferred to a 1.5 ml screw cap tube with 0.5 g ceramic beads. 200 µl buffer RLT with β-ME was then added to each sample. Tissue was homoginized using Precellys®24 Homogenizer. Samples were incubated on ice and centrifuged at 4ºC, 12000 rpm for 10 minutes. The supernatant was transferred to a new eppendorf tube and incubated on ice. The pellet and beads were discarded.

RNA samples were prepared using the RNeasy Mini Kit (Qiagen). An

equal volume of ethanol was then added to each sample, and samples were mixed gently by pipetting. Up to 700 µl of this mixture was then applied to the spin column. The spin column was then centrifuged for 15 seconds at 10000 rpm. The flow-through was discarded and the remaining sample applied to the spin column. This was centrifuged for 15 seconds at 10000 rpm. 700 µl buffer RW1 was added to each spin column. The spin column was centrifuged at 10000 rpm for 15 seconds and the flow-through discarded. 500 µl buffer RPE was added to the spin column and then centrifuged at 10000 rpm for 2 minutes. Spin columns were placed in new collection tubes and centrifuged at full speed for 1 minute to remove excess liquid. 20 µl ribonuclease (RNase)/ deoxynucleotide (DNase) PCR grade water was added to each spin column and samples were further centrifuged at 10000 rpm for 1 minute to elute the RNA. The elutant was transferred to the spin column again and centrifuged at 10000 rpm for 1 minute to increase RNA yield. RNA concentration was measured using Nanodrop.

Quantitative real-time polymerase chain reaction (qPCR) was used to detect target sequences for Noggin and Lrig1. The Stratagene Affinity Script Multiple Temperature cDNA synthesis kits were used to reverse transcribe RNA to cDNA. 0.1 µg random primers were added to the reaction components alone with 1 µg of RNA sample made up to a total volume of 15.7 µl with diethyl pyrocarbonate (DEPC) treated H_2O. Thermal conditions were as follows: 65°C for 10 minutes, then the reaction was cooled at room temperature for 10 minutes. The following

reaction components were added: 2 µl 10x buffers, 0.8 µl dNTP mix, 0.5 µl RNase block and 1 µl reverse transcriptase (RT). The solution was mixed and incubated at 25°C for 10 minutes for primer extension, and then incubated at 42°C for 60 minutes for cDNA synthesis.

The reaction was terminated by incubation at 70°C for 15 minutes and the resulting cDNA stored at -20°C. Alongside each set of reactions, two control samples were also included, one no enzyme control without RT and one no template control without RNA. In addition, human reference total RNA (Stratagene, Cheshire, UK) was used in quadruplicates as a calibrator for the RT reaction.

The controls and calibrators were included in each subsequent PCR. Sample preparation and real-time PCR were performed as described previously (Cudmore et al., 2011). For each sample, 1 µg RNA in 10µl DEPC-treated water was prepared. This was made up to a total reaction volume of 25 µl in DNase- RNase free thin walled 0.2 ml tubes by the addition of 12.5 µl (2x SYBR) Green qPCR master mix (Brilliant, Stratagene, Cheshire, UK). ROX reference dye 0.375 µl 30 nM (Quantace, London, UK), 0.75 µl forward primer, 0.75 µl reverse primer, and 0.625 µl DEPC treated water. Samples were incubated at 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds for denaturation, primer annealing at 60°C for 1 minute and polymerisation at 72°C for 1.5 minutes. A melting point curve was performed to verify the specificity of amplicons. Each reaction was conducted in duplicate and

the two controls from the RT step included as well as a no template control. Copy number was calculated using a fold change prepared by serial dilution of placental cDNA, and was expressed relative to the mean copy number for four internal calibrators, which were included with each reverse transcriptase reaction. Primers specific for Noggin, Lrig 1, and β-actin are listed in table 3.3 below. The mean threshold cycle for Noggin and Lrig 1 were normalised to β-actin, which was used as a control for equal amounts of template cDNA in the reaction.

Table 3.3 Primer used for qPCR.

Primer	Forward primer	Reverse primer
Noggin	5′-CGAGAGAGTCAGTGGTTTCCATG-3′	5′-GGCATCCGAAATTACTCCAGG-3′
Lrig1	5′-CACATCAATGGAACCTGGGTATTTTGAC-3′	5′-GTTTCGGTTCAATTCGAGATGTTGCAGTT-3′
β-actin	5′-AGCCATGTACGTAGCCATCC-3′	5′-CTCTCAGCTGTGGTGGTGAA-3′

3.1.9 *In vitro* angiogenesis tube formation

Tube formation is a powerful tool for screening to discover whether a protein has anti or pro-angiogenic effect on cultured cells. Assessment of *in vitro* capillary formation used growth factor-reduced Matrigel (Becton Dickinson, Oxford, UK). The cells were cultured on plates coated with collagen (Zhao et al., 2011; Holland et al., 1996; Jaffe et al., 1973). Matrigel is a basement membrane matrix composed primarily of collagen

IV, laminin, entactin, and heparan sulfate proteoglycans (Emonard et al., 1990).

The Matrigel matrix was thawed, gently mixed to homogeneity using cooled pipettes, and diluted 1:1 with culture medium. Diluted Matrigel (350 µl) was placed into each well of a 24 well culture plate at 4°C and allowed to polymerize by incubation at 37°C for 30 minutes. After polymerization of the Matrigel suspension, 5 x104 cells were plated to each well in culture medium containing 10% FBS. After cells had attached to the Matrigel (2 hours at 37°C), the FBS-containing media was removed, and the agonist or the vehicle alone in media containing 0.2% BSA was added and incubated for 4 hours at 37°C. Wells that were pre-treated with antagonist were stimulated at 37°C for 30 minutes prior to stimulation with agonist.

The tubular network growth area was compared to control and effector treated Matrigel matrix. In parallel experiments, Trypan Blue exclusion showed that cell viability was >90%. Cells were observed with a Nikon inverted microscope and experimental results recorded with an Optima's image analysis software (Microscope Service, Surrey, UK). NIH image was used to measure the total tubule length by parameters such as tube length, and the number of loops formed on the gel.

3.2.10 Estimation of protein concentration

The amount of total protein present in cell and tissue lysate was

measured by a Bio-Rad protein assay to ensure equal amount of lysates protein loading per sample. Protein assay standards were prepared from a stock solution of 20 mg/ml BSA by diluting in distilled water to give a range of concentrations of 0.2–1.4 mg/ml. The sample proteins were diluted in a volume ratio of 1:5 in radioimmunoprecipitation assay (RIPA) buffer, and 5 µl of each sample, along with the BSA standards, were added in triplicates to a 96 well plate. The standard assay protocol was adopted, which is part of the Biorad Protein Assay kit. The absorbance was measured within 20 minutes using a Multiskan Ascent 96 well plate. The plates were read at 690 nm and results were obtained by plotting the protein BSA standard concentration against the optical density to give a straight-line graph. Each sample was aliquoted to 30 µg of total protein from tissue homogenate prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for Western blot analysis (WB).

3.2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

3.2.11.1 Gel preparation

Gels were prepared in advance and were dependent on the size of protein being assessed. Most commonly 10% acrylamide gels, for separation of bands between 40-120 kDa, were prepared (see appendix III. Addition of 10% ammonium persulphate (APS) and Tetramethylethylenediamine (TEMED) initiated polymerisation. Gels

were poured into the plate assembly, taking care to prevent the formation of air bubbles. The gel was then overlaid with 70% ethanol, to prevent gel shrinkage and formation of a meniscus, and allowed to polymerise. 5% acrylamide stacking gels were prepared as above, and were poured carefully onto the polymerised separating gel. Sample loading combs were inserted into the stacking gel and removed once the gel had set. The gels were then transferred to the running apparatus, and the upper and lower chambers of the running apparatus filled with an electrophoresis running buffer.

3.2.11.2 Loading of protein samples

50 µg of total cell protein samples were diluted to a final volume of 25 µl using 2x reducing or non-reducing sample buffer containing bromophenol blue as a tracking dye. Immunoprecipitates were routinely resuspended in 50 µl of 2x reducing sample buffer. The reducing buffer contained dithiothreitol (DTT) and 200 mM mercaptoethanol, which chemically reduces disulphide chains and allows separation of individual peptide chains, whilst the non-reducing buffer allows detection of full weight proteins. All samples were boiled for 5 minutes to reduce disulphide bonds or dissociate protein, and then cooled and spun for 1 minute at 5000 rpm prior to loading equal 25 µl volumes onto the SDS-PAGE gel against a pre-stained Kaleidoscope of molecular weight markers ranging from 17 to 208 kDa. Gels were electrophoresed at 50 or 60 volts until samples had passed through the 5% stacking gel for 30 minutes, and the

voltage was then increased to 120 volts for 90 minutes. Electrophoresis was stopped when the bromophenol blue tracking dye reached the bottom of the gel.

3.2.11.3 Western blotting protocol

Following protein electrophoresis, the semi-dry electrophoresis transfer cell was used to transfer proteins from acrylamide gels to nitrocellulose membranes (BioRad). Hybond Enhanced chemiluminescence (ECL) nitrocellulose membrane was cut to the dimensions of the gel, and moistened in transfer buffer for 2 minutes. In addition, two pieces of thick filter paper were cut to the dimensions of the gel and completely saturated in transfer buffer. The blot transfer cell unit was assembled for protein transfer to occur; a current was passed from the cathode to the anode that was proportional to 0.8 mA/cm^2 (the area of the nitrocellulose membrane in cm^2).

Following transfer of the protein samples onto the nitrocellulose membrane, non-specific binding sites were blocked by immersing the membrane in blocking solution composed of 5% skimmed milk and 0.1% BSA in tris-buffered saline-tween-20 (TBS-T). Membranes were blocked for 1 hour at room temperature on an orbital shaker. After blocking, membranes were washed in 0.1% TBS-T for 15 minutes at room temperature followed by two further 10 minutes washes in fresh TBS-T. The membrane was then incubated with a primary antibody. The dilution of primary antibody used in this study and sources are listed in table 3.4

below.

Prior to incubation with the secondary antibody, the membrane was washed with TBS-T buffer as mentioned previously. Secondary antibodies were made depending on the species in which primary antibody was raised; the dilution of secondary antibody used in this study and sources are listed in table 3.4 below. The membrane was incubated with the secondary antibody, which was diluted with 5% milk, for 1 hour at room temperature. The membrane was further washed in TBS-T. For the detection process, the membrane was transferred in the dark room using red safelights and drained of TBS-T. ECL detection solutions A and B (Biological Industries) was mixed at a 1:1 ratio, added to the membrane prior to application and incubated for 1 minute at room temperature. Thereafter, the detection mixture was drained off; the membrane was covered with Saran Wrap, and exposed protein side up to a sheet of autoradiography film (Kodak, XOMAT AR) in an autoradiographic film cassette. Various exposure times were used for each membrane to achieve the best results for each WB.

Table 3.4 Dilutions of primary and secondary antibodies used for WB.

Antibodies	Species raised in	Optimum Dilution	Source
β-actin	Mouse monoclonal	1:1000	Cell Signaling Tech, Herts, UK
Noggin	Rabbit polyclonal	1:2000	Abcam, UK

SFkn	Rabbit polyclonal	1:1000	Abcam, UK
HO-1	Rabbit polyclonal	1:5000	Abcam, UK
BVR	Rabbit polyclonal	1:5000	Abcam, UK
Peroxidase anti-mouse IgG	Mouse polyclonal	1:2000	Abcam, UK
Peroxidase anti- rabbit IgG	Goat polyclonal	1:5000	Abcam, UK

3.2.12 Enzyme-linked immunosorbent assay

The Enzyme-linked immunosorbent assay (ELISA) was first developed in the early 1970s as a replacement for radioimmunoassays (Robinson and Lau., 2012). ELISAs are quick and simple to carry out, and since they are designed to rapidly handle large numbers of samples in parallel. An ELISA was used to measure sFkn, release. This method is both cost and time-efficient. The protocol was modelled on the 'In-Cell ELISA Colorimetric Detection Kit' (Thermo fisher scientific). Cells were plated at a density of 1X104 /well in a 96 well culture plate. Antibodies used for ELISA shown in table 3.5 below. The primary antibody was monoclonal murine anti-human Fkn, and the secondary antibody was biotinylated polyclonal goat anti-Fkn.

Table 3.5 Antibodies used in ELISA.

Antibodies	Species raised in	Optimum Dilution	Source
SFkn	Monoclonal Mouse	1:2000	R&D systems, UK
Biotinylated	polyclonal goat	1:5000	Abcam, UK

3.2.12.1 Enzyme-linked immunosorbent assay protocol

Enzyme immunoassays were carried out using microwells strips (Corning, UK). Each well was coated by 100 μl of capture antibody in PBS (pH7.4), covered with a plastic film, and left in the dark at room temperature overnight at 4°C on a microplate shaker set at 100 rpm. The following day microwells strips were washed three times with PBS

containing 0.05% tween-20 (PBS-T), which was the washing buffer, and then blocked for 1 hour using 200 μl of 1 % BSA in PBS, which was the blocking buffer, on a microplate shaker. After three PBS-T washes to remove residual BSA, samples were added at 100 μl/well and microwells strips plate incubated with agitation for 2 hours at room temperature on a microplate shaker. After washing, 100 μl/well of the secondary antibody in PBS-T was added, and incubated with agitation for 2 hours at room temperature on a microplate shaker. After washing the secondary antibody solution, streptavidin-HRP (1:200) in PBS-T, was added for 20 minutes with agitation then washed off. The hydrogen peroxide colour substrate solution (R&D systems, UK) was added to the microwells strips (see manufacturer's instructions), incubated for 30 minutes until sufficient colour change was observed and the reaction stopped using 2 molars (2 M) Sulfuric acid (H_2SO_4).

Presence or absence of the protein of interest was determined by reading the optical density (OD) at 450 nm, with a correction of 540 nm (for wavelength correction) in a Multiscan Ascent 96 well of microwells strips plate reader, and subtracting the blank value (sample diluent only) from the sample absorbance values.

3.2.13 Immunohistochemistry

Immunohistochemistry (IHC) of placental tissue was performed on formalin fixed, paraffin wax embedded samples. Routine sections of 5 μm

thickness mounted onto Fisher scientific (U.K) glass slides and dried (carried out at the Queen Mary Hospital Pathology department, Edinburgh for wax block preparation and tissue sectioning). Slides were placed into a 250 ml bath of xylene before being transferred to absolute alcohol for no longer than 60 seconds to remove all embedding wax. Overexposure of the slides to xylene can reduce the antigenicity of proteins. The slides were then removed from xylene, rinsed by immersion in 100% ethanol and placed in a bath of gently running water. The slides were incubated in 3% hydrogen peroxide in methanol for 10 minutes at room temperature to block endogenous peroxidase. The slides were then rinsed in distilled water and placed in PBS for a further 5 minutes before being transferred to 3 L of boiling citrate buffer (pH 6.0) in a 15-lb pressure cooker. Slides were then rinsed in TBS (pH 7.4) and incubated with 5% milk in PBS for 1 hour at room temperature to block non-specific protein recognition, followed by one 5 minute wash with PBS.

Slides were incubated with the primary antibody, diluted optimally in 10% (v/v) non-immune goat serum in PBS, for 1 hour at room temperature or overnight at 4°C. Negative controls included incubation of the section with either non-immune serum, or by pre-absorption of the primary used for IHC shown in table 3.6 below. After incubation, the slides were rinsed in TBS and incubated with secondary antibody in 1:300 dilutions (Jackson immunoResearch, West Grove, PA) for 60 minutes. After incubation, the slides were rinsed in TBS followed by avidin biotin staining (Vectastain Elite ABC, Vector Laboratories, Burlingam, CA) for 35

minutes. They were then rinsed in TBS, and incubated with 3, 3-diaminobenzidine/nickel sulphate as chromogen solution for 10 minutes. The slides were then rinsed in tap water; the primary antibodies confirmed the specificity of staining. Specific staining was evaluated semi-quantitatively in a blinded fashion by examining six fields per slide, and subjectively scoring on a scale from 0 (no staining) to 5 (intense blue-black staining) the intensity of the chromogen deposited in the placental villous trophoblast, stromal and endothelial cells.

Table 3.6 Antibodies used for IHC.

Antibodies	Species raised in	Optimum dilution	Source
Noggin	Rabbit	1:300	Abcam, UK
Biotinylated Goat anti-Rabbit IgG (H+L)	Rabbit	1:1000	R&D systems, UK
LRIG1	Goat	1:200	Abcam, UK
Biotinylated Goat Anti-Rabbit IgG (H+L)	Goat	1:1000	R&D systems, UK
CD31	Goat	1:200	Abcam, UK
Cyt-7	Goat	1:100	Abcam, UK

For illustration purposes, sections were developed using Vector NovaRed (Vector) chromogen and were counterstained with hematoxylin. The staining was analysed using a Nikon inverted

microscope and an Image Pro Plus image analysis software (Media Cybernetics).

3.2.14 Statistical analysis

All data was analysed and graphs were generated with GraphPad Prism 5 (GraphPad Software Inc.), and the parametric one-way analysis of variance ANOVA was employed for ELISA. Tube formation formed in the collagen gel were identifiable under high magnification microscopy and photographed in 5 different random fields (×10). The total tube length (mm/mm^2) was quantified with NIH image analysis system, and one-way ANOVA was used to compare the difference in mean between samples compared to the control. One-way ANOVA was used to compare the difference in mean between qPCR results. Image Pro was used for densitometry analysis for WB to observe differences in band density between samples. Image J was used on the same film to calculate significant differences in expression levels. Slides for IHC were randomised and density was scored 1 to 5 depending on density. Least dense was scored 1 and most dense as 5. This was then passed blindly to another colleague to ensure that scoring was fair prior to sample identification. Data was presented as mean ± SEM (standard error of the mean). Statistical significance was classified where $*p<0.05$.

CHAPTER FOUR: SOLUBLE FRACTALKINE: A POTENTIAL NEW TARGET FOR PREECLAMPSIA

4.1 Inflammatory cytokines and soluble Fractalkine release in endothelial cells

4.1.1 Introduction

Pregnancy is associated with changes in cytokines and other inflammatory markers (Friis et al., 2013; Björkander et al., 2012; Coussons-Read et al., 2007; Teran et al., 2001). The amniotic fluid of pregnant women contains various cytokines, including IL-1, IFN-γ, TNF-α and IL-6 (Bowen et al., 2002; Heikkinen and Alanen., 2001; Baud et al., 1999; Simpson, 1999; Lauw et al., 1999). Studies have shown that pro-inflammatory cytokines, such as TNF-α and IFN-γ, are related to the severity of illness, and are associated with the activation of NF-κB (Soundravally et al., 2013; Bai et al., 2003). Additionally, these cytokines have been shown to cause harmful effects during pregnancy (Chaouat et al., 1990).

PIGF is considered as pro-inflammatory as it acts as a chemoattractant for monocytes and stimulates pro-inflammatory cytokine secretion (Chaballe et al., 2010). In pregnant rats, inhibition of PlGF and VEGF is required to produce PE-like symptoms (Maynard et al., 2003). It has been shown that PE may arise due to loss of VEGF activity, which occurs because of elevation of sFlt-1 (Ramma et al., 2012; Ahmad and ahmed, 2004; Ahmed et al., 1997). TNF-α is reported to stimulate sFlt1 release from placental explants (Cudmore et al., 2007). Studies in smooth muscle cells found that TNF-α and IFN-γ induce small amounts of sFkn (Ludwig et al., 2002). However, the up-regulation of sFkn in smooth muscle cells is time dependent, and only increases at 24 hours upon stimulation with TNF-α and IFN-γ (Ludwig et al., 2002). It is unclear whether sFkn release

is associated with a rise in circulating anti-angiogenic factors. The anti-inflammatory compound, Honokiol, has a variety of pharmacological activities including anti-tumor (Hu et al., 2008), anti-inflammatory (Matsuda et al., 2001) and anti-oxidative activity (Dikalov et al., 2008; Lo et al., 1994).

In this chapter, the relationship between pro-inflammatory cytokines and release of sFkn from the endothelial cells was assessed. We investigated whether a direct relationship could be demonstrated between anti-angiogenic factors and the release of sFkn. In addition, we tested the effect of Honokiol on sFkn release from endothelial cells in the presence and absence of IFN-γ.

4.1.2 Results

4.1.2.1 TNF-α and IFN-γ induce soluble Fractalkine release from endothelial cells

To ascertain whether the pro-inflammatory cytokines, TNF-α (10 ng/ml), IFN-γ (10 ng/ml) and IL-1β (10 ng/ml), could stimulate sFkn release from HUVECs over 24 hours, conditioned media was assayed by sandwich ELISA to measure the release of sFkn. sFkn is constitutively released from endothelial cells under normal growth conditions. The graph shows that TNF-α and IFN-γ increased the release of sFkn, compared to the controls, by 7-fold, respectively. However, IL-1β had no effect on sFkn release compared to the controls (Figure. 4.1). Our study has corroborated with the observations made by Isozaki et al. (2008) on sFkn release.

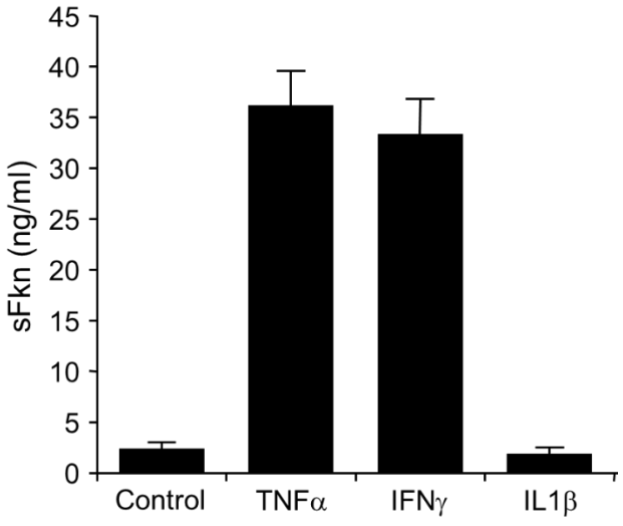

Figure 4.1 Effect of cytokines on soluble Fractalkine release in endothelial cells. Cytokines TNF-α (10 ng/ml), IFN-γ (10 ng/ml) and IL-1β (10 ng/ml) promote level of sFkn release from HUVECs following 24 hours treatment as measured by ELISA. Data is expressed as mean ± ± S.E.M of one experiment carried out in duplicate.

4.1.2.2 Concentration of TNF-α and IFN-γ on soluble Fractalkine release

To determine the concentration of TNF-α and IFN-γ required for sFkn release in endothelial cells, HUVECs were incubated with increasing concentrations (1, 10 and 100 ng/ml) of TNF-α and IFN-γ alone, or combinations of both were added under identical conditions over 24 hours. ELISA was performed on the conditioned media to determine the level of sFkn release. The result demonstrated that at the lowest concentration, 1 ng/ml of TNF-α and IFN-γ, a 5-fold increase in sFkn release was observed, whereas the combination of TNF-α and IFN-γ resulted in a 7-fold increase in sFkn release compared to unstimulated controls (Figure 4.2). TNF-α and IFN-γ effects were observed on sFkn release at a concentration of 10 ng/ml. In the presence of 10 ng/ml of both TNF-α and IFN-γ, sFkn release was increased by approximately 7-fold compared to unstimulated controls. The level of sFkn release was further elevated with 100 ng/ml concentrations of TNF-α and IFN-γ alone compared to unstimulated controls.

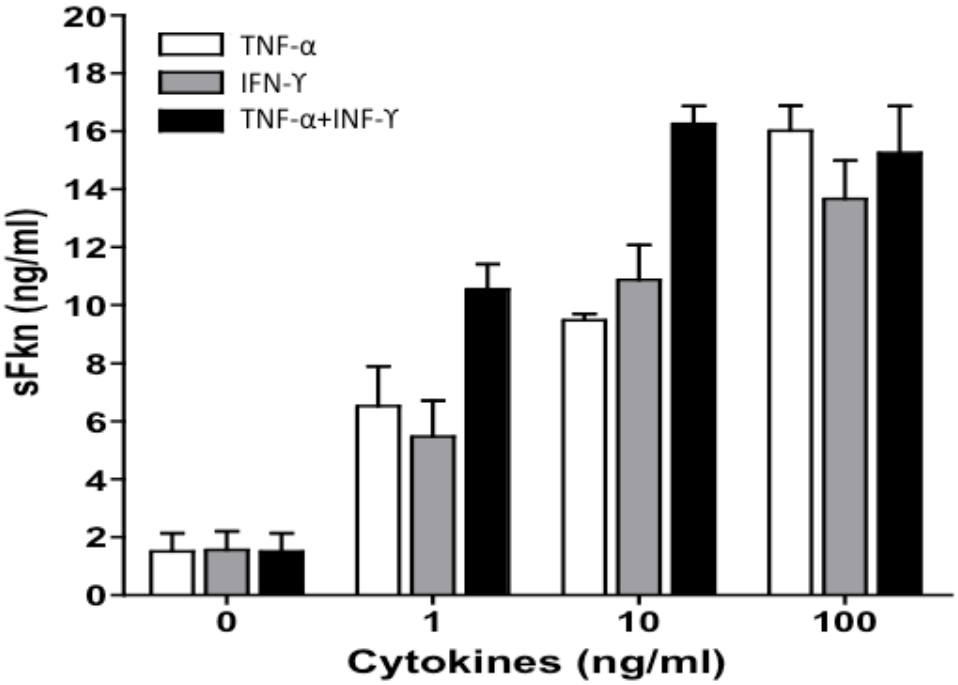

Figure 4.2 Concentrations of TNF- α and IFN-γ needed to stimulate soluble Fractalkine release from endothelial cells. Confluent HUVECs were incubated with increasing concentrations (1, 10, and 100 ng/ml) of TNF-α and IFN-γ alone or in combination, for 24 hours. ELISA was used to measure level of sFkn release in the cell culture supernatants. Data is expressed as mean ± S.E.M of one experiment carried out in duplicate.

4.1.2.3 Time-dependent TNF-α and IFN-γ mediated soluble Fractalkine release

In order to further characterise sFkn release, HUVECs were stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 0, 25, 75, and 100 hours. ELISA was used to detect the level of sFkn release. The graph shows that sFkn was up-regulated as early as 8 hours after TNF-α and IFN-γ stimulation. Also, sFkn release reached peak levels after 24 hours, and this threshold was maintained for at least 96 hours (Figure 4.3).

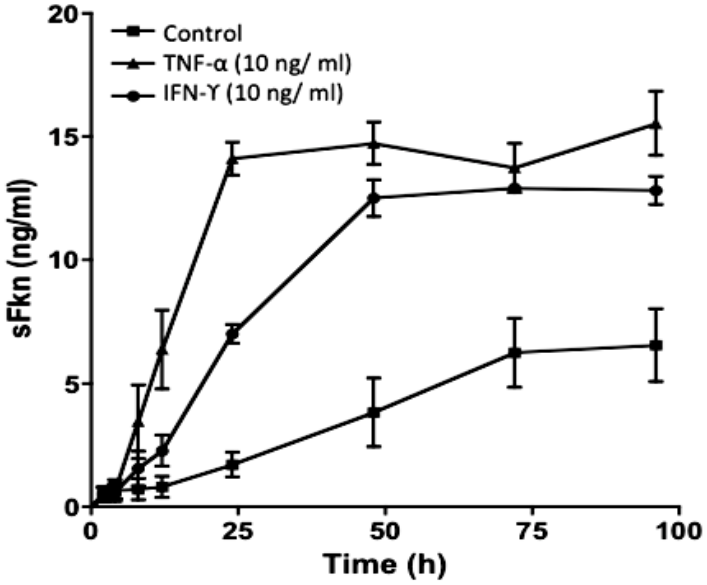

Figure 4.3 Time-dependent TNF-α and IFN-γ mediated soluble Fractalkine release in HUVECs. HUVECs were stimulated for different time periods and cell supernatant were collected from control unstimulated cells, TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) stimulated cells at each time point. Level of sFkn were measured from the cell culture supernatants using an ELISA. Data is expressed as mean ± S.E.M of one experiment carried out in duplicate.

4.1.2.4 Effect of angiogenic factors on release of soluble Fractalkine

PlGF is present in very low concentrations in quiescent endothelial cells. However, when endothelial cells are activated in pathological conditions, the level of PlGF is increased (Carmeliet et al., 2001). Therefore, we investigated whether angiogenic factors affect sFkn release in HUVECs. HUVECs were incubated with PlGF (10 ng/ml and 50 ng/ml), VEGF-A (10 ng/ml and 50 ng/ml), and VEGF-E (10 ng/ml, 50 ng/ml) for 24 hours. ELISA was used to detect the level of sFkn release. The results showed that sFkn release is not induce upon stimulation angiogenic growth factors (Figure 4.4).

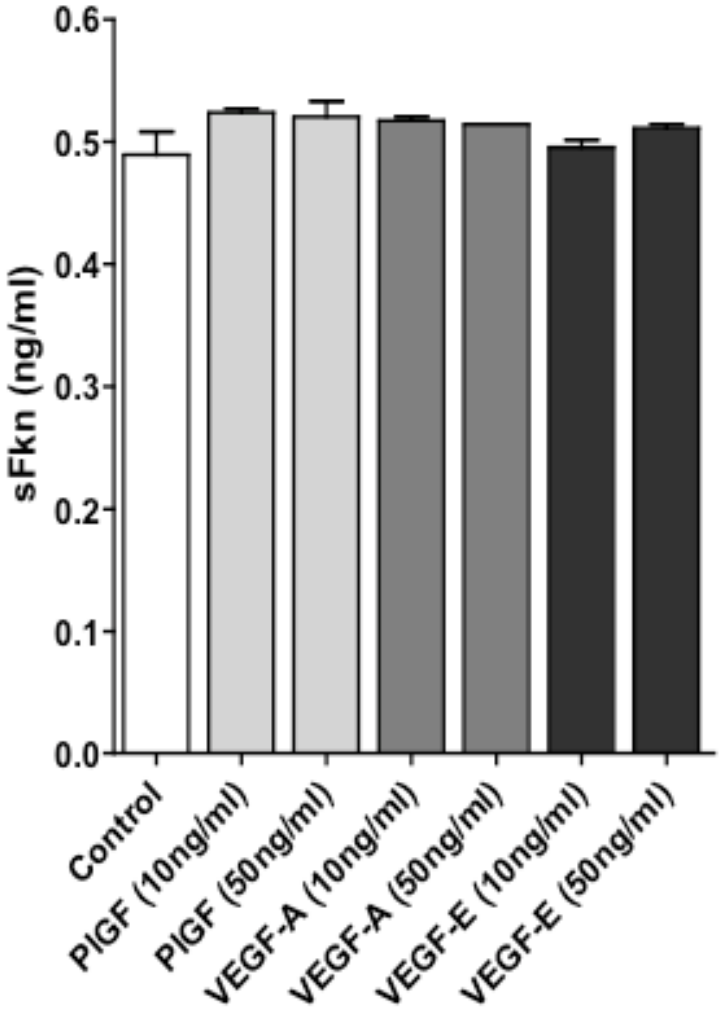

Figure 4.4 The effect of angiogenic factors on soluble Fractalkine release in HUVECs. HUVECs were treated with PlGF (10, 50 ng/ml), VEGF-A (10, 50 ng/ml), and VEGF-E (10, 50 ng/ml). Level of sFkn release was measured from the cell supernatants using an ELISA for 24 hours. Data is expressed as mean ± S.E.M of three independent experiments performed in triplicate.

4.1.2.5 Honokiol inhibits the release of soluble Fractalkine

Using Honokiol in inflammatory conditions is still controversial. We evaluated whether Honokiol could reduce sFkn release in HUVECs. HUVECs were treated with Honokiol at two different concentrations, 2 µg/ml and 20 µg/ml, for 1 hour followed by incubation with IFN-γ (10 ng/ml) for 24 hours. ELISA was used to measure sFkn release. Using Honokiol at 2 µg/ml with IFN-γ in HUVECs resulted in a significant reduction in the release of sFkn compared to untreated cells. Moreover, increasing the concentration of Honokiol to 20 µg/ml further reduced sFkn release compared to untreated cells (Figure 4.5).

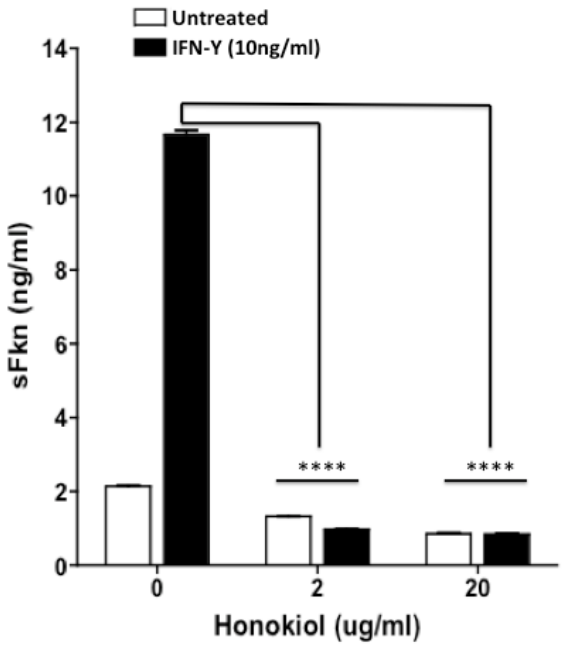

Figure 4.5 Effect of Honokiol on soluble Fractalkine release in HUVECs. HUVECs were incubated with IFN-γ in the presence or absence of Honokiol. Levels of sFkn were measured from the cell supernatants using an ELISA. Data is expressed as mean ± S.E.M of three independent experiments performed in triplicate (**** $P \leq 0.0001$).

4.1.3 Discussion

The results presented here demonstrate that pro-inflammatory cytokines, in particular TNF-α and lFN-γ, significantly up-regulate sFkn release in a concentration-dependent manner in primary cultured endothelial cells. TNF-α appeared to induce greater levels of sFkn release as compared to IFN-γ. This result is also supported by Ahn et al. (2004) and Chandrasekar et al. (2003). Unexpectedly, we found that IL-1β had no effect on sFkn release in HUVECs compared to the control. Earlier two studies had reported that IL-1β induces the expression of Fkn (Szukiewicz et al., 2013; Jones et al., 2010). It has also been reported that in the human intestinal epithelial cell line T-84, sFkn was steadily released over 24 hours under the control of IL-1β (Muehlhoefer et al., 2000). This suggests that there could be a difference between the effects of IL-1β on different cell types (epithelial cells vs. HUVECs).

Several lines of evidence have found that TNF-α and IFN-γ activate endothelial cells, which in turn up-regulates adhesion molecules in endothelial cells, hence amplifying the leukocyte endothelial cell interaction and the inflammatory process (Ley and Reutershan., 2006; De Assis et al., 2000; Lush and Kvietys., 2000). Cytokines are also involved in several events during pregnancy, such as ovulation, implantation, placentation, and parturition (Bowen et al., 2002), and a successful pregnancy requires a balance between anti-inflammatory and pro-inflammatory cytokines (Kim et al., 2015; Hunt, 2006). In this study, we observed a direct positive correlation between increased

concentrations of TNF-α or IFN-γ with sFkn release when compared with controls. We also found that a combination of TNF-α and IFN-γ at low concentration (1-10 ng/ml) increased sFkn release, more than treatments with either TNF-α or IFN-γ alone. An earlier study has reported that TNF-α levels are higher in the plasma of preeclamptic women compared to women with normal pregnancies (Barrera et al., 2015). On the other hand, the serum level of TNF-α and IFN-γ is lower in preeclamptic women compared to healthy pregnant women in the first and second trimesters (Serin et al., 2002).

Results from our laboratory have demonstrated that TNF-α and IFN-γ induced sFkn release in HUVECs as early as 8 hours. We found that TNF-α and IFN-γ induced maximal sFkn release in HUVECs after 24 hours. In contrast, Ludwig et al. (2002) could not detect sFkn when smooth muscle cells were co-stimulated with TNF-α and IFN-γ for 8 hours. These differences must reflect cellular differences in response to stimuli and sFkn production.

Our data demonstrates that the angiogenic factors PlGF, VEGF-A and VEGF-E failed to induce the release of sFkn from endothelial cells. Studies have previously shown that sFkn encourages endothelial cell migration in tube formation on Matrigel and synovial angiogenesis by interacting with CX3CR1 (You et al., 2007; Lee et al., 2006; Yoneda et al., 2003; Volin et al., 2001). Moreover, higher levels of sFkn release are indicative of endothelial damage or vascular inflammation (Matsunawa et

al., 2006).

Our pre-clinical study found that Honokiol strongly inhibits sFkn release in HUVECs stimulated with IFN-γ. We also found that Honokiol significantly reduced sFkn release mediated by IFN-γ. Both 2 μg/ml and of 20 μg/ml of Honokiol caused a similar reduction in sFkn release. This is likely due to an inhibitory effect on Akt activity (Kim and Cho., 2008). Honokiol is not toxic, and has not shown any pathological changes in the kidney, bone marrow, brain, heart, pancreas, liver, lung, intestines, and spleen after oral or systemic administration (Arora et al., 2012). Honokiol should be more systematically evaluated in PE. Honokiol is used in cancer therapy by drinking Magnolia teas prepared from the tree bark (Williams, 2009). Honokiol could be a valuable treatment for PE however, further work is necessary to ensure its safety as well as efficacy.

We can conclude from these findings that TNF-α and IFN-γ stimulate sFkn release in a concentration and time-dependent manner. However, angiogenic factors may not play a causative role in the level of sFkn release in endothelial cells. Honokiol appears to have an anti-inflammatory effect through the down-regulation of sFkn release, however this requires further clarification. These results suggest that Honokiol could be a novel treatment for PE.

4.2 HO-1 reduces soluble Fractalkine release mediated by TNF-α and IFN-γ in endothelial cells

4.2.1 Introduction

HO-1 is up-regulated by stress stimuli such as heme, heavy metals, peroxynitrite, endotoxin, hypoxia, hyperoxia, NO, and various cytokines (Dulak et al., 2008). A few studies have demonstrated that up-regulation of Fkn induces proliferation of endothelial cells (Perros et al., 2007; McDermott et al., 2001). None have directly addressed whether Fkn induced endothelial cell injury can be prevented by the induction of HO-1, BVR, CO and heme in the endothelium. Recently, HO-1 has also been shown to play a role in angiogenesis (Dulak et al., 2004), and the anti-inflammatory response of HO-1 has been shown in a few disease models (Otterbein et al., 2000; Poss and Tonegawa, 1997; Willis et al., 1996; Otterbein et al., 1995).

Metalloporphyrins act as competitive HO inhibitors (Lin et al., 2008; Amersi et al., 1999). Chromium protoporphyrin (CrPP) has been shown to inhibit HO activity *in vitro* (rat and human spleen) and *in vivo* (rat liver and spleen explant) (Schulz et al., 2012; Drummond and Kappas, 1984; Drummond, 1982).

In addition, tin protoporphyrin IX (SnPP) has been shown to be effective in inhibiting HO-1 activity *in vivo* and *in vitro,* and preventing the development of neonatal hyperbilirubinemia immediately after birth in rats (Drummond and Kappas, 1981) Inhibition of HO-1 results in decreased endothelial cell proliferation and capillary formation *in vitro* (Volti et al., 2005). Over-expression of HO-1 has been shown to enhance

endothelial cell proliferation (Deramaudt et al., 1998). In addition, over-expression of HO-1 decreases production of CXCL10, which results in stimulation of angiogenesis and vasculogenesis (Seog Seo et al., 2015).

In the previous sectian, we have shown that the cytokines TNF-α and IFN-γ induce the release of sFkn from endothelial cells. In the present study, actinomycin D, cycloheximide, CrPP and SnPP treatments were used with one or both of TNF-α and lFN-γ to study sFkn release. Here we hypothesized that inhibition and stimulation of stress-responsive enzyme HO-1 activity may influence sFkn release from endothelial cells. To support this study, we investigated whether HO-1 metabolites affect sFkn release from endothelial cells.

4.2.2 Results

4.3.2.1 Soluble Fractalkine release is reduced in the presence of the transcriptional inhibitor actinomycin D in endothelial cells

To check whether the transcriptional inhibitor actinomycin D inhibits sFkn release, HUVECs were pre-treated with actinomycin D (1 µg/ml) for 1 hour, and then stimulated with one or both of TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 24 hours. The supernatants were harvested and the level of sFkn release was measured by ELISA (Figure 4.6). We found that the level of sFkn release was inhibited completely in HUVECs treated with actinomycin D and TNF-α, IFN-γ and TNF-α plus IFN-γ, in comparison to untreated HUVECs.

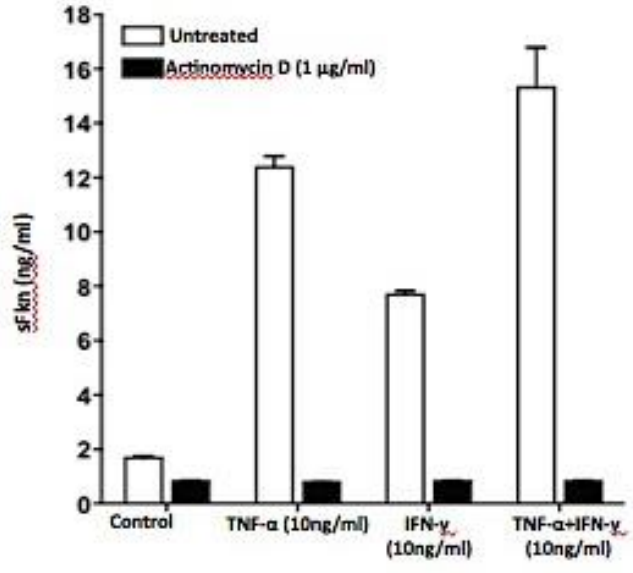

Figure 4.6 Actinomycin D inhibits soluble Fractalkine release from HUVECs. HUVECs were pre-incubated with actinomycin D and TNF-α, IFN-γ or a combination. sFkn levels were measured from the cell culture supernatants using ELISA. Data is expressed as mean ± S.E.M of one experiment carried out in duplicate.

4.2.2.2 Cycloheximide decreases release of soluble Fractalkine from endothelial cells

The effect of the protein synthesis inhibitor cycloheximide on sFkn release was investigated. HUVECs were treated with or without cycloheximide (5 µg/ml) for 1 hour, and then incubated for 24 hours with one or both of TNF-α (10 ng/ml) and IFN-γ (10 ng/ml). The supernatants were harvested and sFkn levels were measured by ELISA. The results indicated that following treatment with cycloheximide the level of sFkn release was decreased in cells stimulated with one or both of TNF-α and IFN-γ compared to HUVECs treated with cycloheximide only (Figure 4.7). In the presence of cycloheximide, sFkn release was reduced by 3-fold in HUVECs stimulated with TNF-α alone, by 2-fold in HUVECs stimulated with IFN-γ alone, and by 5-fold in HUVECs stimulated with TNF-α plus IFN-γ.

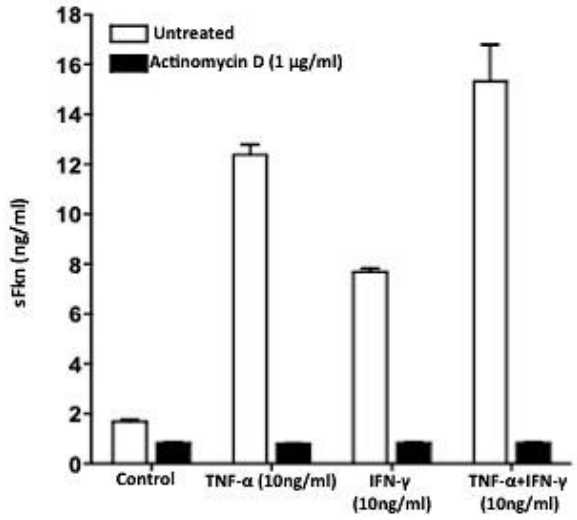

Figure 4.7 Cycloheximide inhibits soluble Fractalkine release in HUVECs stimulated with one or both of TNF-α and IFN-γ. Cycloheximide (5 µg/ml) and one or both of TNF-α and IFN-γ were used to treat HUVECs. sFkn release was measured by ELISA after 24 hours. The results showed that cycloheximide inhibited sFkn release. Data is expressed as mean ± S.E.M of one experiment carried out in duplicate.

4.2.2.3 Chromium protoporphyrin increases soluble Fractalkine release in HUVECs

We examined the effect of the HO-1 inhibitor CrPP on sFkn release. HUVECs were incubated with CrPP (40 µM) for 10 minutes, and then TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) were added for 24 hours. The supernatants were harvested and levels of sFkn release were measured by ELISA. The results showed that sFkn release was increased almost by 2-fold, in HUVECs incubated with CrPP alone compared to untreated HUVECs. Moreover, sFkn release was increased in HUVECs stimulated with CrPP and cytokines (TNF-α and IFN-γ), compared to HUVECs treated with cytokines alone (Figure 4.8).

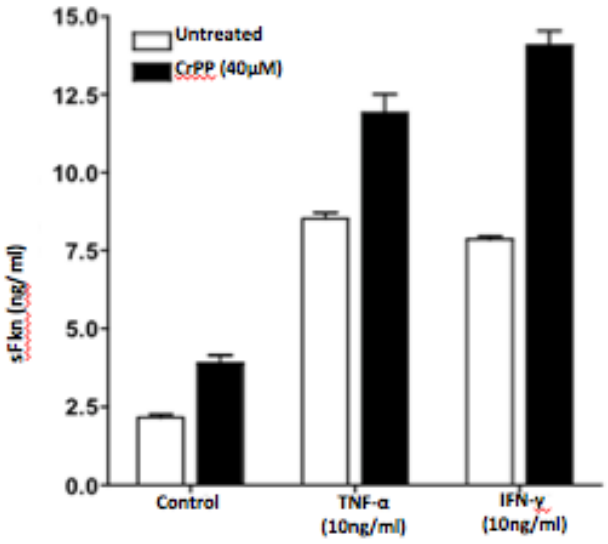

Figure 4.8 Inhibition of HO-1 activity by Chromium protoporphyrin increases soluble Fractalkine release. HUVECs were seeded on a 24 well plate and were incubated with CrPP (40 µM), along with either TNF-α or IFN-γ. Levels of sFkn were measured using ELISA. Data is expressed as mean ± S.E.M of two experiments carried out in duplicate.

4.2.2.4 Soluble Fractalkine release is reduced by tin protoporphyrin IX in HUVECs

We then investigated the effect of another HO-1 inhibitor, SnPP, on sFkn release. SnPP is a metalloporphyrin formed by a chelate of tin with the porphyrin ring, and has proven to be one of the most efficient inhibitors of HO-1 both *in vitro* and *in vivo* (Jozkowicz et al., 2003). SnPP (50 µg/ml) was added to HUVECs for 10 minutes, and then one or both of TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) were added for 24 hours. The supernatants were harvested and the levels of sFkn were measured by ELISA. The graph showed that sFkn release was decreased by 3-fold in HUVECs incubated with SnPP compared to untreated HUVECs. In addition, sFkn release was reduced by a fold in HUVECs incubated with SnPP and cytokines (TNF-α, IFN-γ and TNF-α plus IFN-γ), compared to HUVECs incubated with cytokines alone (Figure 4.9).

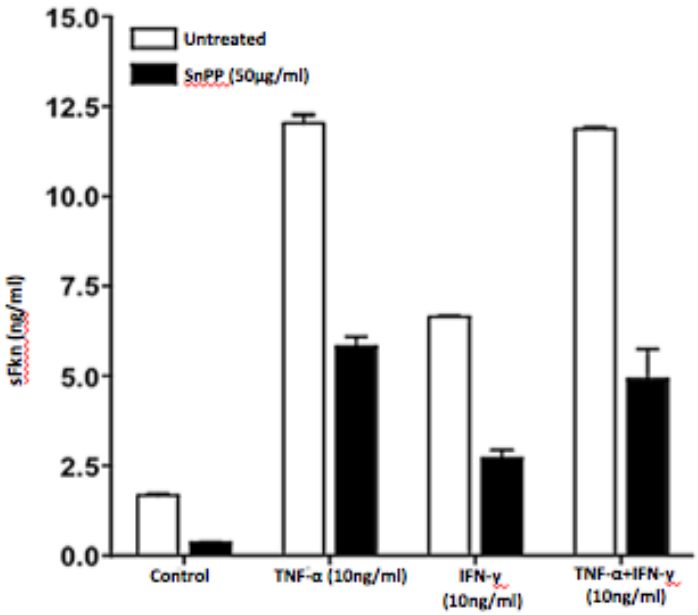

Figure 4.9 Tin protoporphyrin IX reduces soluble Fractalkine release in the presence and absence of cytokines. SnPP (50 µg/ml), together with one or both of TNF-α and IFN-γ, was used to treat HUVECs. Levels of sFkn were measured using ELISA. The results showed that SnPP inhibited sFkn release by a fold. Data is expressed as mean ± S.E.M of two experiments carried out in duplicate.

4.2.2.5 Hemin decreases soluble Fractalkine release in endothelial cells

We tested whether the hemin, HO-1 hemin, has the ability to reduce sFkn release. HUVECs were treated with hemin (5 µm/ml) following which TNF-α (10 ng/ml), IFN-γ (10 ng/ml) or both TNF-α and IFN-γ (10 ng/ml) were added for 24 hours. The level of sFkn release was measured using ELISA. The results indicated that hemin reduced sFkn release in HUVECs, as compared to untreated HUVECs (Figure 4.10).

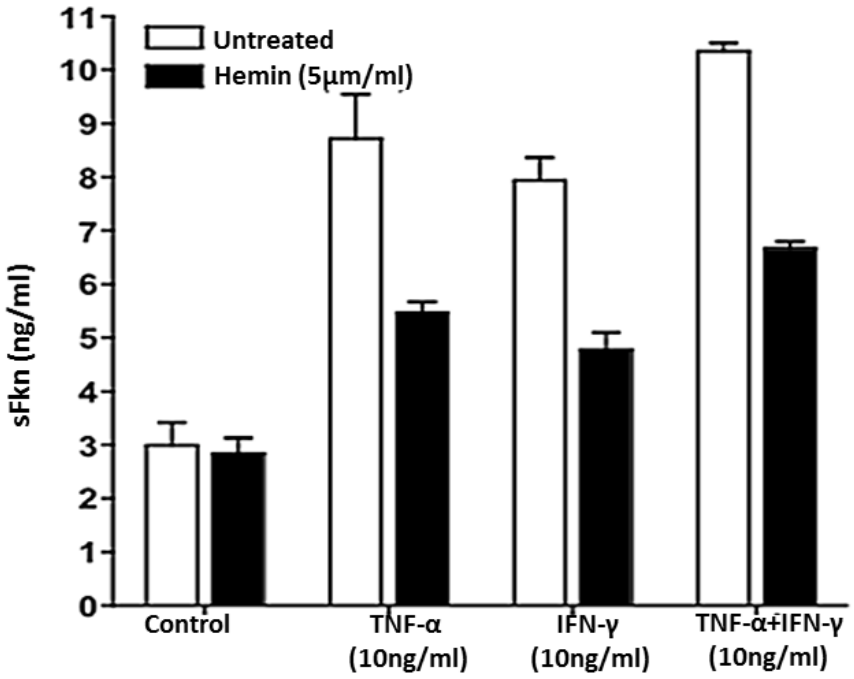

Figure 4.10 Hemin decreases soluble Fractalkine release in HUVECs. HUVECs were incubated with hemin (5 µm/ml) and TNF-α, IFN-γ or TNF-α plus IFN-γ. ELISA was used to measure sFkn levels in the cell culture supernatants. The data showed that hemin reduced sFkn release compared to untreated HUVECs. Data is expressed as mean ± S.E.M of two experiments were carried out in duplicate (*P ≤ 0.05).

4.2.2.6 Bilirubin reduces soluble Fractalkine release in endothelial cells

We then investigated the effect of bilirubin on sFkn release. HUVECs were treated with bilirubin (10 ng/ml), following which TNF-α (10 ng/ml), IFN-γ (10 ng/ml) or both TNF-α and IFN-γ (10 ng/ml) were added for 24 hours. Levels of sFkn were measured by ELISA. Our results showed that treatment with bilirubin had no effect on sFkn release in HUVECs under basal conditions. However, treatment with bilirubin, in the presence of TNF-α and IFN-γ and with a combination of TNF-α and IFN-γ, reduced the release of sFkn compared to the untreated controls (Figure 4.11).

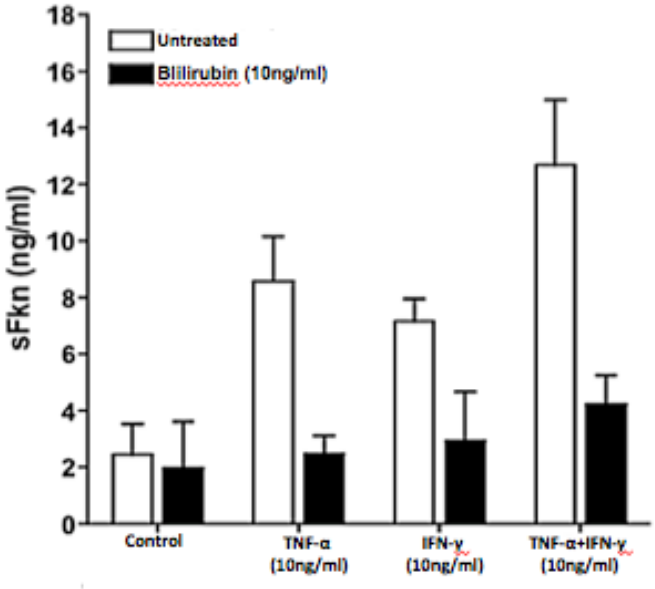

Figure 4.11 Bilirubin reduces soluble Fractalkine release in endothelial cells. HUVECs were incubated with bilirubin (10 ng/ml) along with TNF-α, IFN-γ or both. ELISA was used to measure sFkn release in the cell culture supernatants. Bilirubin reduces sFkn release by a fold compared to untreated HUVECs. Data is expressed as mean ± S.E.M of two experiments carried out in duplicate.

4.2.2.7 Carbon monoxide decreases soluble Fractalkine release in endothelial cells

Heme breakdown by HO-1 releases CO, which is a vasodilator and anti-inflammatory gaseous molecule (Ahmed et al., 2000; Bergmann et al., 2010). To determine whether the effects of Ad-HO-1 transfection are due to the enzyme itself or due to the CO product of the HO-1 enzyme, CORM was used. CORM is a molecule known to release biologically active CO. We investigated the effect of CORM on sFkn release. HUVECs were incubated with CORM (10 µM), following which TNF-α (10 ng/ml) or IFN-γ (10 ng/ml) were added for 24 hours. An inactive form of CORM (iCORM-2) was used as a negative control. These data revealed that sFkn release was decreased by over 50% in HUVECs treated with CORM compared to untreated HUVECs under basal and cytokine conditions. Cells treated with iCORM did not affect sFkn release under control of cytokine stimulated condition. The use of CORM has highlighted the possibility of CO having an important protective function in inflammation (Figure 4.12).

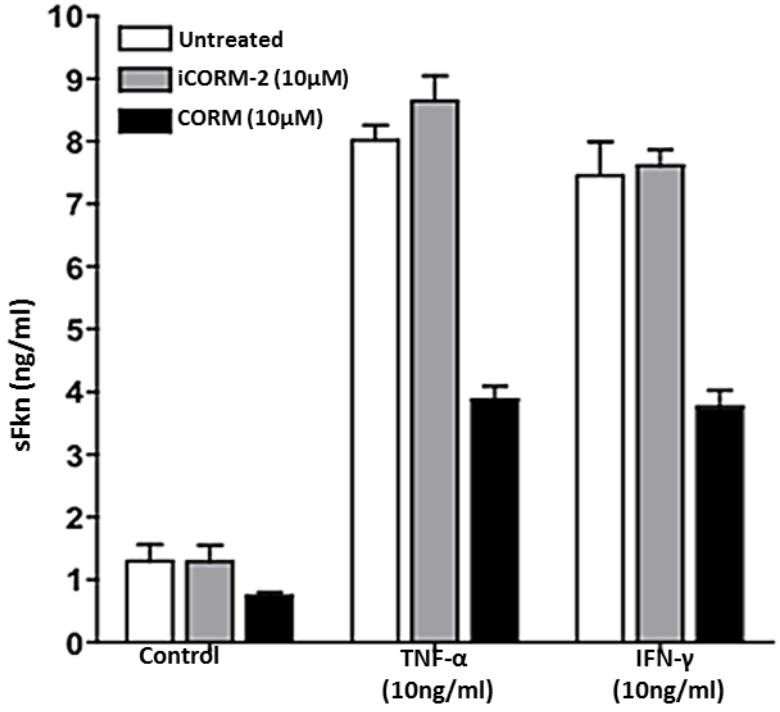

Figure 4.12 CORM decreases soluble Fractalkine release in HUVECs. Endothelial cells were incubated with iCORM (10 µM) in combination with TNF-α or IFN-γ for 24 hours. ELISA was performed to measure sFkn release in the cell culture supernatants. Data is expressed as mean ± S.E.M of two experiments were carried out in duplicate (*P ≤ 0.05).

4.2.3 Discussion

Several inflammatory mediators enhance the production of Fkn (Muehlhoefer et al., 2000). In this study, we found that sFkn release from endothelial cells was reduced by an inhibitor of RNA synthesis, actinomycin D, in the presence and absence of the TNF-α and IFN-γ.

We further found that cycloheximide, a protein synthesis inhibitor, greatly inhibited sFkn release in the presence of TNF-α and IFN-γ. We found that CrPP, an inhibitor of HO-1, increased sFkn release in endothelial cells stimulated with TNF-α and IFN-γ compared to the controls. Several studies have shown that loss or reduced expression of HO-1 contributes to several diseases including PE (Suliman et al., 2017; Tong et al., 2015; Chaiworapongsa et al., 2014; O'Hara et al., 2006).

SnPP is a widely used inhibitor of HO-1 activity (Pae et al., 2008). SnPP is a metalloporphyrin that efficiently inhibit of HO-1 (Jozkowicz et al., 2003; Brouard et al., 2000). We showed that SnPP decreased sFkn release in the presence and absence of TNF-α and IFN-γ in endothelial cells.

We also treated endothelial cells with hemin, which is a pharmacological inducer of HO-1 (Bussolati et al., 2004; Jozkowicz et al., 2003). Hemin induces HO-1 expression (Hualin et al., 2011), and increases HO-1 activity. We found that hemin reduced sFkn release in the presence of TNF-α and IFN-γ.

Interestingly, Noda and colleagues (2010) have shown that sFkn can induce expression of HO-1 in microglia; sFkn exerts a neuroprotective function in damaged neurons by binding to CX3CR1. This activates the c-Jun N-terminal kinases (JNK) Mitogen-activated protein kinases (MAPK) signalling pathway leading to recruitment of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor, which leads to HO-1 expression (Noda et al., 2010). A recent study by Jansen and colleagues (2010) demonstrated that the protective effect of pharmacological over-expression of HO-1 was almost completely abrogated in BVR-silenced cells, indicating that BVR is essential in HO-1 induced cytoprotection. Moreover, Baranano and colleagues (2002) found that knockdown of BVR caused depletion of bilirubin, and increased the levels of reactive oxygen species, which led to apoptotic cell death. Based on this, we can also speculate that the cytoprotective functions of both HO-1 and BVR may be due to bilirubin. Indeed, bilirubin has previously been shown to protect against oxidative stress and lipid peroxidation (Neuzil and Stocker, 1994). We found that bilirubin greatly reduced sFkn release in endothelial cells in the presence of TNF-α and IFN-γ.

Our results demonstrated that CORM inhibits sFkn release in the presence of TNF-α and IFN-γ. There is mounting evidence that CO confers cytoprotection against tissue and cellular injury (Dulak et al., 2008; Otterbein et al., 1999). Therefore, CO could be a new therapy in

pregnancy disorders. However, before it can be taken into the clinic, it is important to determine the mechanism of action and whether CO adversely affects fetal outcome. It is possible that CO, produced by HO-1 activity, mediates the protective effects of HO induction through modulation of these pathways. Another important finding from this study was that CO and bilirubin reduced sFkn release in endothelial cells stimulated with TNF-α and IFN-γ, whereas abrogation of HO-1 activity increased sFkn release. Brouard and colleagues (2000) showed that HO-1 has an anti-inflammatory effect, due to the production of CO in endothelial cells expressing HO-1, and that CO might likely act as an intercellular signalling molecule to preserve those endothelial cells which do not express HO-1.

Our present findings may have important implications not only for understanding the mechanisms regulating the protective effects of HO-1, but also for the development of therapeutic approaches to suppress these inflammatory reactions. Future research should be carried out to determine whether there is a variation of HO-1 in placenta using co-culture experiments.

4.3 Soluble Fractalkine levels in tissues of Hmox1 homozygous knockout and wild-type mice

4.3.1 Introduction

The Hmox1 gene codes for the HO-1 protein, which plays a role in angiogenesis and is highly expressed in the placenta. Hmox1 has been shown to be reduced in human PE placenta (Ahmed et al., 2000). Bainbridge and Smith (2005) confirmed that a reduction in placental Hmox1 expression was associated with recurrent miscarriages, spontaneous abortions, and PE. HO-1 expression in the placenta of mice increases gradually during the second trimester before steadily declining until term, a pattern that is directly correlated to the level of placental tissue oxygenation, which increases to midgestation before gradually declining until term (George et al., 2014). Additionally, Zenclussen and colleagues (2006) have reported that the level of HO-1 during placental development is reduced in mice with sonic stress or IL-12-induced abortions.

A recent study demonstrated that the pattern of Fkn gene expression in mice is similar to sFkn (Yao et al., 2015). In cultured rat cardiac cells Fkn promotes myocardial injury and accelerates heart failure (Xuan et al., 2011). In additional, levels of sFkn were significantly higher in patients with ST-elevation myocardial infarction (Yao et al., 2015). Furthermore, Fkn has been shown to be induced by transforming growth factor-β (TGF-β) in mouse kidney dendritic cells (Kassianos et al., 2015), and Fkn expression is induced in the kidney of mice through the NF-κB pathway (You et al., 2015).

In this section we investigated whether our *in vitro* findings would correlate *in vivo,* using a HO-1 knockout mouse model. In addition, we assayed levels of sFkn release in the conditioned media of TNF-α and IFN-γ by using the heart and lung tissue of HO-1 knockout mice.

4.3.2 Results

4.3.2.1 Elevated levels of soluble Fractalkine in Hmox1^{-}/$^{-}$ mice lung tissues

In the first part of this thesis we showed that over-expression of HO-1, as well as substances known to up-regulate HO-1 expression, reduced sFkn release. We then investigated the effect of knocking out HO-1 on sFkn release from the lung tissues of mice. These tissues were obtained from wild-type (WT) (Hmox1^{+}/$^{+}$) (control), heterozygous (HET) (Hmox1^{+}/$^{-}$) and homozygous knockout (KO) (Hmox1^{-}/$^{-}$) mice. We measured sFkn release in supernatants of the homogenised lung tissue using an ELISA. The results showed that KO mice had greater sFkn release compared to the other groups (Figure 4.13).

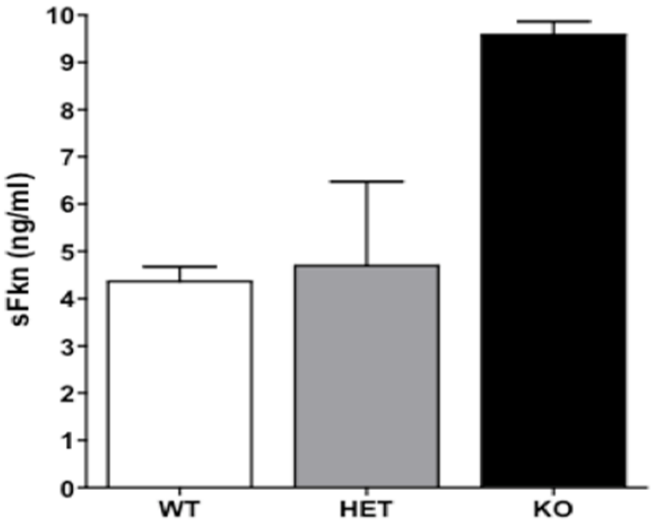

Figure 4.13 HO-1 deficiency is associated with increased soluble Fractalkine release in lung tissue of mice. Lung tissues of WT, HET and KO mice were incubated in medium for 24 hours. ELISA was used to measure sFkn levels in the explant lung tissue supernatants. Data is expressed as mean ± S.E.M. of two experiments carried out in duplicate.

4.3.2.2 Effect of TNF-α and IFN-γ on Soluble Fractalkine levels in murine lung tissue explants

We have shown that the pro-inflammatory cytokines TNF-α and IFN-γ increased sFkn release in endothelial cells *in vitro*. We assessed the level of sFkn release in the presence of TNF-α and IFN-γ in the conditioned medium of KO lung mice. Explanted lung tissue was incubated with TNF-α and lFN-γ (20 ng/ml) for 48 hours. This experiment involved KO ($Hmox1^{-/-}$), WT ($Hmox1^{+/+}$) (control), and HET ($Hmox1^{+/-}$) mice tissues. Levels of sFkn in the conditioned medium of the lung tissues were measured using ELISA. The results showed that there were higher levels of sFkn in the conditioned medium of KO compared to the corresponding WT explant tissue (Figure 4.14). However, we found that levels of sFkn in the conditioned medium of HET explant tissue were lower compared to WT mice when exposed to these cytokines.

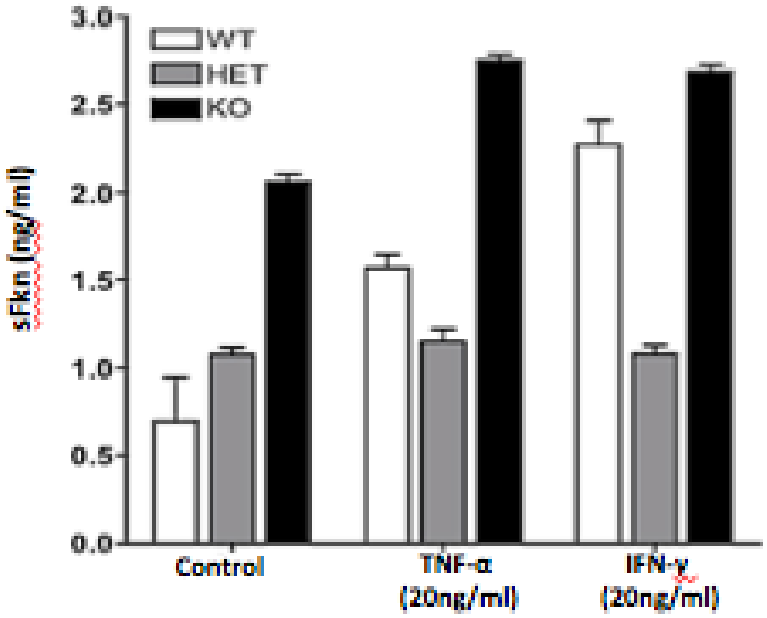

Figure 4.14 Soluble Fractalkine release is increased in the lung tissue of Hmox1$^{-/-}$ mice in the presence of TNF-α and IFN-γ. Lung tissues explants of WT, HET and KO mice were incubated with TNF-α and IFN-γ. ELISA was performed to measure sFkn. Data is expressed as mean ± S.E.M. of two experiments carried out in duplicate.

4.3.2.3 Elevated levels of soluble Fractalkine release in the heart tissue of HO-1 homozygous knockout mice

We then determined the effect of HO-1 deficiency on sFkn release in the heart tissue of KO ($Hmox1^{-/-}$), WT ($Hmox1^{+/+}$) (control), and HET ($Hmox1^{+/-}$) mice. ELISA was used to measure sFkn release in supernatants of homogenised heart tissue. Our data showed that an absence of HO-1 in KO mice resulted in increased release of sFkn, compared to the corresponding WT mice (Figure 4.15).

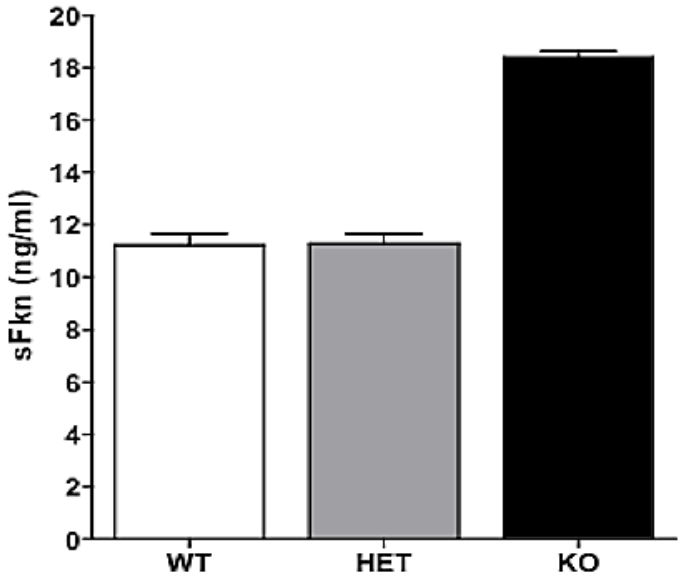

Figure 4.15 HO-1 deficiency is associated with increased soluble Fractalkine release in the heart tissue of $Hmox1^{-/-}$ mice. Heart tissues from WT, HET and KO mice were used. sFkn was measured from the tissue supernatants using ELISA. Data is expressed as mean ± S.E.M. of two experiments were carried out in duplicate.

4.3.2.4 Soluble Fractalkine levels are elevated in the conditioned medium of the hearts of mice

We further studied the effect of HO-1 deficiency on sFkn release in the presence of TNF-α and IFN-γ in the heart tissue of mice. We used heart tissue from KO ($Hmox1^{-/-}$), WT $Hmox1^{+/+}$ (control), and HET ($Hmox1^{+/-}$) mice. As with the lung tissue, explanted heart tissue was incubated with TNF-α and IFN-γ (20 ng/ml) for 48 hours. ELISA was then used to measure sFkn release in the supernatants of the homogenised heart tissue. We observed that sFkn release was increased slightly (not statistically significant) in the conditioned medium of KO mice in comparison to WT mice (Figure 4.16). Interestingly, there was a slight reduction in sFkn release in the conditioned medium of HET mice compared to the corresponding WT and KO mice.

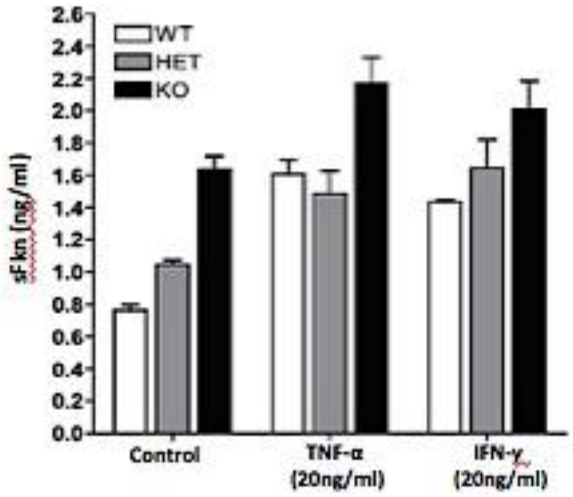

Figure 4.16 Soluble fractalkine release is increased in the heart tissue of $Hmox1^{-/-}$ mice in the presence of TNF-α and IFN-γ. Heart tissue from WT, HET and KO mice were incubated with TNF-α and IFN-γ. ELISA was used to measure sFkn. Data is expressed as mean ± S.E.M. of two experiments were carried out in duplicate.

4.3.3 Discussion

There is compelling evidence, produced by two separate groups, that Fkn plays an important role in atherogenesis, fueling further interest in Fkn and its receptor as potential therapeutic targets (Barlic and Murphy, 2007; Lesnik et al., 2003). One study has demonstrated that Fkn could up-regulate HO-1 gene expression in a macrophage cell line (Inui et al., 2010). Our studies indicate that there is a difference in sFkn levels between $Hmox1^{-/-}$ and $Hmox1^{+/+}$ mice. The use of mouse models has shown that a partial deficiency of maternal Hmox1 induces growth restriction in both the placenta and fetus, resulting from defects in the placental vasculature probably result in growth restriction of the fetus (Zenclussen et al., 2011).

We found that there was increased sFkn release in both the lung and heart tissue of KO mice compared to WT mice. In the reported case of HO-1 deficiency in man increased expression of vascular adhesion molecules and more severe inflammation were observed compared to HO-1 knockout mice (Kawashima et al., 2002).

HO-1 also inhibits the endothelial release of pro-inflammatory chemokines. Determining whether pre-existing inflammatory conditions could contribute in aggravating PE symptoms could be an important step in the management of complicated pregnancies. It has previously been shown that IFN-γ and TNF-α regulate Fkn expression in smooth muscle cells and arterial endothelial cells (Ludwig et al., 2002). Our data showed

that the levels of sFkn were increased by approximately 2-fold in cultured lung tissue explants and cultured heart tissue explants of KO mice, compared to WT, in the presence of TNF-α and IFN-γ.

The role of smooth muscle cell derived Fkn in vascular inflammation is dependent on the synergism of TNF-α or IFN-γ, as well as the activity of Fkn cleaving metalloproteinase (Ludwig et al., 2002). Endothelial expression of membrane associated Fkn may be an important contributing mechanism by which monocytes are selectively recruited to inflamed tissue. Our KO mouse model completely lacked HO-1 expression, however, this is rarely observed in humans (Araujo et al., 2012).

The data presented here provides evidence to support the essential anti-inflammatory function of HO-1 in supperssing of sFkn release. This data opens up the possibility that this very early decrease in HO-1 could lead, at least in part, to elevated inflammation.

Although results derived from mouse explant tissues of lung and heart cannot be completely extrapolated to humans, our model may provide insights into the mechanisms by which inter-individual variations can cause placental insufficiency. Future studies could investigate other mechanisms by which HO-1 is thought to confer cellular protection in response to inflammatory cytokines.

Our study has demonstrated that the anti-inflammatory action of HO-1 plays a critical role in regulating sFkn release in the lung and heart tissue of mice. Our *in vivo* data supports our *in vitro* findings that TNF-α and IFN-γ stimulate sFkn release. Taken together, our endothelial cell and mouse tissue studies point to HO-1 as a negative modulator of sFkn during inflammation.

4.4 Soluble Fractalkine in human placenta and maternal plasma for normal and complicated pregnancies

4.4.1 Introduction

Several theories suggest that PE stems from maternal endothelial malfunction (Roberts and Redman, 1993; Roberts et al., 1989). The endothelium consists of a thin layer of cells lining the inner surface of the blood surface of blood vessels. The endothelium is important for immune function, and control of volume and electrolyte content of the intravascular and extravascular spaces. Endothelial dysfunction occurs due to loss of NO bioavailability (Kinzler et al., 2004; Chambers et al., 2001).

Excessive inflammation may cause early-onset PE (Redman et al., 1999). Indeed, Redman et al. (1999) showed that the inflammatory changes in peripheral blood leukocytes associated with normal pregnancy and PE were similar to sepsis. A pro-inflammatory cytokine secreted by both activated leukocytes and endothelial cells is elevated in severe PE (Szarka et al., 2010; Tosun et al., 2010; Clark et al., 1998B). Moreover, an earlier study had demonstrated that neutrophil activation is confined to the maternal circulation in pregnancy induced hypertension, where it may contribute to vascular damage (Greer et al., 1991). Thus, the idea that inflammation causes PE has persisted without solid causational evidence (Hill, 1965).

Current knowledge on placental derived Fkn and its implications on pregnancy is limited and based on a small number of studies (Siwetz et al., 2014). These studies have found that Fkn may be considered as an

inflammatory chemokine, which is expressed in activated endothelial and epithelial cells, as well as in dendritic cells, neurons, osteoblasts, lymphocytes, and microglial cells (Siwetz et al., 2014; Corcione et al., 2012; Lucas et al., 2001). Indeed, Siwetz et al. (2014) showed that Fkn is released by uterine glands from the syncytiotrophoblast into the maternal circulation via metalloprotease dependent shedding in human placenta.

The aim of this study was to determine whether a direct relationship could be demonstrated between the level of sFkn release and complicated pregnancy from human placenta. In addition, we investigated whether sFkn release is elevated in the plasma of complicated pregnancy.

4.4.2 Results

4.4.2.1 Patient selection for soluble Fractalkine level

The clinical characteristics of complicated and uncomplicated pregnancies included in our study are shown in table 4.1. The study population consisted of (n=14) women with normal pregnancies. Pregnancies had negative urine dipstick protein reading, and 48 women with complicated pregnancies preterm (n=12), PE (n=13), IUGR (n=9) and HELLP (n=14). Data in the table expressed as mean ± SEM. The exclusion criteria in the five patients groups included the presence of infectious diseases, pre-existing inflammatory conditions and autoimmune disorders.

Table 4.1 Clinical characteristics of patient groups used in the study

Characteristic	Term Control (n=14)	Preterm (n=12)	PE (n=13)	IUGR (n=9)	HELLP Syndrome (n=14)
Patient age at delivery (years)	32 ± 0.8	28± 0.8	34±1.8	33±1.1	30±1.1
Weight (kg)	57 ± 1.9	67.1	67.1	55±0.8	65.3±1.7
Gestational age (weeks)	30 ± 0.8	31.7±0.7	31.7±0.9	29.2±0.4	31.6±0.9
Systolic blood pressure > 20 weeks (mm Hg)	118.1 ±3.4	165.3±3.8	165.3± 3.9	170±1.2	170.9±7.2
Diastolic blood pressure > 20 weeks (mm Hg)	65.3 ±1.6	102.3±2.9	102.3± 30	113±0.4	106.9±7.6
Proteinuria (24Hr) (mg/mmol)	126.8±12.3	2491.5±569.8	2493.5 ±569.8	2100	2684.4±1141.1
BMI(kg/m^2)	25.1±1.2	28.5±0.9	28	22±1.9	21.6±1.3

4.4.2.2 Level of soluble Fractalkine release in human placenta of complicated pregnancies

To demonstrate a direct relationship between placental derived sFkn release and the incidence of pregnancy complications, the following groups were investigated: term (n=12), preterm (n=12), PE (n=13), IUGR (n=2), and HELLP (n=14). Levels of sFkn in the placental lysates of patients were measured using ELISA. The data illustrates that the level of sFkn release in the placental lysates was not significantly increased in complicated pregnancies compared to normal pregnancies (Figure 4.17).

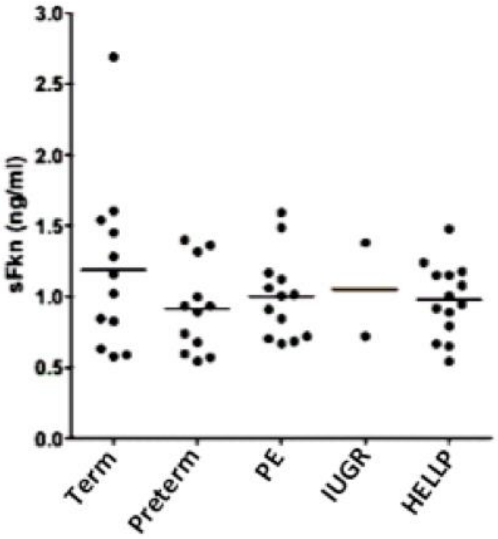

Figure 4.17 Circulating level of soluble Fractalkine in normal and abnormal human placenta. Levels of sFkn release in placenta explant supernatants for 24 hours were assessed by ELISA in term (n=12), preterm (n=12), PE (n=13), IUGR (n=2), and HELLP (n=14). Each point represents an individual patient sample Data is expressed as mean ± S.E.M of three independent experiments.

4.4.2.3 Level of soluble Fractalkine release in the plasma of complicated pregnancies

Further blood samples were obtained from the same patients to see whether there was a difference in sFkn level in the maternal plasma of normal and complicated pregnancies. The level of sFkn release was compared, using ELISA, in the maternal plasma of the following groups: term (n=16), preterm (n=9), PE (n=12), and IUGR (n=9). Although not significant, this data showed that there was a trend towards increased sFkn release in the maternal plasma of patients with complicated pregnancies and normal pregnancies (Figure 4.18).

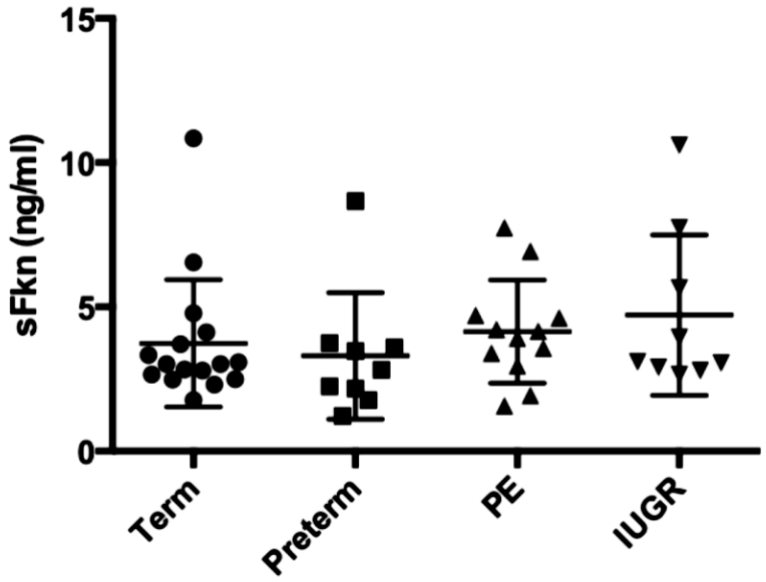

Figure 4.18 Level of soluble Fractalkine in the plasma women with normal and complicated pregnancies. Level of sFkn release in the maternal plasma women was assessed by ELISA in term (n=16), preterm (n=9), PE (n=12), and IUGR (n=9) patients.. Data is expressed as mean ± S.E.M of three independent experiments. Each point represents an individual patient sample.

4.4.3 Discussion

At early time points, Fkn (expressed in amniotic fluid during pregnancy) is amongst the most abundantly expressed chemokine in the uterine cavity during pregnancy (Shimoya et al., 2003). Fkn acts as a soluble chemoattractive factor and transmembrane adhesion molecule (Siwetz et al., 2014). The chemokines, Fkn, CCL14, and CCL4, promote human trophoblast migration at the foetus maternal interface (Hannan et al., 2006). Based on placental explant experiments, Fkn is not only expressed, but is also released from villous trophoblasts (Siwetz et al., 2014). Immunohistochemical staining of sections of the human placenta in the first trimester and at term showed localised Fkn at the apical microvillous plasma membrane of the syncytiotrophoblast (Siwetz et al., 2014). Hannan et al. (2004) showed that there was weak CX3CR1 staining in the villous trophoblast layer of first trimester placenta. However, the profile of sFkn is largely unknown.

This study found that there was no relationship between the level of sFkn release and term complicated pregnancies in human placental lysates compared to the controls. Stepanian and colleagues (2009) also found that Fkn and sFkn levels were significantly increased in the maternal plasma of PE patients compared to the controls; this increase was detected in very early-onset PE, diagnosed between 25 and 29 weeks of gestational age, rather than later-onset PE. However, we found that sFkn levels were not significantly increased in the maternal plasma of term PE patients compared to the controls.

In some cases, high Fkn release has been suggested to contribute to maternal inflammatory responses (Szukiewicz et al., 2014). Inflammation is required for normal pregnancy, but excessive inflammation may contribute to complicated pregnancies. Indeed, NK cells, dendritic cells, and macrophages infiltrate the decidua and accumulate around the invading trophoblast during the first trimester. Depletion of immune cells, instead of helping the pregnancy, terminates the pregnancy. Thus, deletion of macrophages, NK cells, or dendritic cells has deleterious effects on placental development, implantation, or decidual formation. In other studies, it was shown that in the absence of NK cells trophoblasts are not able to reach the endometrial vasculature, leading to termination of the pregnancy (Mor et al., 2011). These studies suggest that uterine natural killer cells are critical for trophoblast invasion in the uterus during normal pregnancy.

Redman and colleagues (1999) have postulated that PE is the consequence of an excessive inflammatory response to pregnancy, and that the intravascular inflammatory response is not an epiphenomenon, but is in fact the cause of the clinical syndrome of PE. Studies have shown that Fkn plays an important role in inflammatory vascular diseases, including atherosclerosis and rheumatoid arthritis (Lesnik et al., 2003; Ruth et al., 2001). As such, it cannot be ruled out that placental Fkn may contribute to low grade systemic inflammatory responses observed in the third trimester of normal pregnancy (Siwetz et al., 2014).

In summary, although our results showed that in the placental explant supernatants sFkn release does not appear to be significantly affected by complicated pregnancy (PE, IUGR, and HELLP) compared to normal pregnancy, our results also showed that there was a trend towards slightly increased sFkn release in the maternal plasma of patients with PE. However, the underlying mechanisms of sFkn release require further investigation, and the evidence suggests that sFkn may not be a key determinant in the cause of PE. Future research should be carried out to determine whether there is a variation in levels of HO enzymes and these should be measured in patients presenting with inflammation.

CHAPTER FIVE: EXPRESSION OF NOGGIN AND LRIG-1 IN PLACENTA

5.1 Introduction

5.1.1 Noggin

Bone morphogenetic proteins (BMPs) have an important role in vascular homeostasis. Noggin, Chordin and follistatin bind BMPs in the extracellular space (Massague and Chen, 2000). The functions of BMPs can be determined by the interplay of at least two groups of BMPs. Noggins are amongst the secreted proteins that bind to BMPs and reduce their bioavailability for interaction with the BMP receptors. Noggin acts by binding with high affinity to BMP2, BMP4, BMP5, BMP7, BMP13 and BMP14, Growth differentiation factor (GDF) 5, and GDF6 (Zimmerman et al., 1996). However, BMP3, BMP6, BMP9, BMP10 and BMP15 signalling seems unaffected (Krause et al., 2011) by other members of the TGF-β family of peptides (Wu et al., 2016; Aspenberg et al., 2001).

Noggin, which is encoded by the NOG gene (Gobeske et al., 2009), is a secreted homodimeric glycoprotein (Smith and Harland, 1992) with a molecular mass of 60 kDa (Wu et al., 2003). It has been confirmed by observations *in vitro* and *in vivo* that Noggin can block BMP function (Schwaninger et al., 2007; Wu et al., 2003).

Noggin affects endothelial cells by two mechanisms (Kang et al., 2009). Firstly, Noggin interrupts BMP-mediated VEGF promoter activation. Secondly, Noggin inhibits endothelial tubulogenesis and migration by down-regulating E-cadherin through disruption of β-catenin/ lymphoid

enhancer binding factor-1 (Lef1) mediated transcriptional regulation of E-cadherin expression, since Noggin has been shown to induce Lef1-mediated transcription (Cornejo et al., 2015; Kang et al., 2009; Dai et al., 2004).

Noggin has been used as a tool to block both types of BMP receptors (Type I and Type II) functionally and directly before BMP has reached its target. This is because it is a relatively specific inhibitor of BMP activity (Zimmerman et al., 1996). Furthermore, evidence has shown that this regulates neural development (Valenzuela et al., 1995). Noggin is known to regulate angiogenesis and vasculogenesis (Kang et al., 2011). Interestingly, in 2007 Schwaninger and colleagues used Noggin as a therapy for osteolytic bone metastases. Furthermore, it has recently been used in improving bone structural environment and balanced bone remodelling. Mice lacking Noggin show elevated BMP levels causing postnatal mortality. Conversely, a recent study showed that over-expression of Noggin counteracts BMP4 activity leading to over-proliferation of neural tissue and neural precursor cells (Krause et al., 2011). Unfortunately, the expression and role of Noggin in the placenta is unknown.

5.1.2 Leucine rich repeats immunoglobulin-like domains

The leucine rich repeats immunoglobulin-like domains (Lrig) family is comprised of three groups: Lrig1 (formerly LIG1), Lrig2, and Lrig3, which is located on chromosome 12q13. In 1996 mouse Lrig1, formerly called

LIG-1 (Suzuki et al., 1996), and in 2002 human Lrig1 were cloned (Hedman et al., 2002). Expression of Lrig1 is required for epidermal homeostasis (Jensen et al., 2009), and Lrig1 is a newly identified negative regulator of the epidermal growth factor (EGF) receptor family of proteins. The EGF receptor (EGFR) family of proteins contains receptor tyrosine kinases (Shattuck et al., 2007). Lrig1 shares a sequence, which antagonises growth factor signalling mediated by EGFR tyrosine kinases (Petit et al., 1997). The mammalian Lrig1 protein contains 15 leucine rich repeats (LRRs) (Goldoni et al., 2007).

Over-expression of Lrig1 in neuronal cells inhibits glial cell line derived neurotrophic factor in an autonomous manner (Ledda et al., 2008). Moreover, Lrig1 acts as a tumour suppressor *in vitro* and *in vivo* (Simion et al., 2014; Wang et al., 2013), and it has been proposed that knockdown of Lrig1 increases proliferation of cultured human keratinocytes (Jensen and Watt, 2006) and negatively regulates EGF (Jensen et al., 2009). However, Lrig1 expression and its role in the normal placenta are unknown.

Gene array profiles conducted in our laboratory identified the presence of these genes in the placenta. For these reasons, the aim of this study was to determine the gene expression and protein localisation of Noggin and Lrig1 in the placenta, specifically in the preeclamptic placenta, by qPCR, IHC, and WB. Furthermore, we wanted to assess the effect of Noggin on angiogenesis in endothelial cells by *in vitro* tube formation.

5.2 Results

5.2.1 Patient selection

The clinical characteristics of uncomplicated and complicated pregnancies included in our study are shown in table 5.1. Detailed clinical data was abstracted from Barcelona's Hospital de Maternitat, and includes clinical information regarding the delivery age, blood pressure, proteinuria and mean birth weight between the studied groups. The study population consisted of (n=9) women with normal pregnancy, (n=6) women with preterm, (n=9) women with PE, and (n=7) women with normotensive IUGR. All expectant mothers were followed prospectively from enrolment to delivery. Control women delivered a healthy infant at term. The exclusion criteria in the study groups were the presence of infectious disease, or medical complications including diabetes mellitus, inflammatory conditions, and autoimmune disorders. In order to maintain an unbiased approach, samples were randomly numbered and the groups were not known until after analysis of the data.

Table 5.1 Clinical characteristics of patient groups included in the study.

Characteristic	First trimester (n=7)	Second trimester (n=7)	Control (n=7)	Preterm (n=6)	PE (n=9)	IUGR (n=7)
Patient age at delivery (years)	29 ± 0.8	35±0.8	32 ± 0.8	28± 0.8	34±1.8	29 ± 1.1
Weight (kg)	63.8 ± 3.0	63.4 ± 2.8	57 ± 1.9	67.1	67.1	66.6± 1.1
Gestational age (weeks)	9±0.9	16 ±0.9	30 ± 0.8	31.7±0.7	31.7±0.9	30.4 ± 0.5
Systolic blood pressure > 20 weeks (mm Hg)	-	-	118.1 ±3.4	165.3±3.8	165.3± 3.9	124.4 ±2.4
Diastolic blood pressure > 20 weeks (mm Hg)	-	-	65.3 ±1.6	102.3±2.9	102.3± 30	71.3± 1.1
Proteinuria (24Hr) (mg/mmol)	-	-	126.8±12.3	2491.5± 569.8	2493.5 ±569.8	229.4 ±19.8
BMI	-	-	25.1±1.2	28.5±0.9	28	22.7± 0.6

5.2.2 Noggin mRNA levels in normal and abnormal human placenta

In the present study, we investigated whether gene expression of Noggin in the human placenta plays a significant role in PE in order to get a better understanding of the pathogenesis of PE, and to discover helpful biomarkers. Real time qPCR was used to confirm expression of Noggin in the placenta. Interestingly, we found an increase in Noggin mRNA in the human placenta. Moreover, we observed an increase of Noggin mRNA expression levels in both PE and IUGR compared with control. Unfortunately, these findings were not statistically significant due to the small sample size, as the sample size software requires at least n=9 to get significant results.

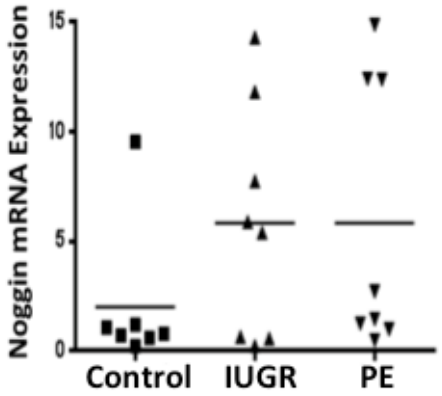

Figure 5.1 Noggin mRNA levels in normal and abnormal human placenta. Noggin mRNA levels were detected in normal (n=7), preterm (9), PE (n=8) and IUGR (n=7). Each point represents an individual patient sample. Experiments were performed in triplicate, and mRNA levels were normalised to β-actin. Data is expressed as mean ± S.E.M.

5.2.3 Pattern of Noggin expression in placenta according to gestational age

We investigated the pattern of expression of Noggin in the human placenta at different gestational ages. We analysed the level of Noggin expression, by WB analysis, in (n=7) human placental sections from the first trimester (9-11 weeks), second trimester (16-26 weeks) and at term (32-42 weeks). Our analysis demonstrated that the expression of Noggin protein in human placenta varied at different gestational ages; Noggin was significantly higher in term compared to first and second trimester, in placental samples, and was detected as multiple bands between 30 and 40 kDa. Interestingly, the density of the protein bands increased throughout the course of gestation. Uniform expression of β-actin was seen, indicating that equal amount of proteins were loaded into each well of the gel for the WB (Figure 5.2).

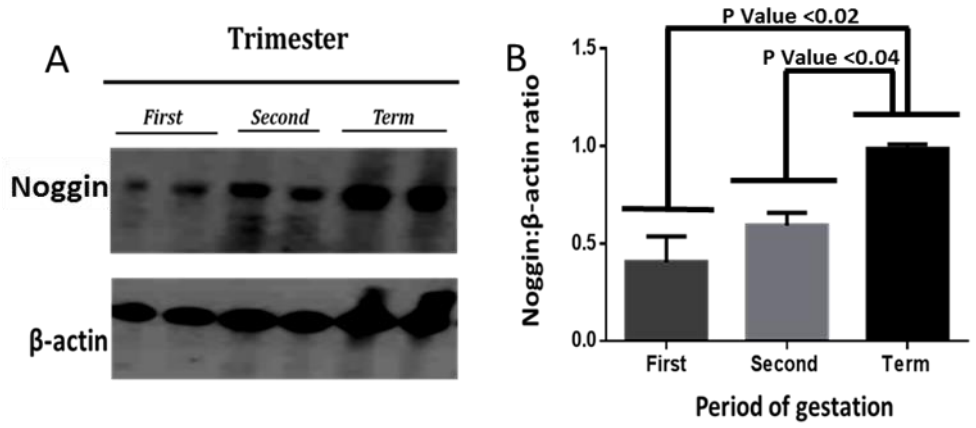

Figure 5.2 Expression of Noggin at different gestational ages of human placenta. The representative WB confirmed expression of Noggin during pregnancy. A: WB confirmed that Noggin, seen as a 30 kDa band, is expressed in humans during the first trimester, second trimester and at term. β-actin levels were used as the control to show equal loading. B: Comparison of Noggin expression in first, second, and term trimester. Data are expressed as mean ± S.E.M. Densitometric analysis of the same film was carried out using Image J. An average of three repeats were used to calculate significant differences in expression levels.

5.2.4 Localisation of Noggin expression in human placenta according to gestational age

Since we found that Noggin expression increases throughout the course of gestation in the human placenta, we further investigated the localisation of Noggin in the human placenta in the first trimester (9-11 weeks), second trimester (16-26 weeks) and at term (32-42 weeks). IHC was performed to determine the localisation of immunoreactive Noggin in human placental sections at different stages of gestation. Brown diaminobenzidine staining in the tissues indicates sites of immunoreactivity. Noggin staining can be observed with or without the light microscope. Negative controls were carried out at each gestational age by incubation of the section with either non–immune serum, or by pre-absorption of the primary antibody with excess synthetic control peptide.

Our analysis demonstrated that Noggin protein expression was observed in the human placenta in the first and second trimester, and at term, but was not significantly increased in the first and second trimester. However, Noggin staining was higher at term compared to first and second trimester. Noggin was localised in the endothelial cells of blood vessels (Figure 5.3). Strong staining for Noggin was predominantly found in the endothelium. Interestingly, the pattern and intensity of immunoreactive Noggin staining in the first and second trimester was weaker than in term placenta sections; Noggin was detected in the bilayer of the trophoblast (syncytiotrophoblast and cytotrophoblast), the endothelium and the

mesenchymal cells within the core of the chorionic villus. Moreover, Noggin staining was also observed in Hofbauer cells. Together our data, from WB and IHC experiments, indicates that Noggin is expressed in the placenta.

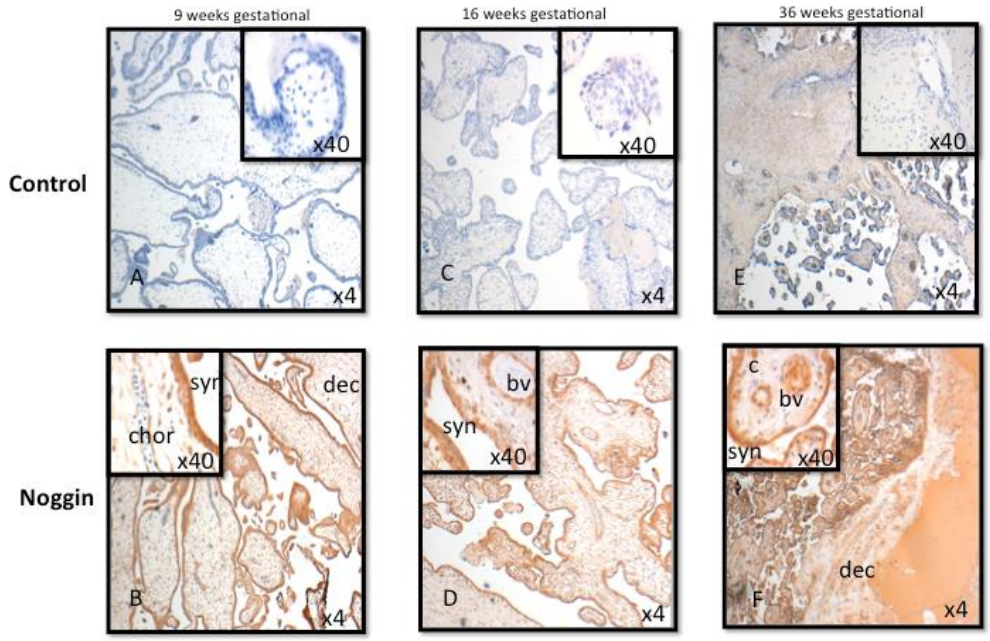

Figure 5.3 Immunolocalisation of Noggin in human placenta according to gestational age. Serial sections of first trimester and second trimester human placenta were stained. A: Control sections of first trimester human placenta were incubated without the primary antibody and showed no immunostaining (9 weeks). B: Intense staining of Noggin was observed in the syncytiotrophoblast (syn), decidual cells (dec) and vascular chorion (chor). C: Control sections of second trimester human placenta incubated without the primary antibody showed no immunostaining (16 weeks). D: Moderate staining for Noggin was detected in the maternal decidua; immunostaining was observed in the trophoblast shell, syncytiotrophoblast (syn) and intense staining in the the surrounding large blood vessels in the perivascular cells stem villi (bv) in the second trimester. B and D: Intense staining of Noggin in the first trimester (9 weeks), compared to the second trimester (16 weeks), was observed in the syncytiotrophoblast. E: Control sections of normal human placenta incubated without the primary antibody showed no immunostaining. F: Immunostaining for Noggin in normal human placenta was detected in the endothelial cells of the large blood vessels in stem villi (bv), the isolated cytotrophoblast (c), syncytiotrophoblast (syn) and maternal decidual cells (dec). Original magnifications: A, B, C, D, E, and F: x4 and x40.

5.2.5 Expression of Noggin is elevated in preeclamptic placenta

We further investigated the differences in Noggin protein expression between normal and PE placenta. To do so, we evaluated Noggin expression in the (n=11) human placental protein lysates by WB. Our results showed that Noggin protein expression almost reached significance in PE compared to matched controls. We also showed uniform expression of β-actin, indicating that equal amount of proteins were loaded into each well of the gel for the WB (Figure 5.4).

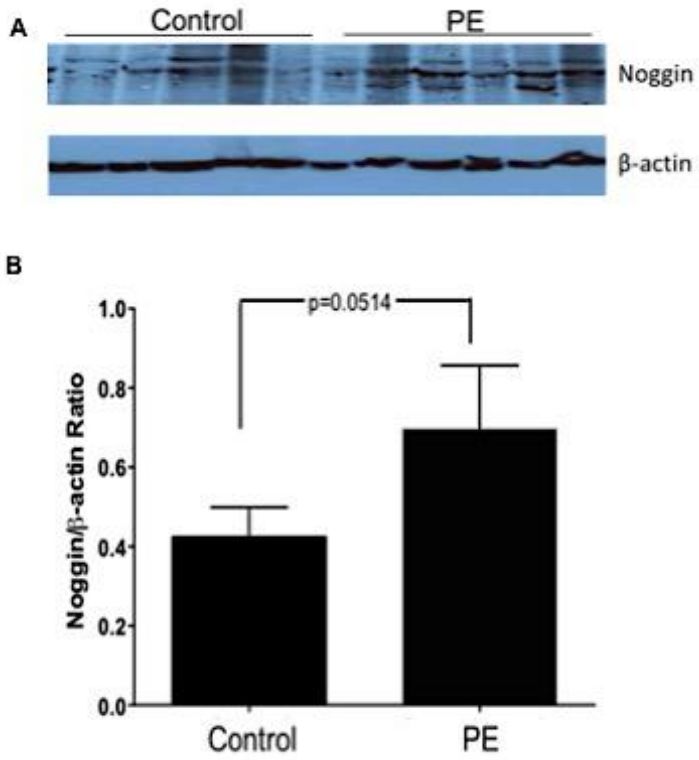

Figure 5.4 Expression of Noggin is high in preeclamptic placenta. A: Expression of Noggin was detected in the normal human placenta at term pregnancies and in PE (n=7), as a specific 30 kDa band. β-actin levels are shown as the control for equal loading. B: Comparison of Noggin expression in normal and PE placenta showed that expression of Noggin almost reached significance P=0.05. Data are expressed as mean ± S.E.M. Densitometric analysis of the same film was carried out using Image J, in order to calculate significant differences in expression levels. An average of three repeats were carried out for densitometric analysis.

5.2.6 Localisation of Noggin expression in preeclampsia placenta

We first evaluated the localisation of the Noggin protein in normal and PE placenta by IHC. We then made comparisons in the staining intensity and localisation of Noggin. Our analysis showed that immunostaining of Noggin was similar in normal term placenta (n=7) (37-42 weeks) compared with PE (n=7) (37-42 weeks) placenta. Greater staining was noted in the syncytiotrophoblast and in endothelial cells in PE placenta compared with normal placenta (Figure 5.5.A). Control sections of normal human placenta were incubated with the primary antibody pre-absorbed with excess control. However, ratio analysis revealed that there were no significant differences in Noggin staining in endothelial cells from the PE (n=7) placenta compared to the normal (n=7) placenta (Figure 5.5.B).

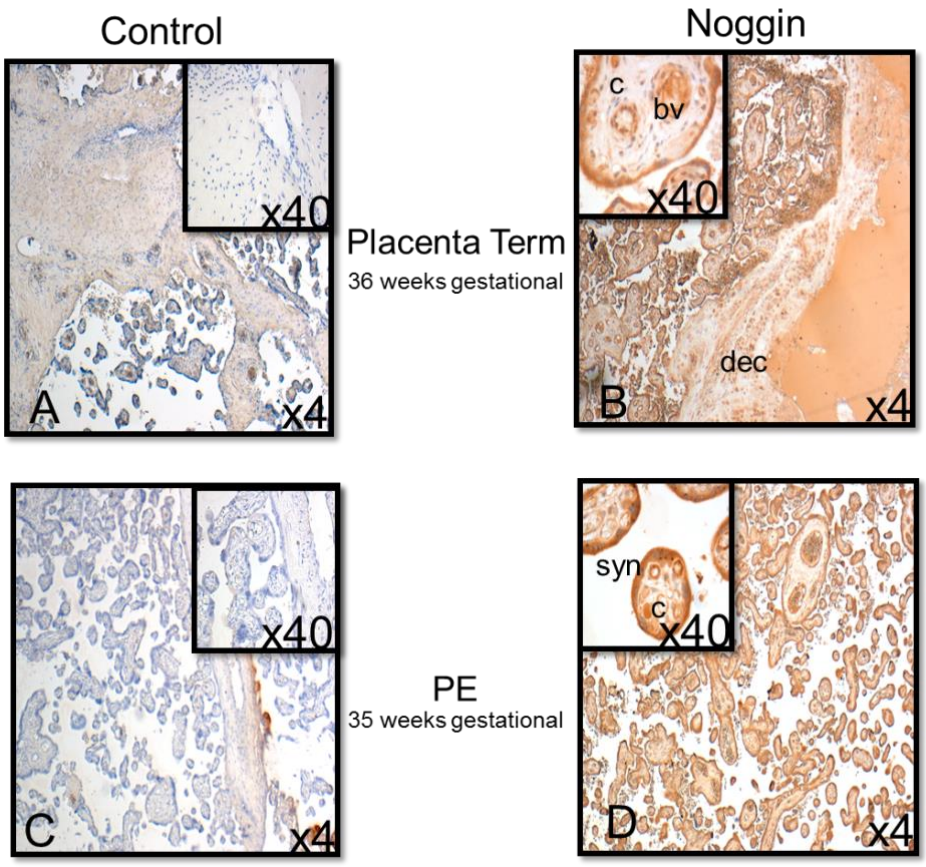

Figure 5.5.A Noggin immunostaining in preeclamptic placenta. Serial sections of term normal and PE placenta were stained (A, C 38 weeks gestational). A: Control sections of normal human placenta incubated without the primary antibody showed no immunostaining. B: In normal human placenta, immunostaining for Noggin was detected in endothelial cells of the large blood vessels in stem villi (bv), the isolated cytotrophoblast (c), syncytiotrophoblast (syn) and maternal decidual cells (dec). C: Control sections of PE placenta incubated without the primary antibody showed no immunostaining. D: In the PE placenta, intense immunostaining for Noggin was observed in syncytiotrophoblast (syn) and cytotrophoblast (c). B and D: (B: 36 weeks gestational) and preeclamptic placenta (D: 35 weeks gestational). A, B, C, and D x4, x40.

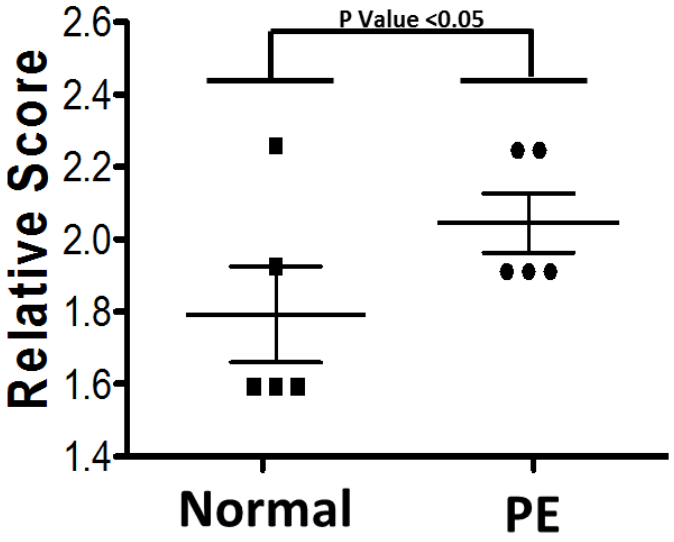

Figure 5.5.B Quantitation of Noggin immunostaining in normal and preeclamptic human placenta. Data was generated using Genie Histology Pattern visual score (horizontal axes). Quantitative results of IHC (0-5) showed that expression of Noggin almost reached significance P=0.05 (n=5) when comparing normal and PE human placenta. Each point represents an individual patient sample.Data is expressed as mean ± S.E.M.

5.2.7 *In vitro* tube formation

We have found that the level of Noggin is not significantly increased in PE. It is important to determine whether Noggin could promote or inhibit angiogenesis. To further investigate this we assessed the angiogenic function of Noggin in HUVECs. Cells were plated on growth factor-reduced Matrigel, in the presence and absence of Noggin or BMP4 or both. We used Noggin (0.5 µg/ml), BMP4 (0.1 µg/ml), and Noggin together with BMP4 to treat HUVECs for 24 hours. Collectively, these findings showed that Noggin, BMP4 and both Noggin and BMP4 act as negative regulators of angiogenesis compared to the control. Quantitative analysis demonstrated that Noggin does not have any effect on angiogenesis (Figure 5.6).

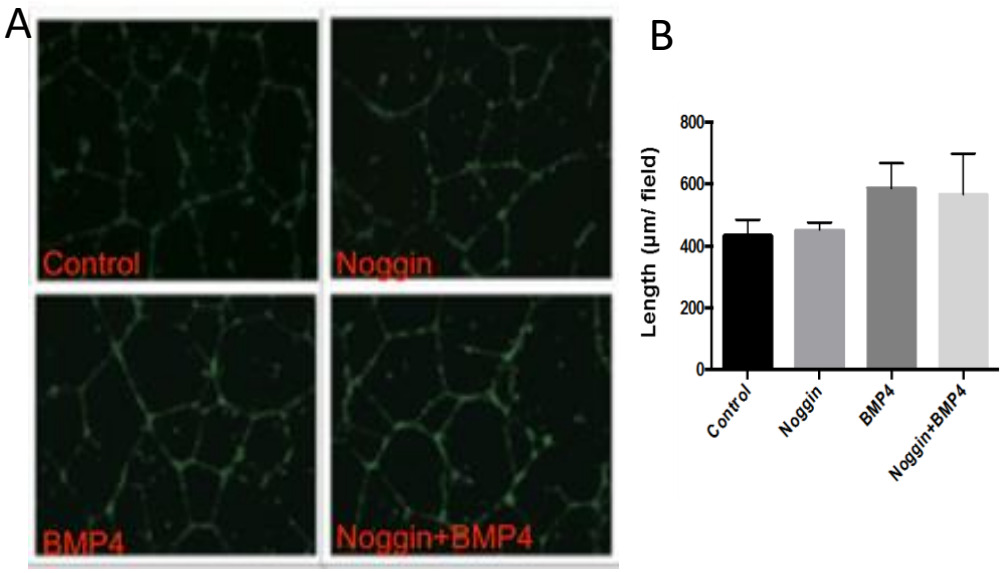

Figure 5.6 Effect of Noggin on *in vitro* angiogenesis. (A) Representative photomicrographs of HUVECs plated on Matrigel and treated with Noggin, BMP4 and both using fluorescent microscopy. (B) Tube formation was measured as total tube length per field. Quantification of tube length was performed using Image-Pro Plus image analysis software and expressed in µm/field. Data are expressed as mean ± S.E.M. Experiments were performed in triplicate.

5.2.8 Expression of Lrig1 mRNA in normal and abnormal human placenta

In the present study, we investigated whether gene expression of Lrig1 in human placenta explants could be important in PE. Real time qPCR was used to confirm expression of Lrig1 in the placenta. Interestingly, Lrig1 mRNA levels were significantly higher in PE compared to control and preterm (Figure 5.7).

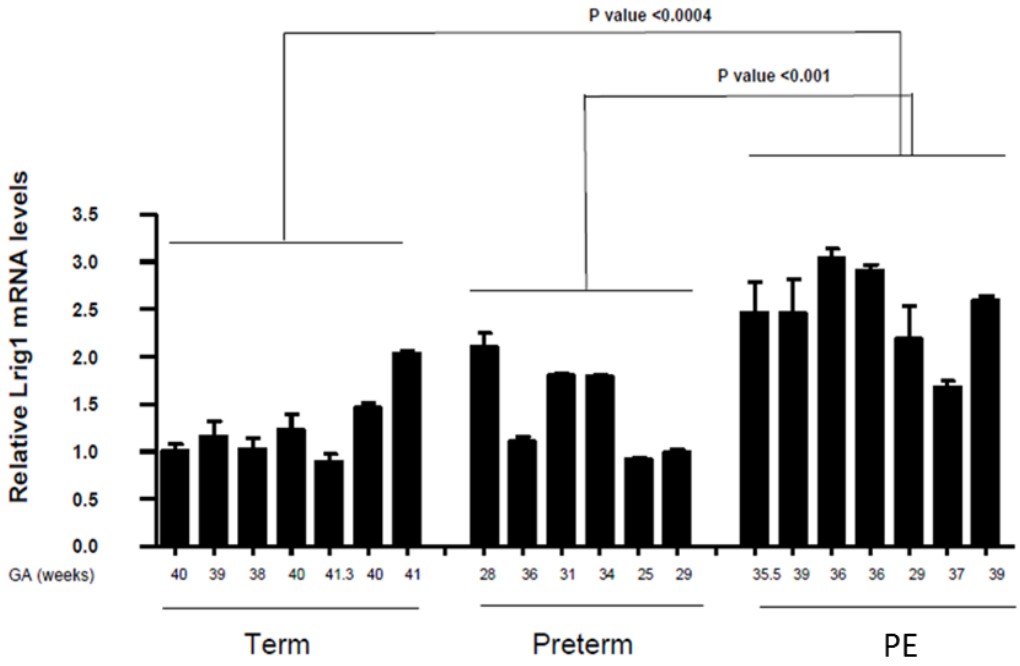

Figure 5.7 Comparison of Lrig1 mRNA expression levels in normal and preeclamptic human placenta. qPCR was performed to analyse Lrig1 mRNA expression in normal (n=7), PE (n=7) and preterm (n=6) placental tissues. Each experiment was performed in triplicate; mRNA values were normalized to β-actin. Data are expressed as mean ± S.E.M. (**P ≤0.001, ***P ≤ 0.0004) compared to the control.

5.2.9 Localisation of Lrig1 expression in preeclampsia placenta

We next investigated the localisation of the Lrig1 protein in normal and PE placentas (third trimester pregnancy) by IHC. We then made comparisons in the staining intensity and localisation. IHC showed that the Lrig1 protein is intensely expressed in the endothelial cells, as well as trophoblasts and Hofbauer cells (Figure 5.8.A). In normal placentas, Lrig1 staining intensity appeared weaker in the endothelial cells (Figure 5.8.A,B) and appeared stronger in trophoblasts. In PE placentas, Lrig1 staining intensity appeared stronger in the endothelial cells (Figure 5.8.A,F), and trophoblasts. The cell adhesion molecule (CD31) was used as a marker of endothelial cells (Figure 5.8.A,C), and cytokeratin 7 (Cyt7) was used as a trophoblast marker (Figure 5.8.A,D). Control sections of normal human placenta incubated with the primary antibody pre-absorbed with excess control. In agreement with our previous results of mRNA fold change expression (Figure 5.7), ratio analysis revealed that Lrig1 in immunostaining was significantly higher in the PE (n=9) placenta compared to the normal (n=9) placenta (Figure 5.8.B).

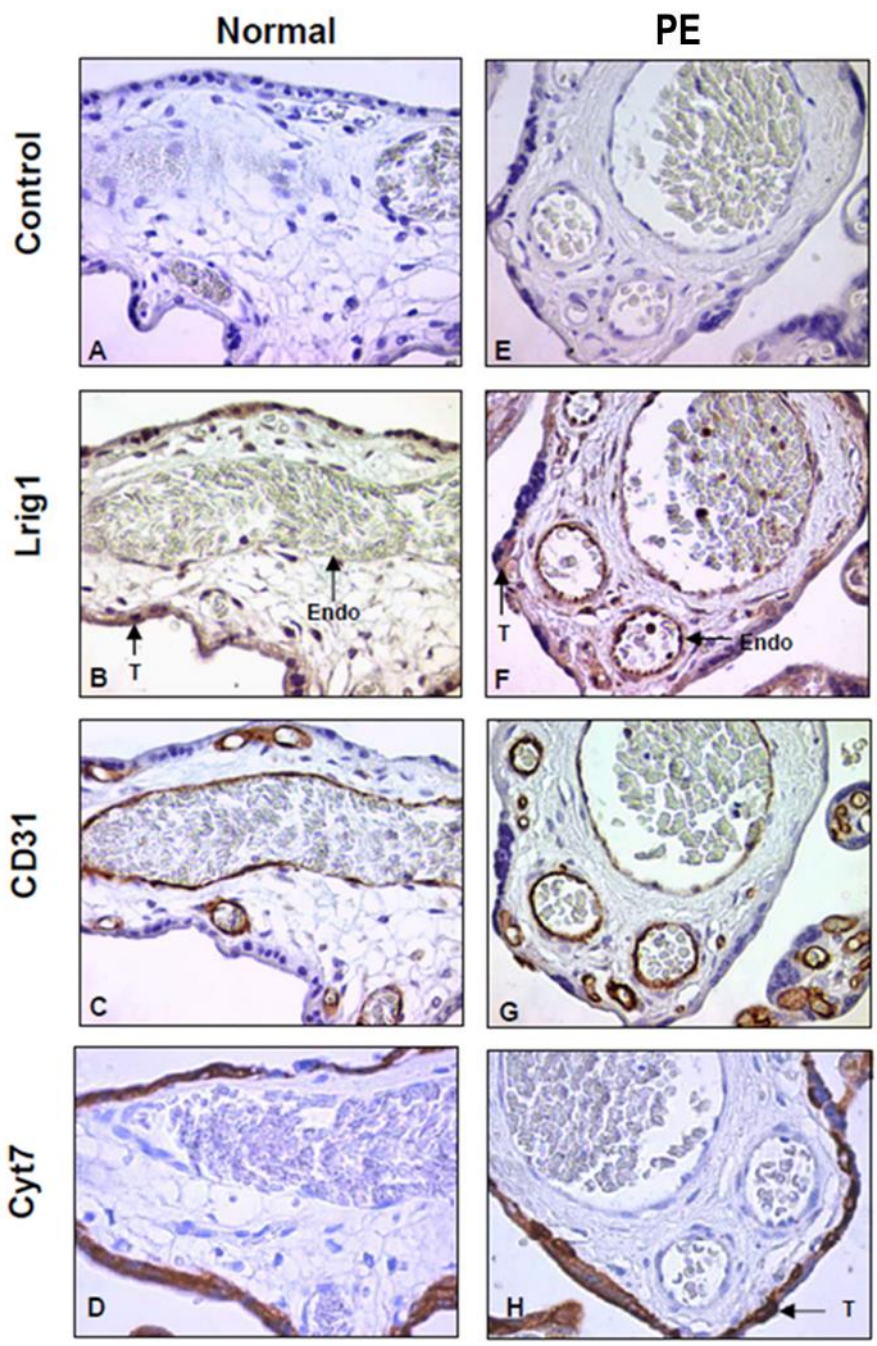

Figure 5.8.A Immunolocalisation of Lrig1 in normal and preeclamptic placenta. Serial sections of term normal (36 weeks gestational) and PE (35 weeks gestational) placenta. A,E: control sections incubated with normal goat serum showed no immunostaining. B,F: sections were incubated with Lrig1 antibody; Lrig1 staining was detected in endothelial cells (Endo) and Trophoblast (T) in PE. C,G: sections were incubated with CD31 antibody. D,H: sections were incubated with Cyt7 antibody; Cyt7 staining was detected in T. Original magnifications: A, B, C, D, E, F, G and H: x4.

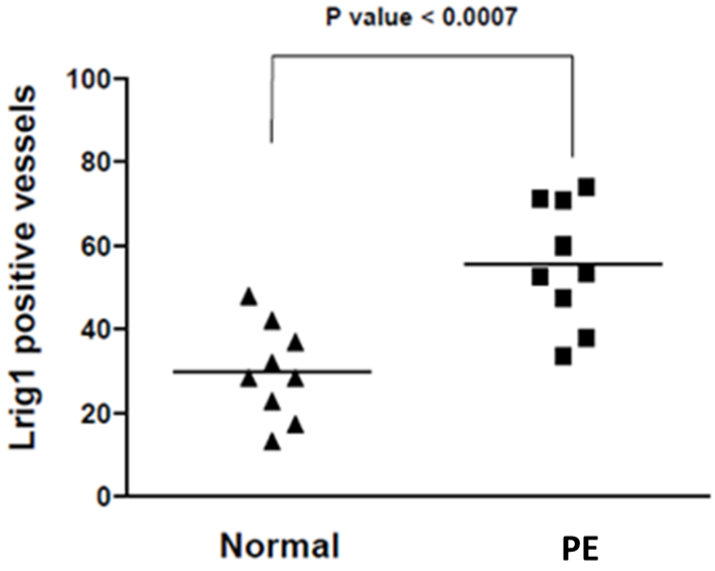

Figure 5.8.B Quantitation of Lrig1 in normal and preeclamptic placenta. The ratio of Lrig1 to CD31 positive vessels in normal (n=9), and PE (n=9). Data was generated using Genie Histology Pattern visual score (horizontal axes). Quantitative results of IHC from (0-5) showed that expression of Lrig1 was significant. Each point represents an individual patient sample. Data are expressed as mean ± SEM or representative of five or more separate experiments performed in triplicate (***P ≤ 0.0007).

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5.3 Discussion

The placenta is the key organ involved in PE. Identification of genes differentially expressed between normotensive and preeclamptic placentas is an important step in understanding the molecular mechanisms involved in the aetiology of the disease, and for the development of novel biomarkers as well as identifying therapeutic targets in complicated pregnancies.

In the present study, WB analysis showed that Noggin was expressed in the placenta during gestation. Interestingly, Noggin protein expression levels appeared to significantly increase with increasing gestational age of the human placenta. In addition, using qPCR, we showed that Noggin mRNA expression levels did not change between normal and PE placenta. However, by WB analyses this study has shown that Noggin protein expression in PE almost reached significant levels compared to controls. Furthermore, we are the first to show that strong expression of Noggin occurs in the trophoblasts (syncytiotrophoblast and cytotrophoblasts), endothelial cells and mesenchymal cells within the core of the chorionic villus, and Hofbauer cells in the placenta of preeclamptic patients. However, the preeclamptic placental sections showed no differences in the staining pattern of Noggin compared to normal term placenta. This should rule out the role of Noggin in PE.

Angiogenesis is a vital process for normal tissue development and wound healing, but is also associated with a variety of pathological conditions.

PE occurs during the second-trimester stages of pregnancy, due to an imbalance of pro-angiogenic and anti-angiogenic molecules that control the villous angiogenesis and maturation of the vasculature of the placenta (Laresgoiti-Servitje and Gomez-Lopez, 2012).

There is ample evidence that Noggin is involved in regulation of vasculogenesis and angiogenesis (Kang et al., 2009). A previous study has shown that Noggin is not expressed in endothelial cells (Kang et al., 2009). In this study, quantitative analysis showed that pre-treatment of HUVECs with Noggin and BMP4 did not prevent the inhibition in tube formation, suggesting that Noggin did not act directly.

Lrig1 is normally expressed in human tissues including the brain, breast, kidney, lung, and other tissues (Suzuki et al., 2002) as two species: 143 kDa and 134 kDa species. Lrig1 is an essential cell surface membrane protein that is expressed by specific cells in various human tissues (Nilsson et al., 2003; Nilsson et al., 2001). We evaluated the expression of the Lrig1 gene and protein in the placenta. Analysis by IHC revealed that the Lrig1 protein is present in both term normal and term PE sections, as seen by a high staining intensity of Lrig1.

In this study, we found that there was higher expression of Lrig1 protein in PE placenta as compared to normal placenta. Thus, expression of Lrig1 seems to play an important role in the placenta, and Lrig1 could be an independent marker in the preeclamptic placenta. Lrig1 has been

shown to inhibit prostate cancer cell proliferation in Swedish patients (Thomasson et al., 2011). Lrig1 was recently shown to be an oestrogen regulated growth suppressor in breast cancer (Krig et al., 2011). Lrig1 also negatively regulates the growth stimulatory EGF receptor (Hedman and Henriksson, 2007). At the molecular level our data demonstrates an apparent difference in the expression of Lrig1, which was significantly up-regulated in PE placenta than normal placenta and preterm placenta. Levels of Lrig1 protein could be of interest as a potential cause for PE. PE does not depend on a single maternal or fetal gene. There are many candidate genes, which are not described here, but are reviewed elsewhere in appendix V (Haram et al., 2014; Williams and Broughton, 2011; Redman and Sargent, 2005; Van Dijk et al., 2005). Due to a lack of supply of placental tissue as well as clinical information about the patients, we could not investigate Lrig1 expression in other types of pregnancy complications such as IUGR. Future studies should investigate this to provide more clarity on the matter. Further studies addressing Noggin expression in the fetal genotype, and the molecular functions of Lrig1 are necessary in order to provide a more comprehensive understanding of the role of these proteins in PE.

Our results highlight that the Noggin and Lrig1 genes are expressed in the placenta. There were no significant differences in the expression of Noggin mRNA and protein between normal and abnormal placenta. However, WB analysis showed that the expression of Noggin protein was not higher in the PE placenta. In contrast, Lrig1 mRNA and protein

expression were significantly higher in the preeclamptic placenta compared to normal placenta.

CHAPTER SIX: CONCLUSION AND FUTURE WORK

6.1 Conclusion and future work

In the 1980s and early 1990s it became apparent that inflammation was linked to PE, as well as other vascular disorders in endothelial cells (Lockshin and Branch, 2006; Saftlas et al., 2005). Cytokines are likely to contribute to endothelial dysfunction or be accountable for the cause of PE. In PE, there are increased circulating levels of TNF-α and IFN-γ (Conrad et al., 1998). Furthermore, intermittent perfusion of the placenta, secondary to reduced trophoblast invasion, causes increased secretion of TNF-α and IFN-γ (Hung et al., 2004).

Our findings document a complex pattern of up-regulation of sFkn release in the presence of cytokines. sFkn release is very low in stimulated endothelial cells, but sFkn release was increased markedly after stimulation of cells by TNF-α and IFN-γ. However, sFkn release was very low or undetectable after stimulation of cells with IL-1β. The mechanisms behind this are not yet understood and require further research. In this study, we also tested whether angiogenic factors have an effect on the release of sFkn. We found that PlGF, VEGF-A and VEGF-E did not affect sFkn release in endothelial cells.

The management of PE involves taking relevant blood samples, collecting urine, and treatment using anti-hypertensive medication. However, this medication may also impact on reproductive events such as ovulation, menstruation, embryo implantation, parturition, and disease conditions such as endometriosis and cancer (Jones et al., 2004).

Delivery of the placenta is the only known cure for PE (Frampton et al., 2016); however, several recent reports underline the importance of cytokines and other mediators of inflammation as therapeutic targets for PE (Rider et al., 2016). As such our study focused on the development of new anti-inflammatory therapeutic strategies for PE. Our study indicates that Honokiol has a significant anti-inflammatory effect. Honokiol significantly reduced sFkn release in endothelial cells stimulated with IFN-γ. However, the signalling pathway for this has not yet been fully investigated.

In addition, we found that pre-treatment of endothelial cells with actinomycin D and cycloheximide had an inhibitory effect on sFkn release from endothelial cells in the presence of TNF-α and IFN-γ. Furthermore, we observed a reduction in sFkn release when endothelial cells were treated with the HO-1 inhibitors, CrPP and SnPP, in the presence of TNF-α and IFN-γ. HO-1 helps to reduce oxidative stress induced factors, such as reactive oxygen species, and has also been proposed to play a protective role in PE (Zenclussen et al., 2011; Ollinger et al., 2007). The HO-1 enzyme system generates three molecules (Biliverdin, Fe^{2+} and CO), which are unique as they all have biological activity. Originally, it was proposal that a lack of HO/CO activity could be a predisposing factor leading to PE (Ryter and Choi, 2013). Furthermore, our study provides the first evidence that bilirubin, CORM and hemin can reduce sFkn release in endothelial cells (Figure 6.1). Previously, it has been shown that women with PE have significantly reduced CO concentrations in their

exhaled breath, compared to women with healthy pregnancies, indicating decreased HO activity (Kreiser et al., 2004). Using a Hmox1$^{-/-}$ mouse model to assess the role of HO-1 in sFkn release we found that sFkn release was increased in the lungs and the heart tissue of KO mice in the presence and absence of TNF-α and IFN-γ. These findings futher suggest that a novel therapeutic approach in the form of HO-1 based therapeutic strategies for the treatment of PE should be explored.

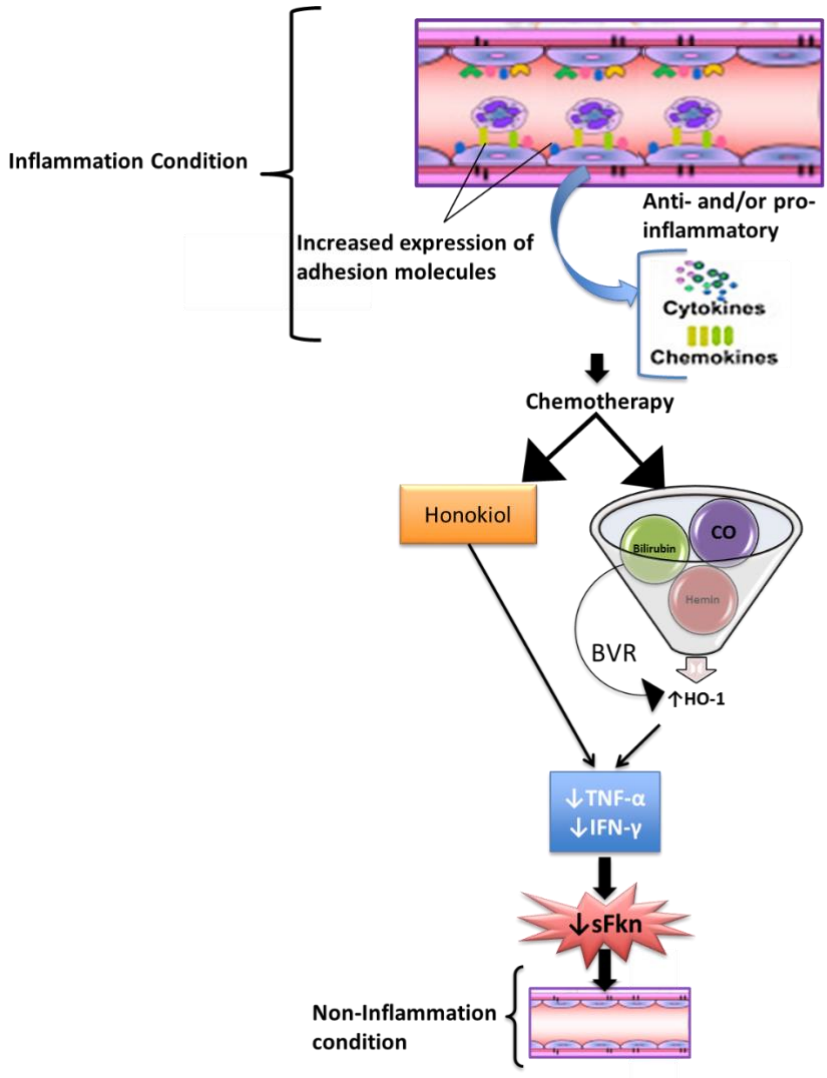

Figure 6.1 Schematic illustration of novel therapeutic strategies to reduce soluble Fractalkine in endothelial cells. Honokiol has protective anti-inflammatory effects on TNF-α and IFN-γ and on sFkn release. Moreover, HO-1, hemin, CO, bilirubin and BVR have anti-inflammatory effects on suppressing the effects of TNF-α and IFN-γ and on sFkn release in endothelial cells.

We have identified high expression of two novel placental factors, Noggin and Lrig1. We further investigated the role of these genes in relation to PE. We identified that Noggin protein levels were increased during gestation. However, no significant changes in the level of Noggin were observed in the PE placenta compared to the normal placenta. In

addition, we found that Noggin does not affect angiogenesis in HUVECs, although further work is needed regarding the role of Noggin in angiogenesis. These studies suggested that up-regulation of Noggin does not necessarily lead to abnormalities associated with disorders like PE.

We are the first to evaluate the expression of the Lrig1 gene in the placenta. Lrig1 is expressed both in normal and PE placenta at the protein and RNA level. We identified that protein and RNA expression of Lrig1 was significantly higher in the PE placenta compared to the normal placenta. This suggests that Lrig1 could be an independent molecular marker for PE, however further work is required to confirm this.

Granted, if there was more time and resources it would have been interesting to test the relation between Noggin release and PlGF in pregnancy. Parallel investigations in trophoblast cells to examine the roles of Noggin and BMP also need to be carried out. Furthermore, the effect of Noggin and BMP in the regulation of angiogenic factors in normal and PE placental explants has to be assessed. Additionally, it will also be important to extend the Lrig studies in order to ascertain its molecular function in the placenta, and the role of Lrig proteins in placental angiogenesis. This may provide further insights into the signalling and metabolic pathways involved in PE, and lead to further studies to establish ways to prevent or treat PE.

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APPENDIX I

Chemical reagents and suppliers

Acetic acid (glacial):	Sigma, Poole, U.K.
Acrylamide solution (40%):	Bio-Rad, Hemel Hempstead, U.K.
Adenovirus:	
Ad-β-gal:	**Gift**: C.Kontos, Duke University, U.S.A.
Ad-HO-1:	**Gift**: J.Alam, Louisiana State University, U.S.A.
Ad-CMV:	Vector Biolabs, Philadelphia, U.S.A.
Ad-Akt:	Vector Biolabs, Philadelphia, U.S.A.
α-defensins ELISA:	Hycult Biotech, Uden, Netherlands
β-mercaptoethanol:	Sigma, Poole, U.K.
Bio-Rad protein assay:	Bio-Rad, Hemel Hempstead, U.K.
Bovine serum albumin:	Sigma, Poole, U.K.
Bromophenol blue:	Sigma, Poole, U.K.
Calcein AM Fluorescent dye:	BD Biosciences, Oxford , U.K.
Calprotectin (MRP8/14) ELISA:	BMA Biomedicals, Switzerland
Cell scrapers:	Sarstedt, Leics, U.K.
Collagenase A:	Boehringer Mannheim, Sussex, U.K.
DABCO:	Sigma, Poole, U.K.
DAKO StreptABC Complex/HRP Duet kit:	Dako, Denmark.
Decon-90:	Phillip Harris, Staffs, U.K.

Dextran:	Sigma, Poole, UK.
Diaminobenzidine:	Sigma. Poole, U.K.
DMEM:	ICN, Basingstoke, U.K.
DMEM (phenol red free):	GibcoBRL, Paisley, UK.
DMSO:	Sigma, Poole, U.K.
DPEX mounting medium:	BDH, Poole, U.K.
DTT:	Pharmacia, Herts., U.K.
ECL detection kit:	Amersham, Buckinghamshire, U.K.
EDTA:	Sigma, Poole, U.K.
Endothelial cell growth supplement	Sigma, Poole, U.K.
EGF:	Peprotech, London, U.K.
Ethanol (99.7-100%):	BDH, Poole, U.K.
Ethyl acetate:	BDH, Poole, U.K.
Fetal calf serum:	GibcoBRL Life Technologies, Scotland.
Formaldehyde:	Sigma, Poole, U.K.
Gelatin:	Sigma, Poole, U.K.
Glycerol:	Sigma, Poole, U.K.
Glycine:	CN Biosciences, Nottingham, U.K.
Goat serum:	Sigma, Poole, U.K.
HAMS-F12:	ICN, Basingstoke, U.K.
Hanks buffered saline solution HBSS:	Sigma, Poole, U.K.
Hematoxylin:	Vector, Burlingame, CA, U.S.A.
Hybond ECL nitrocellulose membrane:	Amersham Int., Buckinghamshire, U.K.

Hydrogen chloride:	Sigma, Poole, U.K.
Hydrogen peroxide:	JT Baker Inc., CA, U.S.A.
Human IL-6 ELISA:	Pierce-Endogen, IL, U.S.A
Isopropanol:	Sigma, Poole, U.K.
Kaleidoscope prestained standards:	Bio-Rad, Hemel Hempstead, U.K.
Kodak, Biomax MR film:	Anachem, Luton, U.K.
L-Glutamine:	Sigma, Poole, U.K.
L-NNA:	CN Biosciences, Nottingham, U.K.
Lipopolysaccharide:	Sigma, Poole, U.K.
Leupeptin:	Sigma, Poole, U.K.
M199 (Earles buffer):	Sigma, Poole, U.K.
Marvel dried milk:	Sainsbury's, U.K.
Mayer's Haematoxylin:	Sigma, Poole, U.K.
Matrigel:	BD Biosciences, Oxford, UK
Methanol:	BDH, Poole, U.K.
MOPS:	Sigma, Poole, U.K.
MMP 2/9 inhibitor:	Calbiochem, UK.
MTT:	Sigma, Poole, U.K.
Percoll:	Sigma, Poole, U.K.
PMSF:	Sigma, Poole, U.K.
Phosphatase Inhibitor Cocktail I:	Sigma, Poole, U.K.
Phosphatase Inhibitor Cocktail II:	Sigma, Poole, U.K.
PBS tablets:	Sigma, Poole, U.K.

PlGF Duoset ELISA:	R&D systems, Abingdon, U.K.
PlGF Quantikine ELISA:	R&D systems, Minneapolis, U.S.A.
Potassium chloride:	Sigma, Poole, U.K.
Protease Inhibitor Cocktail:	Sigma, Poole, U.K.
Resveratrol:	Sigma, Poole, U.K.
RNA easy columns:	Qiagen, West Sussex, UK
Scott's solution:	Sigma, Poole, U.K
SDS:	Sigma, Poole, U.K.
Sodium hydroxide:	Sigma, Poole, U.K.
Sodium iodide:	Sigma, Poole, U.K.
Sodium nitrite:	Sigma, Poole, U.K.
Streptomycin:	GibcoBRL Life Technologies, Scotland.
sVEGFR-1 Duoset ELISA:	R&D systems, Abingdon, U.K
SYBR green:	Quantace, London, UK
TCA:	Sigma, Poole, U.K.
TEMED:	Bio-Rad, Hemel Hempstead, U.K.
Triethanolamine:	Sigma, Poole, U.K.
Tris:	CN Biosciences, Nottingham, U.K.
TRITC-phalloidin:	Sigma, Poole, U.K.
Triton X-100:	Sigma, Poole, U.K.
Trypsin/EDTA:	Sigma, Poole, U.K.
Tween-20:	Sigma, Poole, U.K.
VEGF:	RELIATech, Brauschweig, Germany.

Whatman 3 MM paper:	Whatman, Kent, U.K.
Wortmannin:	Calbiochem, Nottingham, U.K.
Xylene:	JT Baker Inc., CA, U.S.A.

APPENDIX II

Equipment and Suppliers

Cell culture Pipettes (5ml and 10 ml):	Fahrenheit Lab Supplies, U.K.
Centrifuge (Sigma 2K 15):	Sigma, Poole, U.K
Class II cell culture cabinets:	Triple Red, Oxfordshire, U.K.
Conical tubes (15 ml):	GibcoBRL, Paisley, U.K
Coverglass:	Surgipath, St Neots, U.K
Cryovials:	GibcoBRL, Paisley, U.K
Developing Cassettes:	Amersham Int., Buckinghamshire, U.K
Disposable Scalpels:	Appleton Woods, Birmingham, U.K
Eppendorfs:	Sarstedt, Leicester, U.K
Falcon tubes (14 and 50 ml):	Falcon/BDH, Poole, U.K
Filter units (swinnex 47 and 22):	Millipore, Hertfordshire, U.K
Filters (0.22 mm):	Millipore, Hertfordshire, U.K
Flasks (25 and 80 cm2):	GibcoNUNC, Paisley, Scotland
Gilson pipettes:	Anachem, Luton, U.K
Gilson tips (blue):	Appleton Woods, Birmingham, U.K
Gilson tips (yellow):	Sarstedt, Leicester, U.K
Glass Pasteur pipettes 9”:	Fisher Scientific, Loughborough, U.K.
Glassware:	Phillip Harris Scientific, U.K.
Horizontal gel electrophoresis system:	GibcoBRL Life Technologies, Scotland
Intensifying screens:	Amersham, Buckinghamshire, U.K

Micro-centrifuge:	Phillip Harris Scientific, U.K.
Microscope slides (Superfrost):	Fisher Scientific, U.K
Mini sub DNA gel:	Bio-Rad, Hemel Hempstead, U.K.
Mini-monitor (900):	Mini-instruments, Essex, U.K
Modular Incubators:	ICN, Basingstoke, U.K.
Multiwell Plates (6, 12 and 24-wells):	Fahrenheit Lab Supplies, U.K.
NOA 270/280B Analyser:	Analytix, Durham, U.K.
Orbital shaker:	Phillip Harris Scientific, U.K.
Petri dishes (30 and 90 mm2):	GibcoBRL Life Technologies, Scotland.
pH meter:	Corning costar, High Wycomb, U.K.
Polytron Homogeniser PT1200:	Phillip Harris Scientific, U.K.
Rotary shaker (R100):	Luckham, Basingstoke, U.K
Round Petri dishes:	Fahrenheit Lab Supplies, U.K.
Scintillation counter:	Canberra Packard, Pangbourne, U.K.
Shaking water bath:	Grant Instruments, Cambridge, U.K
Sonicator (T460):	Camlab, Cambridge, U.K.
Spectrophotometer 8452A:	Hewlett Packard, Bracknell, U.K.
Spinmix:	Sanyo-Gallenkamp, Leicester, U.K.
Square petri dishes:	Fahrenheit Lab Supplies, U.K.
Syringes (1ml - 50 ml):	Appleton Woods, Birmingham, U.K
Transfer-blot electrophoresis transfer cell:	Bio-Rad, Hemel Hempstead, U.K.
Universals (30 ml):	Phillip Harris Scientific, U.K.
Vertical gel electrophoresis units:	Bio-Rad, Hemel Hempstead, U.K.

Water-Jacketed Incubator:	Sanyo-Gallenkamp, Leicester U.K.
Weight Balance:	Sartonius Limited, Surrey, U.K

APPENDIX III

Solutions, media, and buffers

Antibody Diluent

Tris buffered saline (TBS) pH 7.6

0.5% Bovine serum albumin (BSA)

0.1% Sodium Azide

Blocking Buffer

TBS pH 7.6

5% Fat-free milk

Column Wash Buffer (pH 8.0)

0.05M Sodium dihydrogen phosphate (NaH_2PO_4)

0.01M Imidazole

0.1M Sodium chloride (NaCl)

Gel to Membrane Transfer Buffer

39mM Glycine

48mM Tris

1.3mM Sodium dodecyl sulfate (SDS)

20% Methanol

MBE-medium

DMEM containing phenol red

10% Calf serum

3mM Glutamine

100U/ml Penicillin

100μg/ml Streptomycin

5μM β-mercaptoethanol

1% Non-essential amino acids

20μg/ml equal amounts of bFGF and aFGF (ECGF)

5μg/ml Heparin (increases activity of ECGF)

*Not Fetal bovine Serum (FBS) – Endothelial cells are relatively slow growing and FBS (contains different growth factor content e.g. more PDGF) causes overgrowth of other cell types from the embryo. Culture must be 100% pure endothelial cells before addition of FBS.

Resolving gel 7.5%:

3.64ml of 40% Acrylamide,
2ml of 2% Bis/Acryl,
5ml of 1M Tris
4.06ml Dstilled water (d.H_2O)
5ml of 0.4% Gelatin solution
10µl Tetramethylethylenediamine (TEMED)
100 µl 10% Ammonium persulphate

RIPA Buffer

50mM Tris pH 7.4
1% IGEPAL
0.25% Sodium chloride (NaCl)
1mM Aminopolycarboxylic acid (EGTA)

Running buffer:

0.05% Tris,
0.384M Glycine,
0.1% Sodium dodecyl sulfate (SDS)

Sample buffer 2X:

2mM Ethylenediaminetetraacetic acid (EDTA)
0.02M Tris-HCL pH 8
10% Mercaptoethanol 20% Glycerol Bromophenol Blue to colour
2% Sodium dodecyl sulfate (SDS)

Sample buffer 4x:

0.04 M Tris

4 mM Ethylenediaminetetraacetic acid (EDTA)

4% Sodium dodecyl sulfate (SDS)

40% Glycerol

0.02% Bromophenol blue

Separating buffer:

1.875M Tris

pH 8.8

0.5% Sodium dodecyl sulfate (SDS)

Separating gel:

2.5ml Bis-Acryl

4.55ml 40% Acrylamide

188µl 10% Ammonium persulphate

3.75ml Separating buffer

7.6ml Dstilled water (d.H_2O)

19µl Tetramethylethylenediamine (TEMED)

Solubilisation solution:

0.01% Triton X-100

1mM Bicarbonate buffer pH 7.6

1mM Tetramethylethylenediamine (TEMED)

Stacking gel buffer:

pH 6.8

0.625M Tris

0.5% Sodium dodecyl sulfate (SDS)

Stacking gel:

1.5ml Acrylamide

0.8ml Bis-Acryl

3ml Stacking buffer

9.6ml Dstilled water (d.H_2O)

15ml Tetramethylethylenediamine (TEMED)

150ml Ammonium persulphate.

Transfer buffer:

25mM Tris

190mM Glycine

40% Methanol.

TBS:

0.1M Tris,

0.1% Tween-20 pH 7.5.

0.3M Sodium chloride (NaCl)

APPENDIX IV

The ethical approval for samples.

CLÍNIC
Corporació Sanitària

CLÍNIC
BARCELONA
Hospital Universitari

Dña. Begoña Gómez Pérez , del Servicio de Farmacia del Hospital Clínic de Barcelona y Secretaria del Comité Ético de Investigación Clínica (CEIC)

CERTIFICA:

Que el Comité Ético de Investigación Clínica, según consta en el acta de la reunión celebrada en el día de hoy, ha analizado el proyecto de investigación titulado:

Exploraty study for the development of a combined screening test for the prediction of preeclampsia in the first trimester of pregnacy.

cuyo investigador principal es el Dr. **Gratacós, Eduard**
del Servicio de ***Ginecología y Obstetricia***
entendiendo que dicho estudio se ajusta a las normas éticas esenciales y criterios deontológicos que rigen en este Centro, y, por tanto, ha decidido su aprobación.

Lo que firmo en Barcelona, a 29/01/2009

CLÍNIC
Hospital Universitari
COMITÈ ÈTIC
INVESTIGACIÓ CLÍNICA

Registro: 2009 / 4697

CIF - G-08431173

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APPENDIX V

Introduction

Real-Time PCR, also called quantitative polymerase chain reaction (qPCR), is one of the most powerful and sensitive gene analysis techniques available and is used for a broad range of applications including quantitative gene expression analysis, genotyping, SNP analysis, pathogen detection, drug target validation, and for measuring RNA interference. Frequently, real-time polymerase chain reaction is combined with reverse transcription to quantify messenger RNA (mRNA). There are two main methods used to perform quantitative PCR: dye-based, or non-specific detection, and probe-based, or specific detection. Both methods rely on calculating the initial (zero cycle) DNA concentration by extrapolating back from a reliable fluorescent signal.

The causes of PE are not entirely understood. To better understand the molecular events associated with PE, research determining the novel key genes involved in PE needs to be carried out. This study aimed to identify placental expression of arginine demethylase and lysine hydroxylase (jmjd6), Interleukin 6 (IL6), angiopoietin (ANGPT), 8-Oxoguanine glycosylase (OGG1), Arg1, argininosuccinate lyase (Asl), Biliverdin Reductase A (BLVRA), and argininosuccinate synthase 1 (Ass1) genes in expression in abnormal placenta. In the present study, using the qPCR method, we designed a three stage 8 case study of control and complicated pregnancies as well as 8 cases of severe preeclampsia

(sPE) and 8 cases of IUGR pregnancies. This was carried out to evaluate whether there was a difference between the preeclampsia, and IUGR placenta compared with the controls. Our findings support the hypothesis that preeclampsia shares some underlying genetic cause. We investigated the expression of jmjd6, IL6, Angpt4, OGG1, Arg1, Asl, BLVRA, Ass1 mRNA in preeclamptic placenta. We found that BLVRA and Ass1 in preeclamptic placenta is elevated compared to normal pregnancies. The exclusion criteria in the three study groups were the presence of infectious disease or medical complications including autoimmune disorder, diabetes mellitus and inflammatory conditions.

Table 6.1. Clinical characteristics of patent groups included in the study.

Characteristics	Pregnant control (n=8)	sPE (n=8)
Patient age at delivery (years)	29 (15-40)	27 (14-42)
Weight (kg)	73.90 (62.3-102.7)	87.75 (56.40-149.5)
Parity	1 (0-10)	0 (0-6)
Gestational age at collection (weeks)	29.75 (21.4-33.5)	30.5 (24.1-34.2)
Gestational age at delivery (weeks)	39 (37.20-41)	30.5 (24.1-34.2)
Systolic blood pressure > 20 weeks (mm Hg)	106 (90-140)	168 (130-220)
Diastolic blood pressure > 20 weeks (mm Hg)	63 (50-76)	101 (88-130)
Dipstick proteinuria	-	3 (0-4)
Values shown as median and range (minimum and maximum values) ***P <0.05, Mann-Whitney test**		

Results

To identify genes and genetic variants for preeclampsia using the relative expression levels calculated from the real-time PCR experiments Statistical analysis of intergroup comparisons between control (CON) (n=8), UGR (n=8) and sPE (n=8) were carried out. There was no significant difference in the values in jmjd6, IL6, Angpt4, OGG1, Arg1, Asl genes. However, in BLVRA, and Ass1 genes expression were different between PE and the control group. During the analysis comparisons were made we did not consider the maternal risk factors. The expression patterns for the genes examined by real-time PCR thus in general confirm the trends observed by microarray analysis.

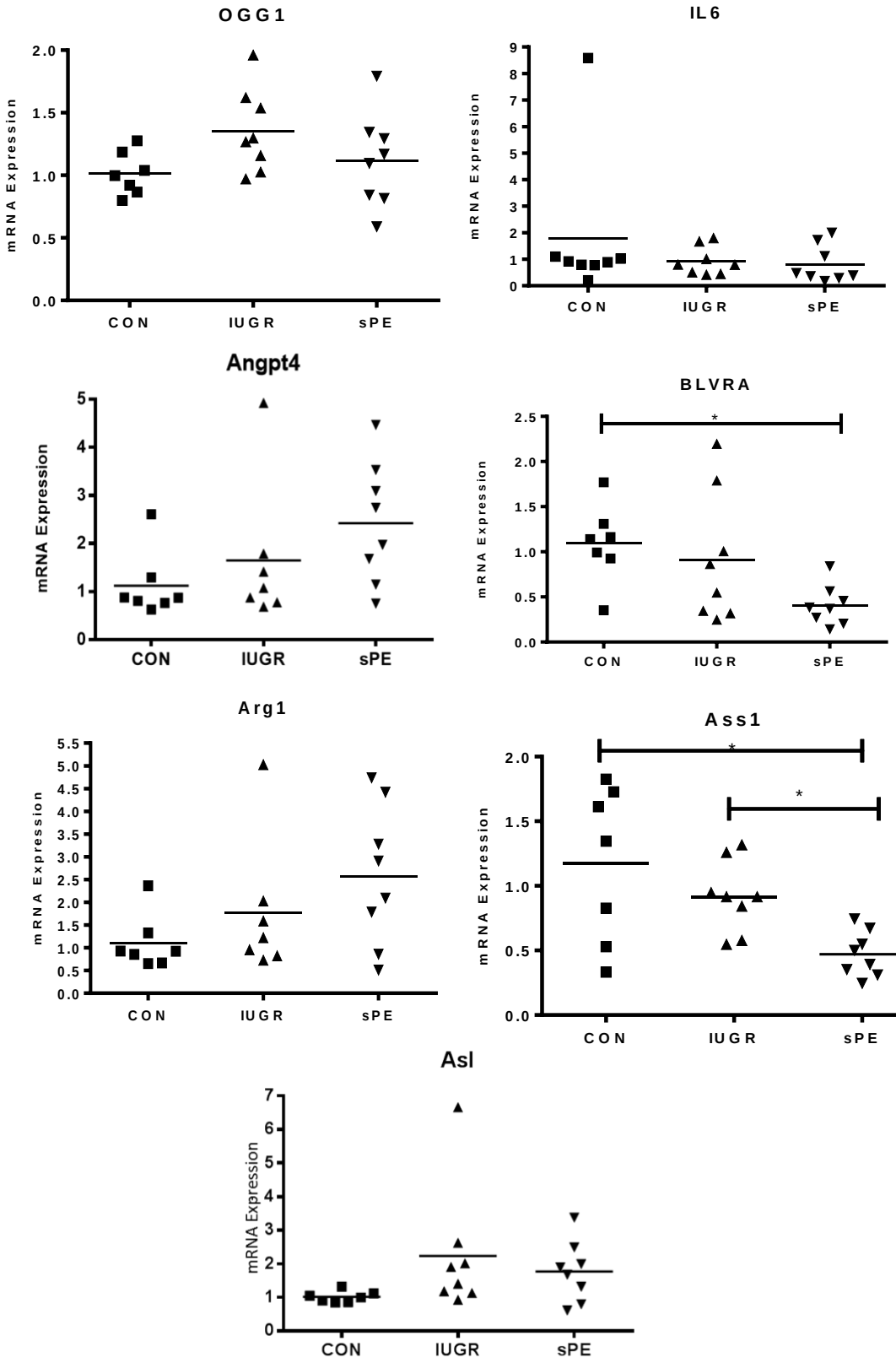
OGG1
mRNA Expression
2.0
1.5
1.0
0.5
0.0
CON
IUGR
sPE
IL6
mRNA Expression
9
8
7
6
5
4
3
2
1
0
CON
IUGR
sPE
Angpt4
mRNA Expression
5
4
3
2
1
0
CON
IUGR
sPE
BLVRA
mRNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
CON
IUGR
sPE
Arg1
mRNA Expression
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CON
IUGR
sPE
Ass1
mRNA Expression
2.0
1.5
1.0
0.5
0.0
*
*
CON
IUGR
sPE
Asl
mRNA Expression
7
6
5
4
3
2
1
0
CON
IUGR
sPE

Figure IV. Comparison of mRNA expression levels in normal, IUGR and preeclampsia placenta: expression of mRNA in placental tissues was detected in normal (n=7), IUGR (n=8) and preeclampsia (n=8). mRNA levels as detected by human primers. Each experiment was performed in triplicate. The expression patterns of the sequences as determined by real-time PCR. Data are expressed as mean ± S.E.M. (**P ≤0.001, ***P ≤ 0.0004) compared to the control.

Discussion

The most interesting feature of this study is the similarity between the arrays and the potential significance of the expression of 7 out of possible 15,000 genes. Preeclampsia is a serious complication that occurs during the second half of human pregnancy. We investigated levels of genes as markers of endothelial cell injury, including BLVRA, and Ass1, which were elevated in preeclampsia as previously demonstrated. The expression of these genes correlated with the activation and release of angiogenic factors to cause preeclampsia. Future studies could be carried out to investigate high blood pressure during pregnancy and preeclampsia.

In conclusion, microarray analysis of the gene expression in pre-ecliptic placenta compared to that of normal controls indicated the regulation of a surprisingly small number of genes. Differences in detection limits and analysis parameters may exist. The clinical characteristics, such as mean gestational age and parity of the included patients, will be of importance as well as the cellular composition of the placental samples. Our control group consists of women that have had uncomplicated pregnancies until delivery.

Furthermore, studies with a focus on different preeclampsia phenotypes are most likely needed. Most importantly, the suggestion that preeclampsia could come in two forms, probably with different genetic backgrounds and underlying pathophysiology must be considered. Sample size clearly needs to be increased since we are currently underpowered to detect association with rare variants and variants with low genetic effects that could be expected for a complex disease such as preeclampsia.

"Allahumma salli `ala Muhammad wa aali Muhammad"

O Allah:
please do bless Muhammad and the Household of Muhammad