THE UNIVERSITY
OF BIRMINGHAM

Master of Philosophy

THE LEVELS OF ANGIOGENIC AND ANTI-ANGIOGENIC MOLECULE CONCENTRATIONS IN PREGNANCY BASED DISORDERS IN THE MATERNAL AND FETAL CIRCULATION

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Abbreviations

Placental Growth Factor	PlGF
Vascular Endothelial Growth Factor	VEGF
Preeclampsia	PE
VEGF Receptor One	VEGFR-1
Soluble VEGF Receptor One	sVEGFR-1
Vascular Endothelial Growth Factor Receptor two	VEGFR-2
Intrauterine Growth Restriction	IUGR
Small for Gestational Age	SGA
Soluble Endoglin	sEng
Human Umbilical Cord Endothelial Cell	HUVEC
Hypoxia-inducible Factor 1, alpha subunit	HIF1α
Heme oxygenase	HO
Hemolysis, Elevated Liver Enzymes, Low Platelets	HELLP
Platelet derived growth factor	PDGF
Mitogen activated protein kinase	MAPK
Stress activated protein kinase	SAPK
Phosphoinositide 3-inase	PI3K
Protein kinase C	PKC
Phospholipase C ɣ	PLCɣ
Dimethyl sulphoxide	DMSO
Bovine serum albumin	BSA
Ethylenediaminetetraacetic acid	EDTA
Fetal calf serum	FCS
Standard error mean	SEM

Pulsatility index	PI
Carbon monoxide	CO
Transforming growth factor beta	TGF_{β}
Porcine Aortic Endothelial cells	PAE
Epidermal growth factor	EGF
Nitric oxide	NO
Hank's Balanced Salt Solution	HBSS
Fibroblast growth factor	FGF
Focal Adhesion Kinase	FAK
L-glutamine, Penicillin, Streptomycin	G/P/S
Extracellular signal-regulating kinase	ERK
Room temperature	RT
Mitogen-activated protein kinase kinase	MEK
Phosphate buffered saline	PBS
Sulphuric acid	H_2SO_4
Optical density	OD
Enzyme-linked immunosorbent Assay	ELISA
Body mass index	BMI
Small interfering RNA	siRNA
Basic fibroblast growth factor	bFGF
Tumor necrosis factor α	TNFα
Interferon y	IFN

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Abstract

OBJECTIVE: To determine if preeclampsia (PE) and intrauterine growth restriction (IUGR) is associated with differing levels of angiogenic and anti-angiogenic growth factors in both maternal and fetal circulation and the effect of heme oxygenase-1 (HO-1) and honokiol, an AKT phosphorylation inhibitor, on endothelial cells (EC) on these anti-angiogenic growth factors.

DESIGN: 1st test ; Nested case-controlled plasma samples from 165 pregnant patients assayed for soluble vascular endothelial growth factor 1 (sVEGFR-1) soluble endoglin (sEng), vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in Spain during 2001-2007. 2nd test; knockdown of HO-1 on EC and assayed to observe the effects on sVEGFR-1 levels released. 3rd test; 24 hour treatment to honokiol, an extract from the magnolia tree, on endothelial cells to observe the effect on sVEGFR-1 and sEng release.

RESULTS: In the PE specimens, maternal sVEGFR-1 levels increased more than 3-fold compared with gestational controls, fetals levels remained unchanged. The levels of sEng, and PlGF did not significantly change and the levels of VEGF 8-fold more than the controls. Free sVEGFR-1 was found to be in 4-fold excess in the PE state compared to the gestational control. siRNA of HO-1 was found to cause an elevation in VEGF activated sVEGFR-1 release. Honokiol was found to decrease VEGF activated sVEGFR-1 release with increasing concentration and increase sEng release in the presence of various growth factors.

CONCLUSION: There is an excess of sVEGFR-1 present in PE. Heme oxygenase 1 mediates sVEGFR-1 release and honokiol decreases sVEGFR-1 release from EC.

Introduction

Preeclampsia

Preeclampsia (PE) is a pregnancy-specific disorder affecting 3-5% of all pregnancies. It is associated with poor placental function due to insufficient endovascular invasion of maternal vessels to the protruding villi of the placenta (Friedman and Fox, 1976). The precise mechanism underlying PE is unknown. Women who have PE are at risk of developing cardiovascular diseases in later life (Bilhartz et al., 2011). Normal women who suffer from PE return to normal following delivery of the placenta suggesting it is a placental driven syndrome.

PE is characterised by the onset of proteinuria and hypertension (>140/90 mmHg) during the third trimester of pregnancy and severe PE can result in small for gestational age babies (SGA). The clinical onset of PE is usually asymptomatic and severe PE can result in progressive forms of PE such as eclampsia and HELLP syndrome (haemolysis, elevated liver enzymes and low platelets). The only current way of alleviating the symptoms is premature delivery in order to preserve maternal health. Early delivery often has consequences on the health of the newborn, particularly before 32 weeks gestation. On the other hand, delay to allow for fetal respiratory maturity, *in utero*, increases maternal risk factors (Ahmed, 2011). PE condition can range from mild to severe and sometimes mild cases are not included in official statistics although over 63,000 maternal deaths worldwide are due to preeclampsia (Noris et al., 2005). PE is associated with placental hypo-perfusion,

which can result in intrauterine growth restriction (IUGR) (Maynard and Hubbell, 2005).

Risk Factors

Previous studies have shown a genetic link attached to PE (Esplin et al., 2001) showing that a first degree relation increases PE risk by up to 4 fold. Although this is a maternal disorder, due to the placenta being a product of both parents, a paternal history of PE can also increase the risk of PE (Esplin et al., 2001).

A medical history of hypertension, diabetes, impaired renal function and obesity have been shown to increase the risk of PE (Duckitt and Harrington, 2005). Smoking during pregnancy has been shown to decrease incidence of PE (England et al., 2002).

Neonatal morbidity is usually as a consequence of early delivery and low birth weight and HELLP syndrome has been linked with a 15% incidence of prenatal mortality due to premature delivery (Roelofsen et al., 2003). PE occurs only in the presence of a placenta and not necessarily in the presence of a fetus (as is the case in a hypatidiform mole) and all symptoms are alleviated following delivery of the placenta (Kanter et al., 2010).

Placental Vascular Remodeling

Early in placentation, the extra villous cytotrophoblasts invade the maternal uterine spiral arteries transforming the maternal vascular endothelial cells, medial elastic tissue, smooth muscle and the neural tissue such that very little smooth muscle remains creating an environment of low blood flow resistance allowing the increase in uterine blood flow through dilated tubes lined by cytotrophoblasts needed to sustain the fetus during pregnancy (Brosens et al., 1972). In PE this invasion occurs either superficially or not at all. This shallow invasion results in an inadequate vascular remodelling, which does not correlate with the fetal blood flow and nutrient demands.

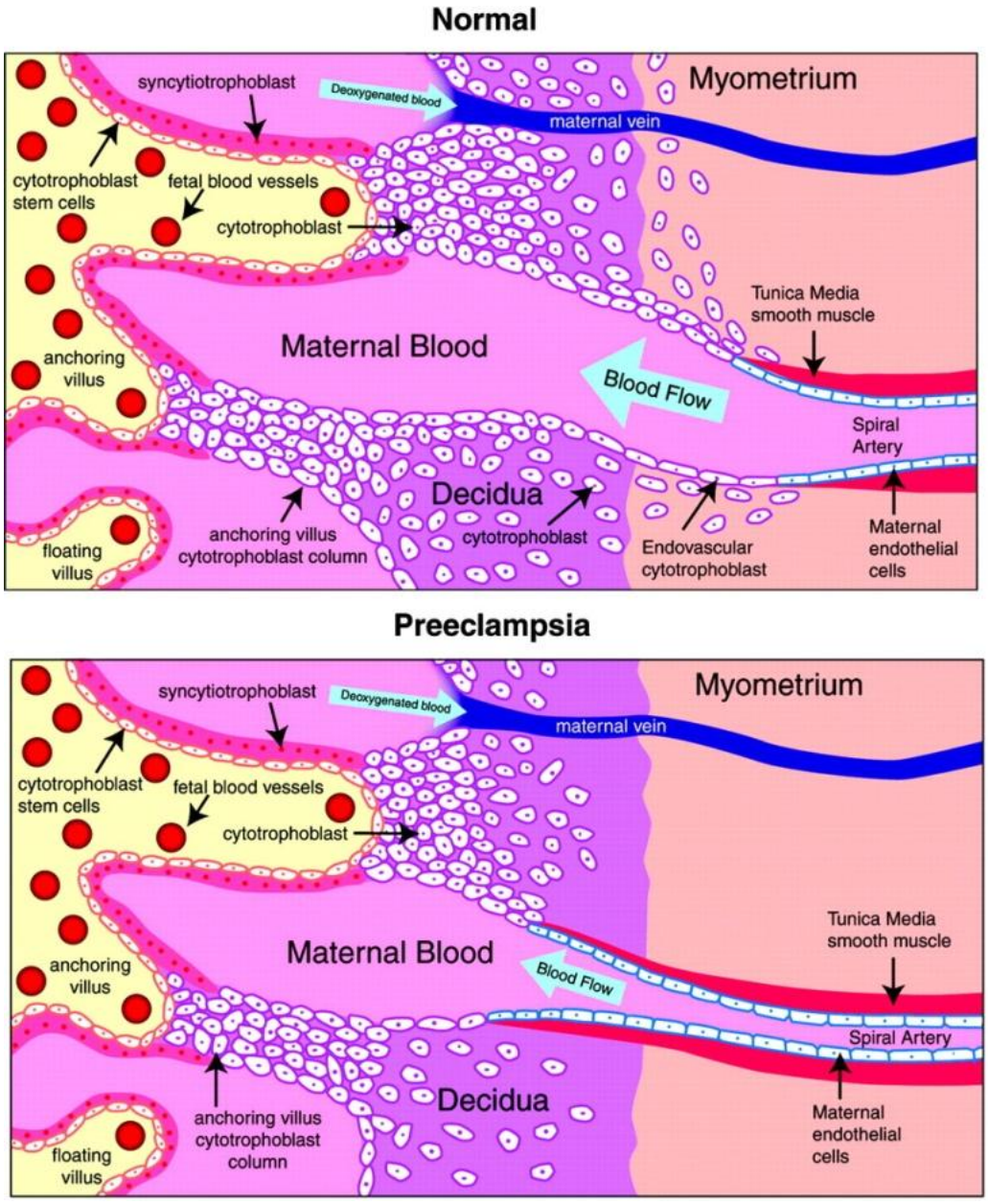

Figure 1: The differences between normal and PE placental invasion. In the impaired preeclamptic state, invasion of the spiral arteries is shallow, and they remain small, highly resistant vessels (Lam et al., 2005).

During normal placentation, the invasive cytotrophoblasts down regulate the epithelial-type adhesion molecules. In PE, these cell surface integrins and adhesion molecules fail to sufficiently invade the maternal myometrial spiral arteries (Zhou et al., 2002). Animal models of PE can be induced by the constriction of uterine blood flow suggesting that PE is as a result of placental ischaemia (Sane et al., 2004).

PE is more common at high altitude suggesting it is hypoxia driven (Zamudio et al., 1995). It has been suggested that placental hypoxia/ischaemia results in the release of products into the maternal circulation that then initiates the maternal circulation into pathophysiological changes resulting in PE.

Vascular Growth Factors and Preeclampsia

The effect of a low oxygen environment is thought to be a major regulator of the balance between VEGF and PlGF function. Khaliq *et al.* showed that hypoxia down regulates PlGF mRNA in a term trophoblast-like choriocarcinoma cell line (Khaliq et al., 1999) and that PlGF-2 inhibited growth of endothelial cells and proliferation (Khaliq et al., 1999).

Trophoblasts produce large quantities of sVEGFR-1 (Clark et al., 1996). Maynard *et al*. demonstrated that overexpression of sVEGFR-1 lead to PE like symptoms

including hypertension, proteinuria, glomerular endotheliosis, suggesting an important role for sVEGFR-1 and the angiogenic factors VEGF and PlGF (Maynard et al., 2003). sVEGFR-1 is elevated in patients with PE as early as 10 weeks into pregnancy in comparison to the gestationally matched control patients (Maynard et al., 2003).

Angiogenesis is a natural adaptive mechanism to redress the imbalance created by a reduction in oxygen tension and nutrient demand but in cancer induction of angiogenesis is unwanted and it is the metastatic response of the tissue to survive.

VEGF and VEGF Receptors

Loss of a single VEGF allele renders an embryonically lethal phenotype due to multiple defects in the cardiovascular system (Carmeliet et al., 1996, Ferrara et al., 1996). Structurally, members of the VEGF family belong to the endothelial cell platelet derived growth factor (PDGF) super-gene family. Various isoforms of VEGF-A include $VEGF_{121}$ (Goerges and Nugent, 2004), $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$. Other members of the VEGF super-family are VEGF-B, VEGF-C, VEGF-D, VEGF-E, PlGF-1 (placental growth factor, also abbreviated to PlGF), PlGF-2 and PlGF-3. VEGF-A_{145} and $VEGF_{183}$ are relatively rare physiologically (Neufeld et al., 1999).

VEGF expression is regulated by changes in oxygen tension, via HIF-1α (Semenza, 2001). PlGF is significantly expressed by endothelium; however has little or no direct

mitogenic or permeability-enhancing activities although it does potentiate the effects of low concentrations of VEGF (Park et al., 1994).

The VEGF family of ligands exercise their direct cellular effects via three dimeric, tyrosine kinase receptors, namely VEGFR-1 (Fms-like tyrosine kinase-1, also sFlt-1), KDR (kinase insert-containing domain receptor, also VEGFR-2, Flk-1 (fetal liver kinase-1) and Flt-4 (VEGFR3). Structurally, VEGFR-1 and VEGFR-2 have seven immunoglobulin-like domains in the extracellular region, a single trans-membrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (Shibuya et al., 1990, Terman et al., 1991). The figure below shows the VEGF family receptors and co-receptors, and the ligands that bind and activate them.

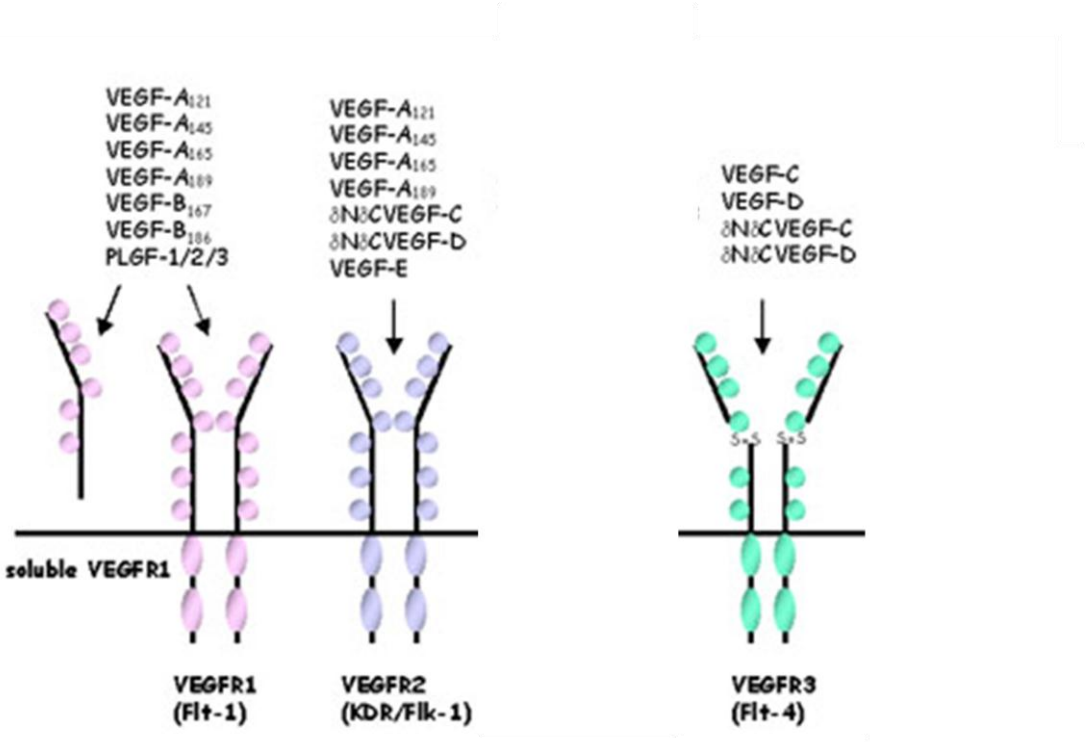

Figure 2 VEGF family and ligands.

The VEGF family has a number of different receptors and ligands, which bind with differing affinity to the receptors. Soluble VEGFR1 (sVEGFR-1) is anti angiogenic having a higher affinity to VEGF than the VEGFR-2 receptor. There is also a VEGF:PLGF heterodimer (not shown). VEGFR-3 has a more dominant role in lymphangiogenesis.

VEGFR3 and its ligands VEGF-C and VEGF-D, which also bind and activate VEGFR-2 but only in processed forms, are mainly involved in lymph angiogenesis (Jeltsch et al., 1997, Makinen et al., 2001, Veikkola et al., 2001). VEGFR-2 is considered to be the receptor most crucial for VEGF-induced mitogenicity, whilst VEGFR-1 has a less clear function. VEGFR-1 has a higher affinity for VEGF than VEGFR-2 but it also has a much lower tyrosine kinase activity therefore signalling function (Shibuya, 2006) has been difficult to interpret, however, it may have a role in endothelial differentiation and in regulation of VEGF availability. Both receptor knock-out phenotypes are embryonically lethal in mice at E8.5 –9.0 due to gross vasculature abnormalities, with VEGFR-2 inactivation leading to an absence of blood vessel development and minimal haematopoiesis (Shalaby et al., 1995) and the VEGFR-1 knock-out displaying a proliferation and disorder of endothelial cells (Fong et al., 1995). This suggests that during embryonic development VEGFR-2 is the positive signal transducer, whilst VEGFR-1, plays a negative regulatory role, a proposal substantiated by the fact that deletion of only the tyrosine kinase domain of VEGFR-1 allows normal embryogenesis in mice (Hiratsuka et al., 1998), indicating that during development VEGFR-1 acts as a ‘VEGF sink’, regulating the availability of VEGF for VEGFR-2.

VEGFR-1 and VEGFR-2 are abundantly expressed by endothelium; although VEGFR-1 is expressed at a ten-fold less abundant on the cell surface than VEGFR-2. VEGFR-1 is also expressed by monocytes and macrophages (Clauss et al., 1990, Clauss et al., 1996), cells that do not produce VEGFR-2, indicating that VEGFR-1 must function as a signalling receptor, at least in this cell type.

VEGFR-1 is also expressed in soluble form and is thought to be important as a negative regulator of angiogenesis (Goldman et al., 1998, Lai et al., 2001). VEGFR-2 is also present on mononuclear and endothelial progenitor cells (Lyden et al., 2001, Asahara et al., 1997, Asahara et al., 1999a, Asahara et al., 1999b, Gabrilovich et al., 1998).

Intracellular Signalling by VEGF

Activation of VEGFR-2 is critical for VEGF-mediated angiogenic response VEGFR-2 stimulation leads to activation of mitogen-activated protein kinases (MAPK); MEK, ERK-1/-2 and p38, stress activated protein kinase (SAPK), protein kinase C (PKC), phospholipase Cγ (PLCγ) and phosphatidyl-inositol 3-kinase (PI3K), resulting in cell survival, proliferation, NO generation and induction of angiogenesis. VEGFR-2 phosphorylation also induces matrix metalloproteinase expression and activation of focal adhesion kinase (FAK); events involved in basal lamina degradation and VEGF induced cell migration (Kliche and Waltenberger, 2001, Larrivee and Karsan, 2000, Zachary, 1998, Petrova et al., 1999).

VEGFR-1 function has been more elusive; it has long been known that VEGFR-1 plays a negative regulatory role in vasculogenesis and angiogenesis. VEGFR-1 acts as a VEGF 'sink' during embryogenesis, inhibiting VEGFR-2 signalling (Hiratsuka et al., 1998) and also VEGFR-1 activation leads to NO release, which negatively regulates VEGFR-2 signalling in HUVEC (Bussolati et al., 2001). It was also shown that VEGFR-1 down regulates VEGFR-2 mediated endothelial proliferation via PI3-kinase dependent pathways (Zeng et al., 2001) and additionally via sensitive G-proteins, Gβγ-subunits, Cdc42 and partly by Rac-1(Zeng et al., 2002). In monocytes, VEGFR-2 is not expressed and VEGFR-1 elicits positive signals, for example, inducing monocyte migration to stimulus (Clauss et al., 1990). However, in endothelial cells positive signalling has also been reported, but in a specific ligand dependent manner. This has been best illustrated in porcine aortic endothelial cells (PAE cells) stably transfected with either human VEGFR-1 or VEGFR-2. VEGF had no effect on VEGFR-1 expressing cells; however PlGF (VEGFR-1-specific ligand) induced MAPK activation and resultant DNA synthesis in these cells. PlGF did not activate PLCγ in PAER-1 cells and thus did not induce them to migrate. But, PlGF did up regulate plasminogen activator protein (PA) by PAER-1 cells and this did not occur in VEGF stimulated PAER-2 cells (Landgren et al., 1998).

Vascular Endothelial Growth Factors

VEGF (vascular endothelial growth factor) was identified as vascular permeability factor in the eighties by Senger *et al*.(Senger et al., 1983) and was then identified to

be the same as VEGF (Leung et al., 1989). The VEGF members are basic glycoproteins.

VEGFR-1

VEGFR-1 is an 180kDa trans-membrane glycoprotein with seven immunoglobulin – like domains in the extracellular region, a single trans-membrane region and a consensus tyrosine kinase sequence (Shibuya et al., 1990). VEGF and PlGF stimulate tissue factor production and chemotaxis in monocytes (Clauss et al., 1990) demonstrating that VEGFR-1 acts as a functional receptor in these cell types.

Pathologically, VEGFR-1 is up regulated and expressed in vessels proximal to healing wounds, suggesting a role in the regulation of vascular repair and maintenance (Peters et al., 1993).

Inactivation of VEGFR-1 in mice leads to embryonic lethality at E8.5 due to disorganised blood vessel formation (Fong et al., 1995) suggesting a role for VEGFR-1 in the organisation of a normal vasculature avoiding overcrowding of endothelial cells. VEGFR-1 has a higher affinity than VEGFR-2 for VEGF-A but has a weak signal transducing activity (Park et al., 1994) resulting in a regulation of VEGFR-2 by sequestering VEGF and controlling its presentation to VEGFR-2.

The VEGFR-1 gene is alternately spliced to produce a 110kDa protein that is shorter and a secreted form labelled as soluble VEGFR-1 (sVEGFR-1). sVEGFR-1 is biologically active in dimer form (Tanaka et al., 1997). It can bind to all isoforms of

VEGF and PlGF and so acts as a VEGFR-2 activation regulator via VEGF (Kendall and Thomas, 1993).

sVEGFR-1 is up regulated by hypoxia and has been found to be significantly expressed by the placenta (Clark et al., 1996, Helske et al., 2001). sVEGFR-1 is elevated in PE and removal of a sVEGFR-1 by immunoprecipitation restores the angiogenic balance in vitro (Ahmad and Ahmed, 2004). VEGF is also increased (Zeng et al., 2002). PlGF is decreased (Karumanchi and Bdolah, 2004). PE may occur due to loss of VEGF activity and this may partly or wholly due to an increase in sVEGFR-1 since adenoviral overexpression of sVEGFR-1 in pregnant rats mimics the clinical characteristics of PE (Maynard et al., 2003). It was shown in 1997 by Ahmed *et al*. that VEGF induces NO release which modulates angiogenesis(Ahmed et al., 1997) and more recently AKT inhibition, which is normally phosphorylated by VEGF via PI3K increased sVEGFR-1 and sEng. It has been previously shown that VEGF induced AKT promotes NO release (Basini et al., 2007).

Placental Growth Factor (PlGF)

PlGF is a 149 amino acid –long protein (Kendall and Thomas, 1993). It is expressed in the villous trophoblasts and elevated levels are present in the murine placenta (DiPalma et al., 1996). In human's PlGF is abundant in the placenta and cultured human umbilical vein endothelial cells (HUVEC),

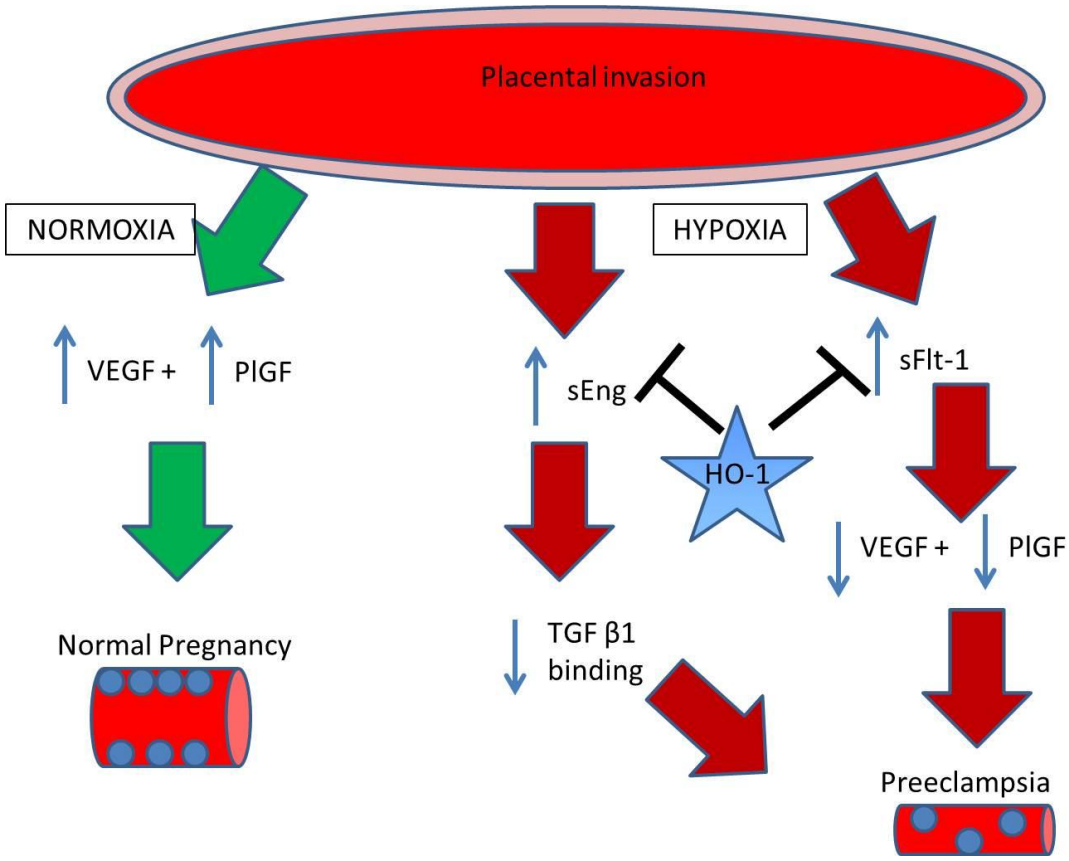

Figure 3. Diagrammatic representation showing the interaction of the various angiogenic factors resulting in preeclampsia.

During the hypoxic preeclamptic state, VEGF and PlGF protective signalling is compromised due to an excess of the sVEGFR-1. This is compounded by the increase in circulation sEng. Heme-oxygenase 1 (HO-1) has been shown to cause a decrease in sEng and sVEGFR-1.

Soluble Endoglin (sEng)

Dysregulation of TGF-β signalling, an anti-inflammatory growth factor, (Robertson et al., 2003), which activates endothelial nitric oxide synthase (Venkatesha et al., 2006) have been reported in preeclamptic patients (Levine et al., 2006). Endoglin is a cell-surface co-receptor for TGF-ß1 and TGF-ß3 isoforms, and it is highly expressed in endothelial cells and the trophoblasts. Endoglin binds to TGF-1 and reduces its bioavailability limiting its activity and in consequence promoting vascular dysfunction (Toporsian et al., 2005). A placenta-derived soluble form of endoglin (sEng) shows anti-angiogenic effects *in vitro* and is elevated in the circulation of preeclamptic patients many weeks prior to clinical onset (Levine et al., 2006). Soluble endoglin has been shown to increase in preeclamptic patients and to be proportional to the increase in levels of sVEGFR-1(Stepan et al., 2007). The same study also showed that there was an increase also observed in IUGR patients. The molecular mechanism regulating sEng release has not been fully explained however HO-1 (Cudmore et al., 2007) and angiotensin type 1 receptor antibodies (Zhou et al., 2008) have been observed to inhibit the release. Not many studies have considered the possible connection between IUGR, SGA and PE and we aimed to shed more light more on these groups.

Heme-oxygenase (HO)

Heme-oxygenase (HO) was described first by Tenhunen and Schmid in 1968 as the gaseous molecule involved in the catalytic breakdown of heme producing biliverdin, which releases carbon monoxide (CO) and iron (Fe^{2+}) (Tenhunen et al., 1968). Although this mechanism is the major pathway for the production of CO, it had remained un-noticed for many years after the discovery of HO. HO being the rate

limiting molecule in the reaction led to researchers trying to understand the mechanism behind HO activity. the specific regulation of HO-1, an inducible isozyme of HO, led to intense research to try to understand the system behind gene regulation by environmental stress.

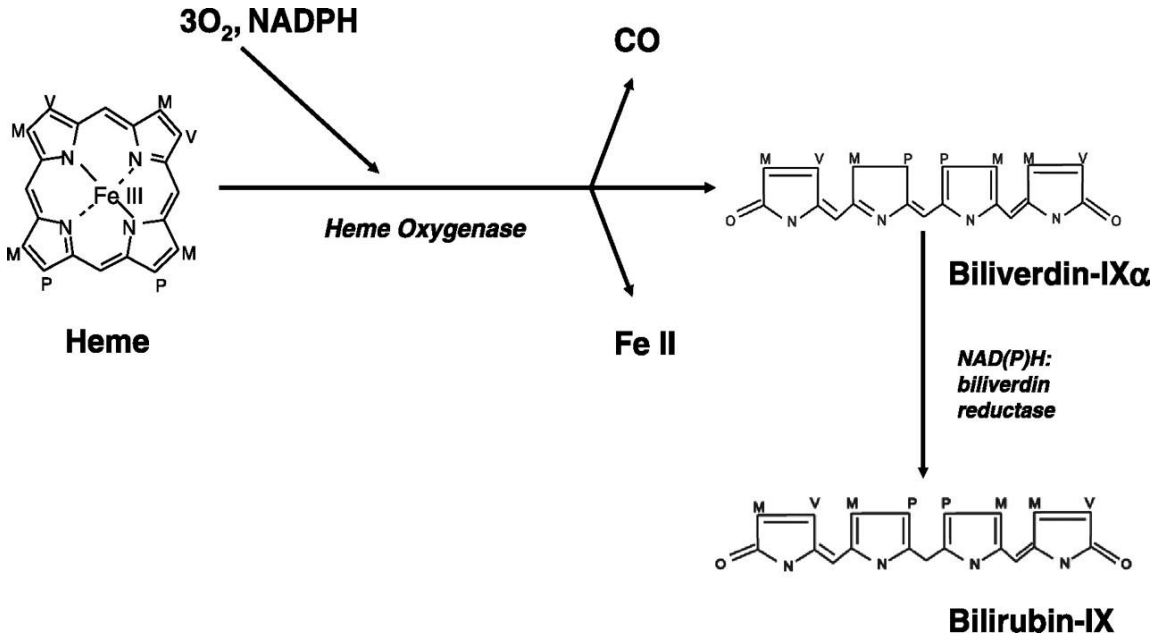

Figure 4. Schematic showing the degradation of Heme by HO in an NADPH dependant manner releasing CO, Fe^{2+} and biliverdin. Fe^{2+}, which is cytotoxic, is stored by ferritin.

Both HO-1 and HO-2 catalyse the identical biochemical reaction. They transform heme into biliverdin with similar substrate specificity and co-factor requirements. In a comparative analysis, HO-1 and HO-2, differential properties with respect to enzyme kinetics and substrate K_m values have been reported in rat as well as differences in apparent thermostability and immune-reactivity (Trakshel et al., 1986, Maines and Trakshel, 1992, Maines et al., 1986). HO-1 is inducible and responds to stress

whereas HO-2 is constitutively active. HO-1 is a crucial vasoprotective mediator via its products exhibiting antioxidant, anti-inflammatory, anti-apoptotic and anti-mutagenic effects (Ollinger et al., 2007). A mentioned previously, Cudmore *et al.* saw a negative regulation of both sVEGFR-1 and sEng release with HO-1 knockdown in placental villous explants (Cudmore et al., 2007).

Honokiol

Honokiol is a biphenolic compound present in the cones, bark, and leaves of the Magnolia tree that has been used in the traditional Japanese medicine. Honokiol has been shown to have pro-apoptotic effects in a number of cancer cell lines (Chen et al., 2011, Ahn et al., 2006). It also modulates the nuclear factor kappa B (NF-κB) activation pathway which is an upstream effector of VEGF. Honokiol has previously been shown to inhibit VEGF-D induced metastasis in a xenograft tumour model (Wen et al., 2009). No previous studies have been carried out regarding the potential role of Honokiol in preeclampsia and any studies with honokiol on endothelial cells have found it to inhibit vascular vessel formation of stem cell-derived endothelial cells (Kim et al., 2012), attenuate vascular contraction in rat aortic rings (Seok et al., 2011) and 5-Formylhonokiol, a derivative of honokiol exerting anti-antiogenic activity(Zhu et al., 2011). I aim to observe the involvement of honokiol on sVEGFR-1 and sEng release from HUVECs.

Aims and Objectives

The aim is to determine the maternal and fetal circulatory levels of angiogenic factors in placental pathologies and to identify any differences or similarities between both the levels and the differing factors of in all the patient groups.

We also aimed to study the effect of HO-1 and AKT on angiogenic and anti-angiogenic factors in endothelial cells As honokiol is an inhibitor of phosphorylation of AKT, we hypothesise that the loss of AKT will result in an increase in sEng since HO-1 negatively regulates the release of sVEGFR-1 and sEng (Cudmore et al., 2007) and loss of AKT activity leads to an increase in sEng release (Cudmore et al., 2011). HO-1 loss was reported to increase sVEGFR-1 and sEng. We hypothesise that the loss of AKT will increase sVEGF-1 and sEng.

Findings from this study could contribute considerably to the field by providing a clearer insight into the understandings of preeclampsia, IUGR and SGA and potential therapeutic agents that could be adopted to alleviate symptoms or the disorder altogether.

Methods

Clinical Data of Maternal and Fetal Samples

This was a nested case-control study including pregnancies at gestation, in which maternal and fetal plasma sVEGFR-1, sEng, VEGF and PlGF concentrations were measured. Patients were recruited from a prospective population who attended the obstetrics department from June 2001 to June 2007 at Hospital de Maternitat, Barcelona. 8-12 months were spent in Barcelona collecting and processing samples prior to their analysis. Plasma samples were stored at - 80 °C.. The study protocol was approved by the Hospital Clinic Ethics Committee (see appendix i), and written parental consent was obtained for all study participants.

The criteria of the International Society for the Study of Hypertension in Pregnancy were used to define PE (Brown et al., 2001). PE was diagnosed if a previously normotensive woman had two repeat (4h apart) diastolic blood pressure measurements of ≥90 mmHg after the 20^{th} week of gestation, together with proteinuria of more than 300 mg in a 24h urine specimen or at least two protein dipsticks in two repeat measurements (4h apart). IUGR was defined as a birth weight below the 10^{th} percentile for gestational age together with a Doppler pulsatility index (PI) in the umbilical artery above the 95^{th} percentile (Soothill et al., 1999, Baschat and Gembruch, 2003). Fetuses with structural or chromosomal abnormalities were excluded. For the purposes of this study, both IUGR and PE were early onset (gestational age under 32 weeks at clinical onset). The cut-off used to define early and late onset was chosen arbitrarily. The gestationally age-matched control group were identified as having had no known complications during pregnancy or at the time of giving birth and went into labour spontaneously with no defects or complications

post-delivery. Gestational age was established based on last menstrual period or early ultrasound evaluation (<20 weeks of gestation) in all cases (Hadlock et al., 1983). Other elements of the diagnosis included IUGR (<10th percentile), persistent neurologic symptoms (headache, visual changes), oliguria (urinary output <500mL/24h), elevated liver enzymes greater than two times the norm and thrombocytopenia (<100,000 cells/µl) (2002). Deliveries were classified as SGA if the birthweight fell below the 10th percentile of the birthweight distribution restricted on: completed week of gestation, year of delivery, sex of the child and parity.

Statistical Analysis

Results are expressed as mean ± SEM. Graphs represent the median, with the interquartile range demonstrated by the box. Comparisons between groups were performed by ANOVA for normally distributed data, respectively.

Tissue and Cell Culture

Maintenance of primary cells and cell lines

All tissue culture media and supplement were purchased sterile or filtered through a 0.22µM pore filter. Distilled water and PBS were autoclaved before use. Tissue culture treated plastics were purchased sterile and all glassware was washed and autoclaved prior to usage. Cell culture media was stored at $4^{o}C$ and used within 3 months. Routine maintenance of cells was carried out in a sterile class II cabinet and cell cultures were maintained in a humidified incubator in 95% air and 5% CO_2 at $37^{o}C$. All media were supplemented with 5-10 or 20% fetal calf serum (FCS) 2mM L-Glutamine 10U/ml penicillin and 0.1 µg/ml of streptomycin. Media was changed every 2-3 days and upon confluency, cells were sub-cultured. Monolayer cultures were washed twice with PBS and cells detached from the culture flasks by addition of 1ml of 0.05% trypsin/0.53mM ethylenediaminetetraacetic acid (EDTA) solution for 3 minutes. Trypsin digestion of the extra cellular matrix was inactivated by addition of 5ml of appropriate growth media and the cell suspension was transferred to a sterile tube and centrifuged at 80G for 5 minutes. The supernatant was aspirated and the cell pellet re-suspended in growth media.

Cells were re-seeded in new culture flasks at lower densities or counted using a haemocytometer and seeded at a specific number per 6, 24 or 96 well plate. Each time cells were detached with trypsin/EDTA, they were assigned an ascending passage number.

Cell cryopreservation

A reserve stock of each cell line was stored for future use by cryopreservation. T-75 flasks containing a confluent monolayer of cells to be preserved were detached with trypsin/EDTA and pelleted by centrifugation prior to suspension at 1 x 106 cells/ml in 70% FCS, 20% media with L glutamine, penicillin streptomycin and 10% dimethyl sulphoxide (DMSO). The cell suspension was transferred to sterile labelled cryovials and freezing was carried out at a rate of -1^{o}C/minute in racks suspended in a vessel (Mr Frosty^{TM}) containing 100% isopropyl alcohol. After 4 hours the cryovials were stored in liquid nitrogen until required. Cryopreserved cells were revived by thawing in a 37^{o}C water bath and immediate transfer to flasks containing appropriate growth media. After attachment of the cells to the flask, the growth media was discarded to remove trace amounts of DMSO and replaced with fresh media.

Details of Primary Cells and Cell Lines

Human Umbilical Vein Endothelial Cell Culture (HUVEC)

Human umbilical cords from obtained from normal term deliveries were washed thoroughly in PBS to remove all traces of blood and a 15-20 cm long section with no obvious clamp trauma was excised. The umbilical vein was cannulised at one end and flushed with PBS to remove traces of internal blood and blood clots. The cord was cannulised at the distal end and infused with 20 ml collagenase (1 mg/ml0 in Hanks

buffered saline solution (HBSS). After a 20 minute incubation at 37^{o}C detached endothelial cells were collected by flushing the vein with medium 199 (M199) containing G/P/S, 20 ng/ml epidermal growth factor (EGF), 2.5 ng/ml fibroblast growth factor (FGF) and 20% FCS (HUVEC growth media). The cell suspension was centrifuged at 100G for 10 minutes and the cell pellet re-suspended in HUVEC growth media. Cells were seeded in flasks coated with 1% gelatine in PBS and allowed to attach overnight. Media was replaced to remove any contaminating erythrocytes and the cells grown to confluence. Cells were sub-cultured 1 in 3 upon reaching confluence and experiments were performed on second or third passage HUVEC. Experiments were carried out on HUVEC in M199 containing 5% FCS and G/P/S and for signalling studies and NO analysis HUVEC were rested in this medium overnight prior to experimentation.

In-vitro Cell Function Analysis

General ELISA Protocol

EIA/RIA PLATES (Corning, UK) were coated with 100 μl/well of capture antibody in PBS pH 7.4, covered with a plastic film and left in the dark at RT overnight. The following day the plates were washed four times with PBS containing 0.05% Tween (PBS-T) and then blocked for 1 hour using 200 μl/well 1% BSA in PBS. After three PBS-T washes to remove residual BSA, samples were added at 100 μl/well and plates were incubated for 2 hours with agitation at RT. After washing, 100 μl of

biotinylated detection antibody in PBS-T was added to each well and incubated for a further two hours at RT with agitation. After washing streptavidin – HRP (1:200) in PBS-T was added for 20 minutes then washed off. The hydrogen peroxide colour substrate solution (R&D systems, UK) was added to the plate (see manufacturer's instructions), incubated until sufficient colour change was observed and the reaction stopped using 2M H_2SO_4. Presence or absence of the protein of interest was determined by reading the optical density (OD) at 450 nm (adjusted at 540 nm) with a Multiskan Ascent 96 well plate reader and subtracting the blank value (sample diluents only) from the sample absorbance values.

Results

The study sought to ask the fundamental question of whether or not there was a difference in the levels of certain angiogenic and anti-angiogenic markers detectable via ELISA in both the maternal and fetal circulation. Although previous studies have published a difference in these markers in the maternal circulation, this has not been investigated in correlation with their correspondingly matched fetus to see whether this difference observed is also passed onto the newborn or in any way related to the fetal circulatory levels.

Clinical Data

Clinical Characteristics of the study population

We analysed plasma samples from mothers and fetuses with singleton pregnancies recruited in the low and high-risk clinics at Barcelona's Hospital de Maternitat between June 2001 and June 2007. All expectant mothers were followed prospectively from enrolment to delivery. There were 48 maternal cases of the gestationally age matched controls, 56 PE of which 42 were actually PE, 2 were Eclampsia and 12 were HELLP syndrome, 25 cases of early-onset normotensive IUGR, 27 cases of PE + IUGR and 9 PE + small for gestational age (SGA). Additionally, each maternal case was matched to the delivered fetus by that patient. Not all maternal samples had a fetal sample and so the number of fetal were 36 gestationally age matched controls, 12 PE, 19 cases of early-onset normotensive IUGR, 24 cases of PE + IUGR and 9 PE + small for gestational age (SGA). Figure 5

represents the differences between the studied groups and the significant differences in their clinical measurement of blood pressure, proteinuria and mean birth weight. In order to maintain an unbiased approach, samples were randomly numbered and the groups were not known until after analysis of the data.

Clinical Characteristics of the study Population

Characteristic	Gestationally Age Matched Control n = 48	PE n = 42	Eclampsia n = 2	HELLP Syndrome n = 12	IUGR n = 25	PE + IUGR n = 27	PE + SGA n = 9
Maternal Age (yr)	32 ± 0.8	34±1.8	33±1.1	30±1.1	29 ± 1.1	29 ± 0.8	35±0.8
Maternal Height (cm)	157±1.8	160±1.2	155±1.2	144.2±1.3	161.9±1.1	160.9±1.2	161.2±1.1
Maternal Weight (kg)	57 ± 1.9	67.1	55±0.8	65.3±1.7	66.6±1.1	63.8 ± 3.0	63.4 ± 2.8
Maternal BP (mmHg)*							
Systolic	118.1 ± 3.4	165.3±3.8[a]	170±1.2 [a]	170.9±7.2 [a]	124.4±2.4	127.3 ± 2.2	174.9 ± 6.3 [a]
Diastolic	65.3 ± 1.6	102.3±2.9[a]	113±0.4[a]	106.9±7.6[a]	71.3±1.1	103 ± 2.8[a]	113.2±6.9[a]
Proteinuria (24Hr)	126.8±12.3	2491.5±569.8[a]	2100[a]	2684.4±1141.1[a]	229.4±19.8	2214.4±489.4[a]	2520.7±730.8[a]
Platelet Count	253 ± 15.7	168.3±18.7[a]	159±5.4[a]	80.3±15.5[a]	221.5±10.6	254 ± 16.6	177 ± 27.1[a]
Smokers	6	7	1	3	4	6	2
BMI	25.1±1.2	28.5±0.9	22±1.9	21.6±1.3	22.7±0.6	25.6±0.9	22.7±0.5
Gestational Age (wk)	30 ± 0.8	31.7±0.7	29.2±0.4	31.6±0.9	30.4 ± 0.5	30 ± 0.7	30.7±1.0
Birth weight (kg)	1.9 ± 0.2	1.7±0.2	1.1±0.8[a]	1.3±0.[7a]	0.9 ± 0.1[a]	0.9 ± 0.1[a]	1.1±0.1[a]

Figure 5 Characteristics of women in the different groups and of their infants.
*Blood Pressure values represent mean of the highest recorded during pregnancy.

PE preeclampsia, IUGR intrauterine growth restriction, HELLP haemolysis elevated liver enzymes and low platelets, SGA small for gestational age.

a $P<0.05$ versus gestationally age matched control.

Sandwich ELISA to detect sVEGFR-1 in human plasma samples

Diluted plasma from the maternal cases was assayed to determine the concentations of sVEGFR-1. From this ELISA it was possible to conclusively state that sVEGFR-1 was present in the human plasma samples. Figure 6 shows the concentration of sVEGFR-1 detected in the plasma samples in the maternal circulation at the time of delivery.

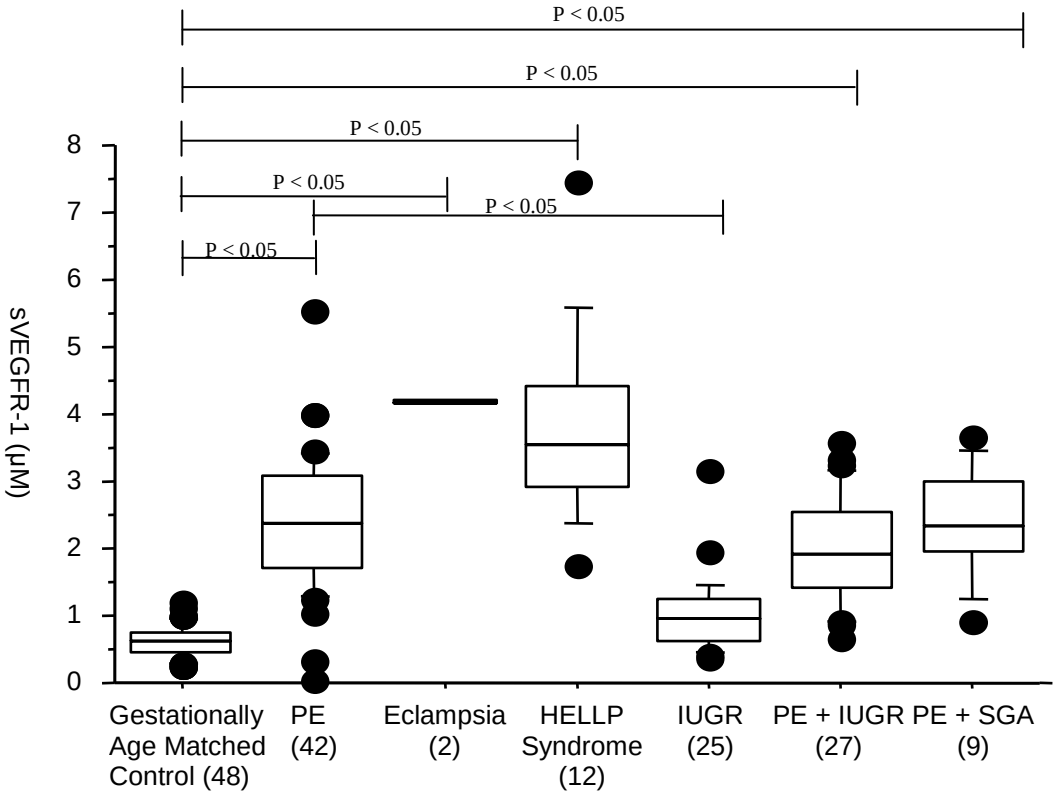

Figure 6. Maternal plasma concentrations of sVEGFR-1 of women in the different groups.

Box and whisker plot of the anti-angiogenic factor sVEGFR-1 in mothers with preeclampsia alone (PE, n=42), eclampsia (n=2), HELLP syndrome (n=12), intrauterine growth restriction (IUGR, n=25) or PE coupled with IUGR (n=27) and PE coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

The sVEGFR-1 levels indicate that sVEGFR-1 is elevated in the preeclamptic maternal plasma samples (PE 2.42 ± 0.16, Eclampsia 4.18 ± 0.10, HELLP syndrome

3.54 ± 0.3 vs. gestationally age matched control 0.62 ± 0.04). These levels were not elevated in the IUGR alone compared to the control group (1.04 ± 0.12). The preeclamptic groups in the presence of IUGR and SGA also had significantly elevated levels of sVEGFR-1 (1.98 ± 0.16 and 2.4 ± 0.16 respectively). To see whether this difference was also present in the fetal circulation, fetal plasma samples were assayed and the levels of sVEGFR-1 was measured.

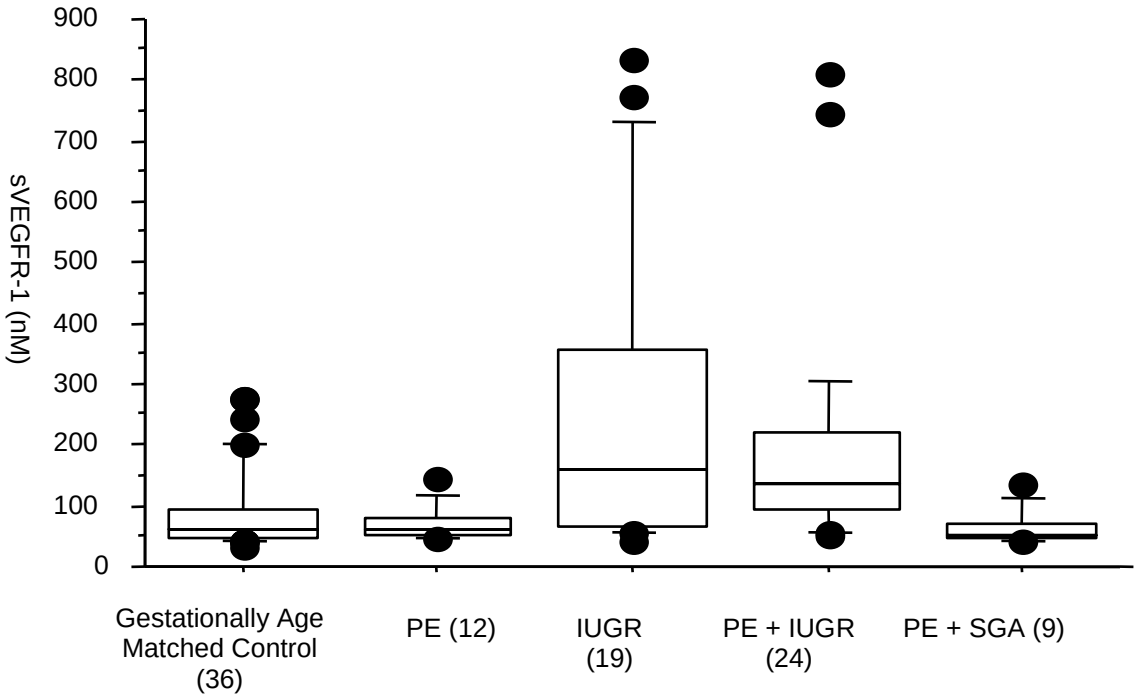

Figure 7. Plasma concentration of sVEGFR-1 present in the fetal circulation at delivery in the different groups.

Box and whisker plot of the anti-angiogenic factor sVEGFR-1 in fetuses whereby mothers had preeclampsia alone (PE, n=12), intrauterine growth restriction (IUGR, n=19) or PE coupled with IUGR (PE+IUGR, n=24) or coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

Interestingly, the concentration of sVEGFR-1 present in the fetal circulation did not vary at delivery in the different groups. No significant differences were found between all groups (PE 60 ± 3, IUGR 200 ± 40, PE + IUGR 190 ± 40, PE + SGA 60 ± 10 VS gestationally age matched control 90 ± 10).

The level of sVEGFR-1 present in the plasma was expected to be elevated in the maternal circulation for the PE affected patients as observed in previous studies (Levine et al., 2004). It was apparent that the levels of sVEGFR-1 are elevated in the maternal circulation and not in the fetal circulation (Figure 7). The levels of sVEGFR-1 in the PE + IUGR fetuses were not as significant as the patients suffering from a form of PE alone and the concentration of maternal sVEGFR-1 tended to increase with increasing severity of the disorder. The IUGR results for both fetal and maternal circulations did not show any significant differences against the gestationally age matched group suggesting a possible different mechanistic cause to the disorder and no relation to the mechanism behind PE.

In order to ascertain whether the disorder was specifically due to sVEGFR-1 in mothers and not another anti angiogenic molecule, we assayed soluble endoglin in a similar manner to determine the concentration on sEng present in the maternal and fetal circulation.

Analysis of Maternal and Fetal Plasma Samples for soluble Endoglin

A Sandwich ELISA was used to detect sEng in human plasma samples. As with sVEGFR-1, sEng, capture and detection antibodies were obtained as a Duoset (R&D Systems). Circulation sEng levels in the plasma were detected and concentrations were compared to a sEng standard curve. Figures 8 and 9 present the levels of sEng present in the maternal and fetal circulation respectively.

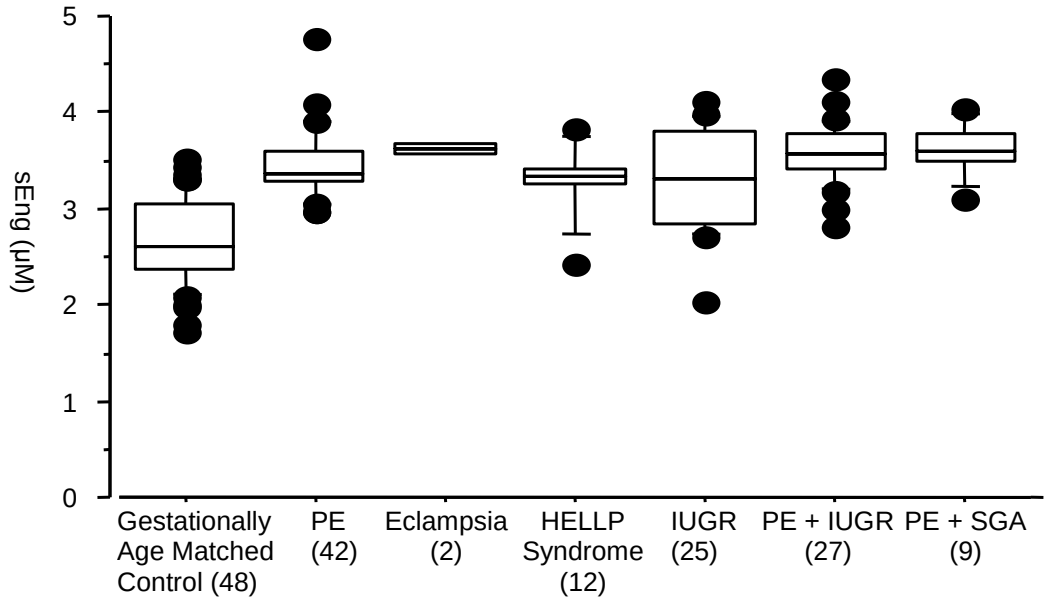

Figure 8. Maternal plasma concentrations of soluble endoglin (sEng) of women in the different groups.

Box and whisker plot of the anti-angiogenic factor soluble endoglin (sEng) in mothers with preeclampsia alone (PE, n=42), eclampsia (n=2), HELLP syndrome (12), intrauterine growth restriction (IUGR, n=25) or PE coupled with IUGR (n=27) and PE coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

The levels of the maternal plasma concentration of sEng present in the maternal circulation at delivery in the different groups didn't vary as much as previous studies had found (Levine et al., 2006). Although there was a slight elevation in the sEng levels in the different preeclamptic states compared to the gestationally age matched

control, there was no statistical difference found (PE 3.47 ± 0.08, Eclampsia 3.62 ± 0.06, HELLP syndrome 3.29 ± 0.1, IUGR 3.28 ± 0.11, PE + IUGR 3.58 ± 0.06, and PE + SGA 3.61 ± 0.09 VS Gestationally age matched control 2.67 ± 0.07). The fetal samples were also assayed to see if there were any differences in the fetal circulatory levels of sEng.

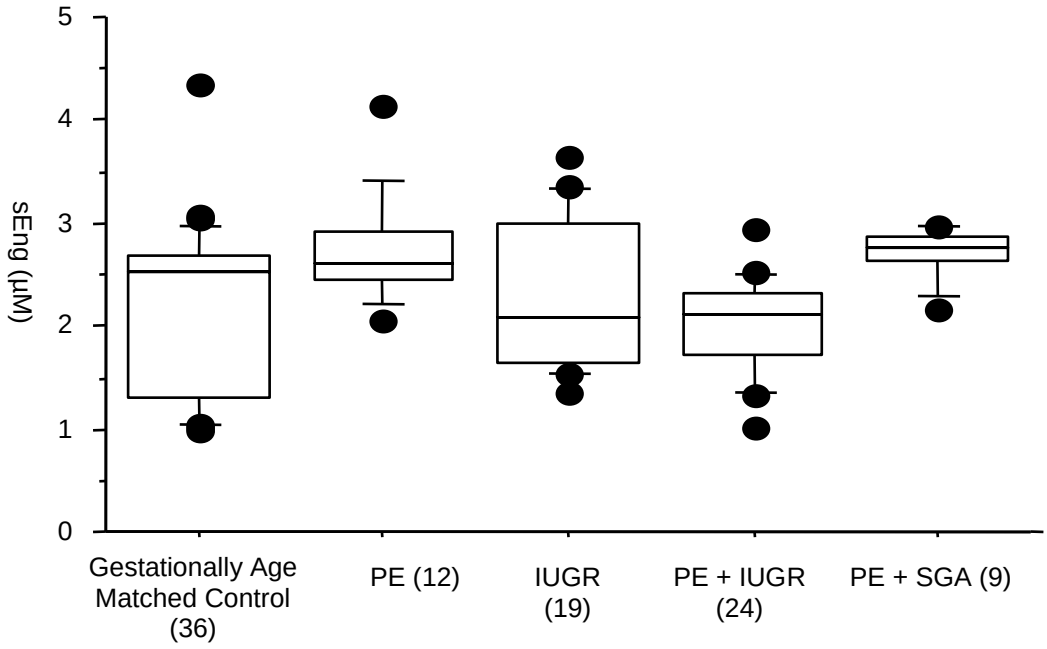

Figure 9. Plasma concentration of soluble endoglin (sEng) present in the fetal circulation at delivery in the different groups.

Box and whisker plot of the anti-angiogenic factor sEng in fetuses whereby mothers had preeclampsia alone (PE, n=12), intrauterine growth restriction (IUGR, n=19) or PE coupled with IUGR (PE+IUGR, n=24) or coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

As we expected, there was a marginal increase in the levels of sEng concentration in the maternal plasma samples and no significant difference in the fetal samples. The differences were not as large as the differences observed in the sVEGFR-1 suggesting that it may not alone cause the pathogenesis of preeclampsia but act secondary to sVEGFR-1 and enhance the pathological symptoms by creating more severe complications as there are more anti-angiogenic molecules present.

Considering the close relationship between sVEGFR-1, VEGF and PlGF, the levels of VEGF and PlGF were also then measured by the same method using the same maternal and fetal samples to see if any correlation could be observed.

Analysis of Maternal and Fetal Plasma Samples for VEGF

A sandwich ELISA was used to detect VEGF in the human plasma samples. As with sVEGFR-1, capture and detection antibodies were obtained as a Duoset (R&D Systems) Circulation VEGF levels in the plasma were detected and concentrations were compared to a VEGF standard curve. The results indicate that there was an increase in the levels of VEGF in the preeclamptic states that were not complicated with IUGR or SGA. These levels were then reduced back to the normal levels when in the presence of the IUGR or SGA fetus. (PE 8.7 ± 0.66, Eclampsia 13.1 ± 0.21, HELLP syndrome 11.73 ± 2.17 VS gestationally age matched control 0.24 ± 0.02) which was not present in the PE + IUGR AND SGA groups (IUGR 0.29 ± 0.01, PE + IUGR 0.38 ± 0.04, PE + SGA 0.43 ± 0.14)

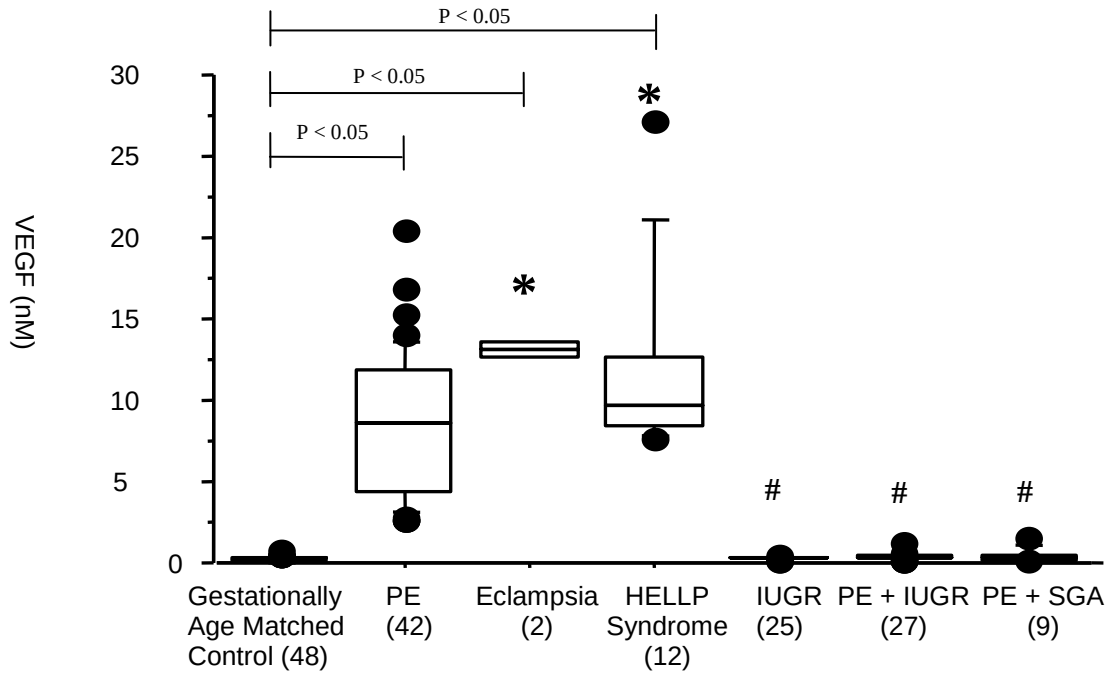

Figure 10. Maternal plasma concentrations of vascular endothelial growth factor (VEGF) of women in the different groups.

Box and whisker plot of the angiogenic factor VEGF in mothers with preeclampsia alone (PE, n=42), eclampsia (n=2), HELLP syndrome (12), intrauterine growth restriction (IUGR, n=25) or PE coupled with IUGR (n=27) and PE coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

Fetal levels were also measured to see if this increase was passed onto the fetus and had any effect on their circulating VEGF levels. This was not to be the case as shown in Figure 11 where we see fairly stable levels of VEGF in all the different groups. We also see that the levels were immensely decreased compared to the maternal

circulating levels showing that this difference is possibly tightly regulated by the placenta to ensure a controlled internal environment for the fetus.

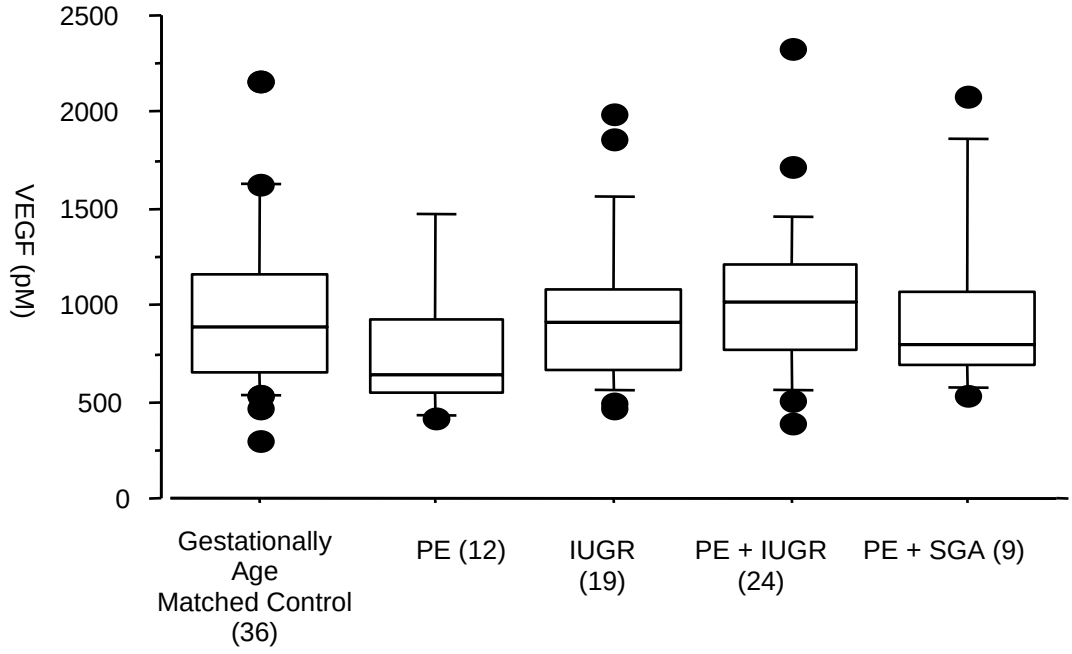

Figure 11. Plasma concentration of vascular endothelial growth factor (VEGF) present in the fetal circulation at delivery in the different groups.

Box and whisker plot of the angiogenic factor VEGF in fetuses whereby mothers had preeclampsia alone (PE, n=12), intrauterine growth restriction (IUGR, n=19) or PE coupled with IUGR (PE+IUGR, n=24) or coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

From this data we can deduce that the levels of VEGF in the maternal circulation were significantly increased in the preeclamptic states alone and not in any of the other groups including the PE + SGA group. The levels of VEGF present in the fetal

plasma samples did not differ in any of the groups in comparison to the gestationally matched control.

Analysis of Maternal and Fetal Plasma Samples for PlGF

A sandwich ELISA was adopted to detect PlGF in human plasma samples. As with sVEGFR-1, capture and detection antibodies were obtained as a Duoset (R&D Systems) and circulation PlGF levels in the plasma were detected and concentrations were compared to a PlGF standard curve. The figures below shows the levels of PlGF present in the maternal and fetal circulation.

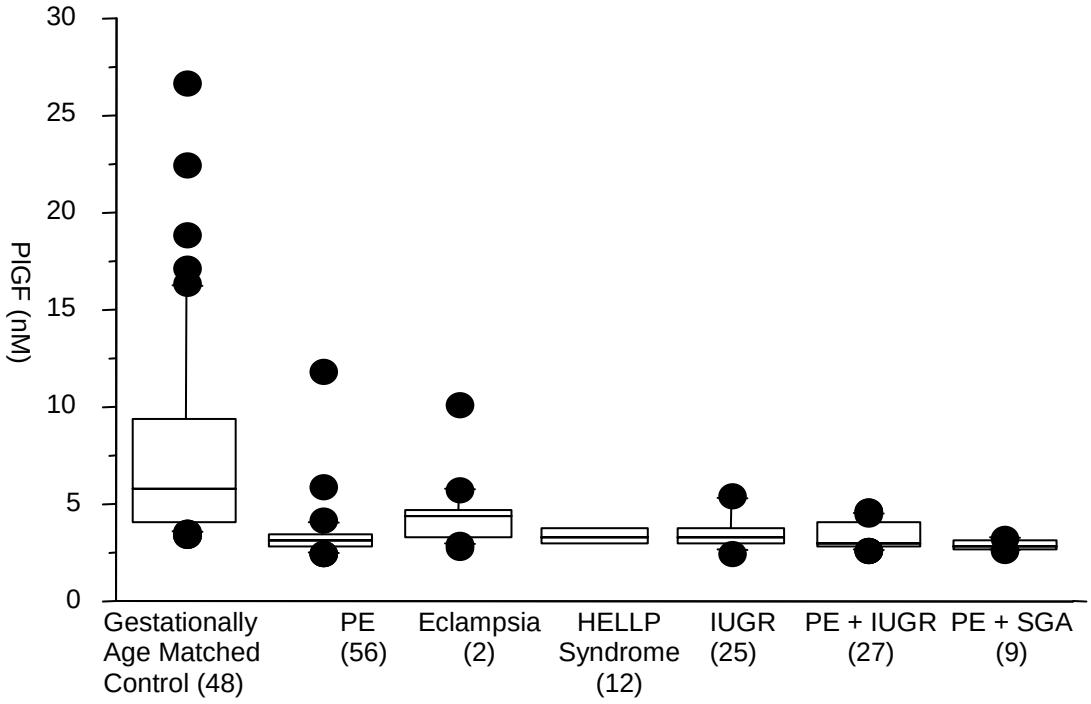

Figure 12. Maternal plasma concentrations of placental growth factor (PlGF) of women in the different groups.

Box and whisker plot of the angiogenic factor PlGF in mothers with preeclampsia alone (PE, n=42), eclampsia (n=2), HELLP syndrome (12), intrauterine growth restriction (IUGR, n=25) or PE coupled with IUGR (n=27) and PE coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

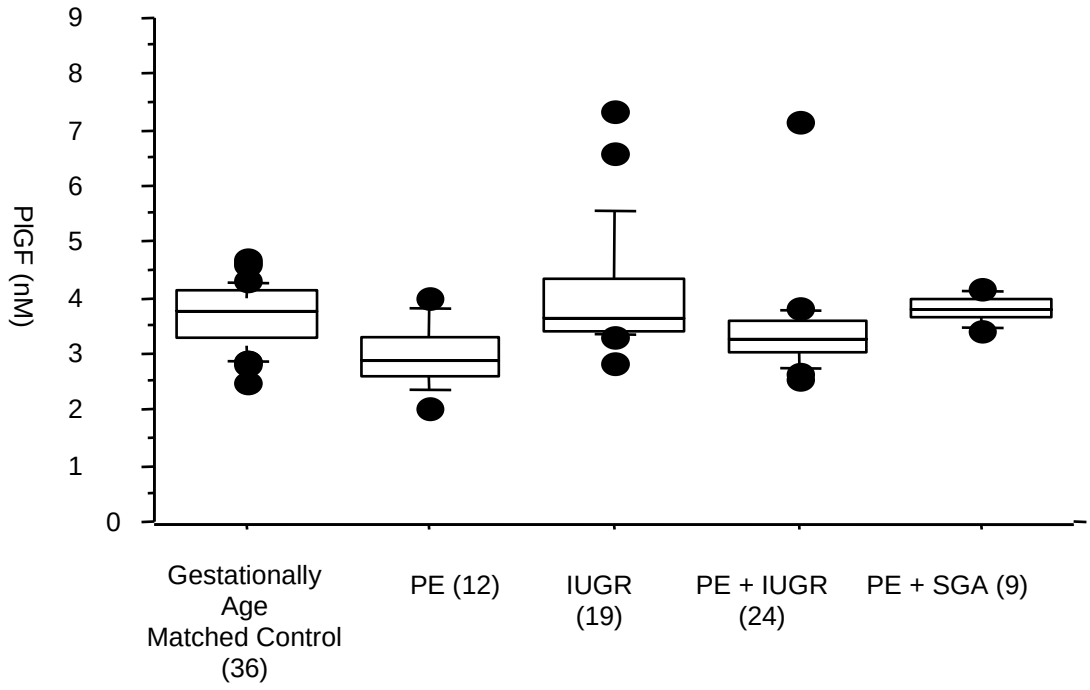

Figure 13. Plasma concentration of placental growth factor (PlGF) present in the fetal circulation at delivery in the different groups.

Box and whisker plot of the angiogenic factor PlGF in fetuses whereby mothers had preeclampsia alone (PE, n=12), intrauterine growth restriction (IUGR, n=19) or PE coupled with IUGR (PE+IUGR, n=24) or coupled with small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests.

No significant difference was found between any of the maternal groups versus the control (gestationally age matched control 7.91 ± 0.78, PE 3.5 ± 0.26, Eclampsia 3.32 ± 0.08, HELLP 3.55 ± 0.13, IUGR 4.38 ± 0.21, PE + IUGR 3.42 ± 0.1, PE + SGA 2.87 ± 0.03). this was also found to be the case for the fetal samples compared to the

age matched control (gestationally age matched control 3.59 ± 0.11, PE 2.88 ± 0.10, IUGR 3.65± 0.25, PE + IUGR 3.1 ± 0.2 and PE + SGA 3.42 ± 0.01).

PlGF levels decreased marginally in the maternal circulation in all the groups versus the gestationally matched control although it was not statistically significant. There was no difference in any of the groups in comparison to the gestationally matched control in the fetal circulation. Despite this being the case, it could have been that the levels were such due to the high levels of circulating sVEGFR-1 in the preeclamptic states and so we tried to calculate if the sVEGFR-1 that was present was in excess or not.

Free sVEGFR-1 presence in the plasma

Concentrations of sVEGFR-1, VEGF and PlGF were obtained by sandwich ELISA as detailed above. PlGF binds to sVEGFR-1 with a higher affinity than VEGF. 16-20 fold molar excess of PlGF is required to displace VEGF (Park et al., 1994). In order to calculate the relative concentration of free sVEGFR-1 present if any without taking into account the binding affinity of each molecule, we calculate how much PlGF and VEGF is present in the plasma and deduct the concentrations of PlGF and VEGF. One molecule of sVEGFR-1 binds two molecules of PlGF, VEGF or one of each. Figure 4demonstrates the concentration of free sVEGFR-1. A value greater than 1 means there is a ratio where there is an excess of sVEGFR-1 present.

The results show that when the concentration of VEGF and PlGF is taken into account with the anti-angiogenic molecule sVEGFR-1, there is an excess of sVEGFR-1 present in the preeclamptic states.

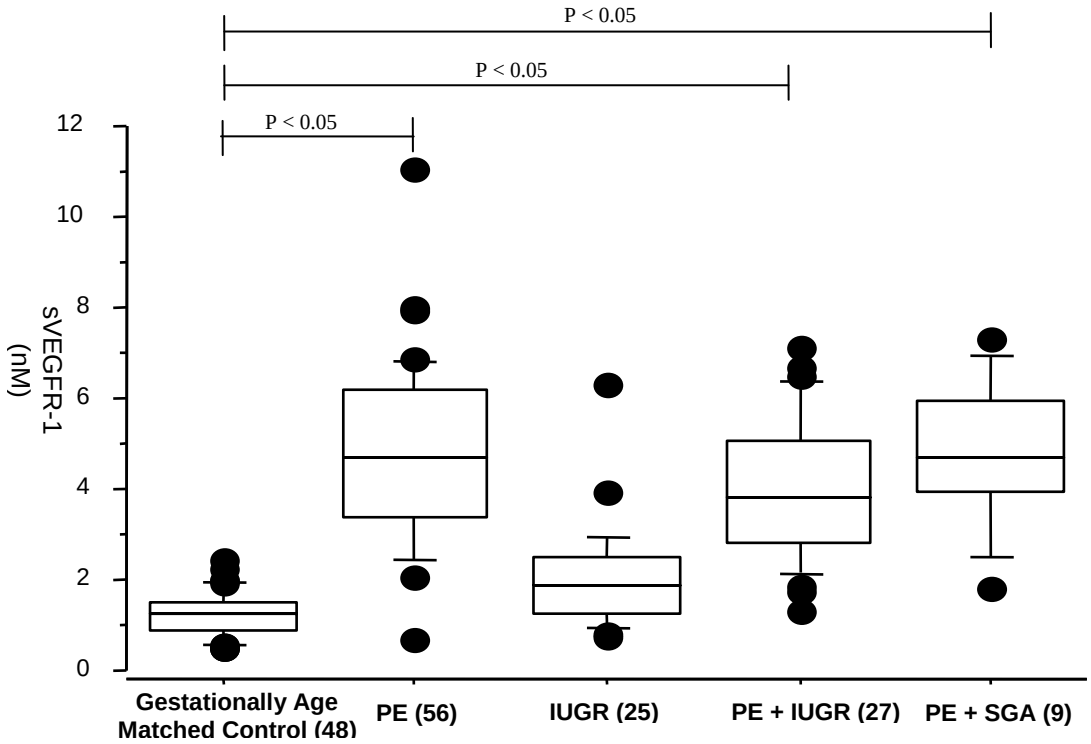

Figure 14. Plasma concentration of free sVEGFR-1 present in the maternal circulation at delivery in the different groups.

Box and whisker plot of the excess anti-angiogenic factor sVEGFR-1 in women with preeclampsia alone (PE, n=12), intrauterine growth restriction (IUGR, n=25) or PE coupled with IUGR and small for gestational age (SGA, n=9). The bottom and top of the box represent the 25th and 75th percentile respectively and the band near the middle is the median. The whiskers represent the 2nd and 98th percentile with the outliers plotted as black dots. Statistical analysis; Kruksal-Wallis ANOVA followed by multiple post hoc Dunn's tests. A value greater than one represent the excess fold presence of sVEGFR-1 as can be observed in all the preeclamptic states.

The levels of free sVEGFR-1 present in the maternal plasma circulation at delivery in the different groups was calculated going by the levels of sVEGFR-1, VEGF and PlGF in each patient sample. Preeclamptic patients had significantly higher levels of free sVEGFR-1 levels than the IUGR alone and gestationally age matched control groups (gestationally age matched control 1.24 ± 0.21, PE 4.10 ± 0.62, IUGR 2.08 ± 0.42, PE+IUGR 3.95 ± 0.76, and PE + SGA 4.8 ± 1.6).

Having noticed a clear elevation in sVEGFR-1 and its correlation with preeclamptic mothers, we then went on to see if it were possible to somehow reduce this excess level of sVEGFR-1 using a knockdown of heme-oxygenase.

ELISA following the Insertion of siRNA of Heme Oxygenase

Heme oxygenase (HO) is an enzyme that catalyses the degradation of Heme to produce biliverdin, iron and carbon monoxide (CO). There are three known isoforms of HO. HO-1 is an inducible isoform in response to oxidative stress, hypoxia and cytokines. HO-2 is expressed under homeostatic conditions. Both are ubiquitously expressed and catalytically active. HO-3 is not active. For this reason, HO-1 and HO-2 were tested. The effect of Heme oxygenase on sVEGFR-1 and sEng was observed by ELISA. HUVECs were trypsinised from T75 flasks and spun down to collect a pellet of cells. The supernatant was discarded and the cells were re-suspended in 100µl of a siRNA solution and pipette into an AMAXA chamber ensuring the suspension was between the metal contacts. The chamber is then placed into the AMAXA machine and allowed to insert the siRNA into the cells. The suspension is

then aspirated and placed into a new gelatinised T75 along with EBM HUVEC medium and incubated for 24hours. After 24 hours, cells were trypsinised and plated at a density of 1 x 10^5 cells per well in Medium M199 20% FCS along with L-glutamine, Pen/Strep, upon a gelatinised 24 well plate and incubated for a further 24 hours. The HUVECs were then washed twice in PBS and re-incubated in M199 5%FCS with various growth factors and then incubated for 24 hours. Medium was collected the next day and frozen at -80^{o}C prior to assaying. An ELISA was prepared by coating the 96-well plate with capture antibody (R&D systems) and detecting using the sVEGFR-1 detection antibody (R&D systems). Concentrations were calculated by comparison to a standard curve to sVEGFR-1 on the same plate as the samples.

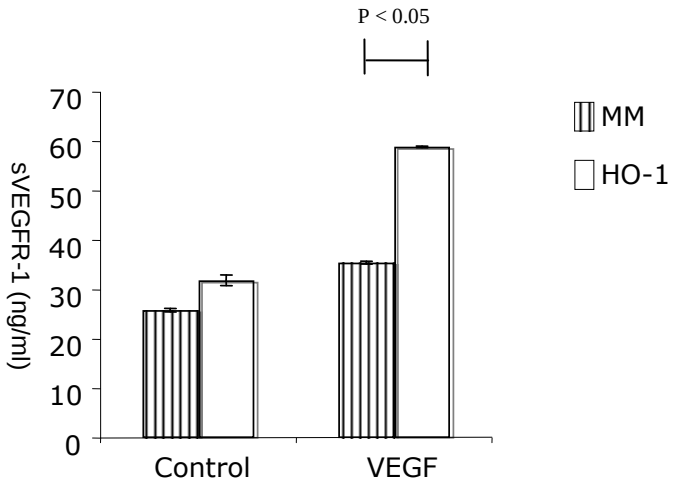

Figure 15. Bar chart showing the levels of anti-angiogenic factor sVEGFR-1 detected by ELISA in human umbilical vein endothelial cells following siRNA knockdown of heme-oxygenase 1 (HO-1) and following 24 hour treatment with VEGF.

VEGF alone did not cause a significant increase in sVEGFR-1 in the control compared to the mismatch but did cause a large release when HO-1 was knocked down showing VEGF induced sVEGFR-1 release is regulated by HO-1 (significance measured by T-test where $P<0.05$, n=3).

Following the administration of VEGF to the HUVECS, we saw a markedly increase in sVEGFR-1 release in the HO-1 siRNA sample compared to the mismatch control when there was no siRNA present for HO-1 (Control MM 25.89 ± 0.35, control HO-1 31.94 ± 1.07, MM VEGF 35.51 ± 0.28, HO-1 + VEGF 58.97 ± 0.14, n=3).

HO-1 seems to be causing an increase in the levels of sVEGFR-1 present in the medium collected from the stimulated HUVECs. To see whether this was specific to HO-1 alone or also by HO-2 we carried out the same test with an siRNA knockdown

of HO-2 and analysed the data to see if any obvious difference could be observed as were with HO-1. A Mismatch (MM) was used as a control.

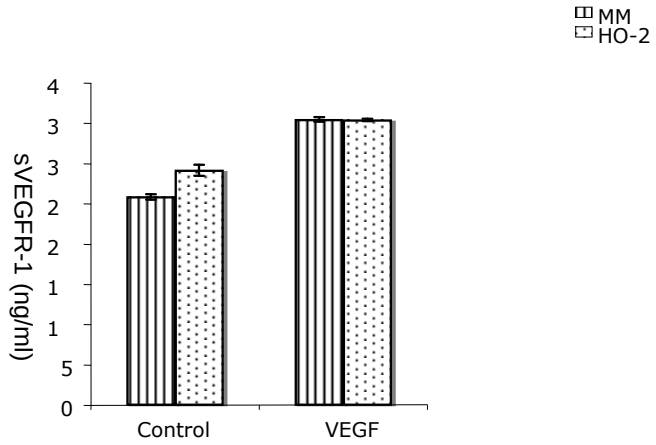

Figure 16. Bar chart showing the levels of anti-angiogenic factor sVEGFR-1 detected by ELISA in human umbilical vein endothelial cells following siRNA knockdown of heme-oxygenase 2 (HO-2) and following 24 hour treatment with VEGF.

VEGF alone did not cause a significant increase in sVEGFR-1 in both the control nor the VEGF induced sVEGFR-1 release, compared to the mismatch control (significance measured by t-test, n=3).

The concentration of sVEGFR-1 detected following siRNA knockdown of HO-2 and stimulation with VEGF for 24hours caused no significant difference to the amount of sVEGFR-1 released by the HUVECS (Control MM 25.89 ± 0.35, control HO-2 29.19 ± 0.68, MM VEGF 35.51 ± 0.28, HO-2 + VEGF 35.43 ± 0.14, n = 3).

HO-2 did not seem to be causing any significant changes in comparison to HO-1. When both HO-1 and HO-2 were used together, the results were similar to the results observed by HO-1 and so it seems that the effect was primarily caused by HO-1 and not HO-2 as demonstrated in Figure 17 (Control MM 25.89 ± 0.35, control HO-1 + HO-2 34.03 ± 0.5, and MM VEGF 35.51 ± 0.28, HO-1 + HO-2 + VEGF 61.26 ± 0.21, n = 3).

$P < 0.05$

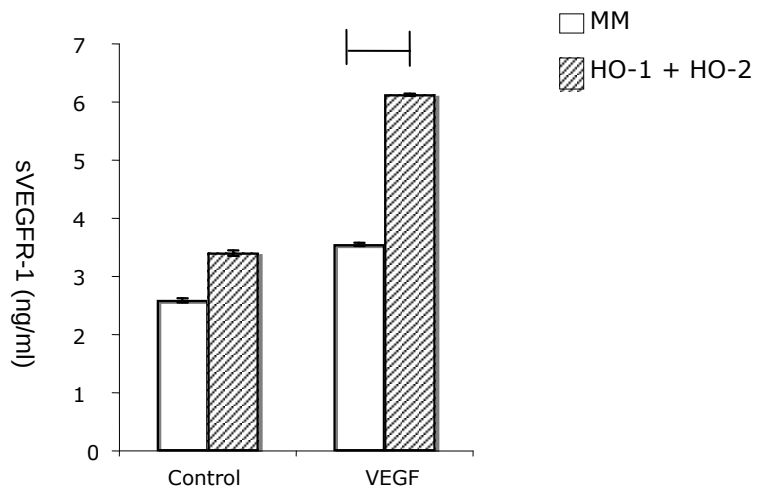

Figure 17. Bar chart showing the levels of anti-angiogenic factor sVEGFR-1 detected by ELISA in human umbilical vein endothelial cells following siRNA knockdown of heme-oxygenase 1 and heme-oxygenase 2 (HO-1 and HO-2) and following 24 hour treatment with VEGF.

VEGF alone did not cause a significant increase in sVEGFR-1 in the control compared to the mismatch but did cause a large release when HO-1 and HO-2 were knocked down showing VEGF induced sVEGFR-1 release is regulated predominantly by HO-1 and not by HO-2 (significance measured by t-test, n=3).

The effect of Honokiol on sVEGFR-1 and sEng release

Honokiol, an extract from the cone, bark and leaves of the magnolia tree has been found to be anti tumourigenic and have potential therapeutic applications since it has been found to be a modulator of the NFκB pathway which is an upstream effector of VEGF and has also been found to inhibit phosphorylation of Akt. We wanted to see if honokiol had any effect on sVEGFR-1 and whether it could be potentially used

therapeutically to alter the increased levels of sVEGFR-1 in the preeclamptic state. We first wanted to see if this would have any effect on the sVEGFR-1 and sEng release on HUVECs and so, similar to the siRNA treatment with HO-1, cells were pretreated with Honokiol for 24 hours and then an ELISA was carried out to see if sVEGFR-1 and sEng levels were affected.

HUVECs were incubated with Honokiol firstly at differing concentrations to determine a dose dependant response and then at 2 differing concentrations (2µg/ml and 20 µg/ml) and then added various growth factors to see if Honokiol had any effect on VEGF induced sVEGFR-1 and sEng release.

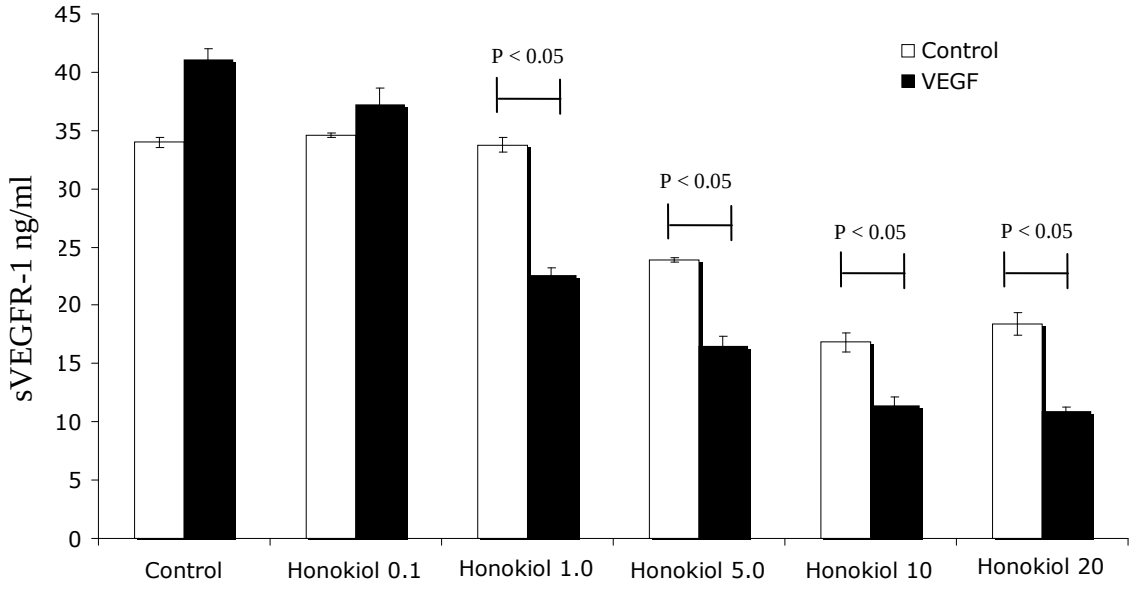

Figure 18. Bar chart showing the dose dependant decrease in sVEGFR-1 release from HUVECs following treatment with honokiol.

Levels of sVEGFR-1 decrease the higher the concentration of honokiol particularly from 1.0-20µg/ml (statistical analysis was carried out using t-test n=3).

Following the observation in Figure 18 where we see that honokiol does indeed decrease sVEGFR-1 release from HUVECS (Control 33.97 ± 0.45, Honokiol 0.1 34.58 ± 0.19, Honokiol 1.0 33.77 ± 0.65, Honokiol 5.0 23.88 ± 0.19, Honokiol 10 16.84 ± 0.84, Honokiol 20 18.4 ± 0.93, VEGF 41.04 ± 0.92, VEGF + Honokiol 0.1 37.23 ± 1.39, VEGF + Honokiol 1.0 22.59 ± 0.6, VEGF + Honokiol 5.0 16.52 ± 0.8, VEGF + Honokiol 10 11.37 ± 0.72, VEGF + Honokiol 20 10.93 ± 0.33, n=4), we then went on to see if it has an effect on sEng release at two particular concentrations, namely 2µg/ml and 20µg/ml since we observed an effect in sVEGFR-1 release at these doses.

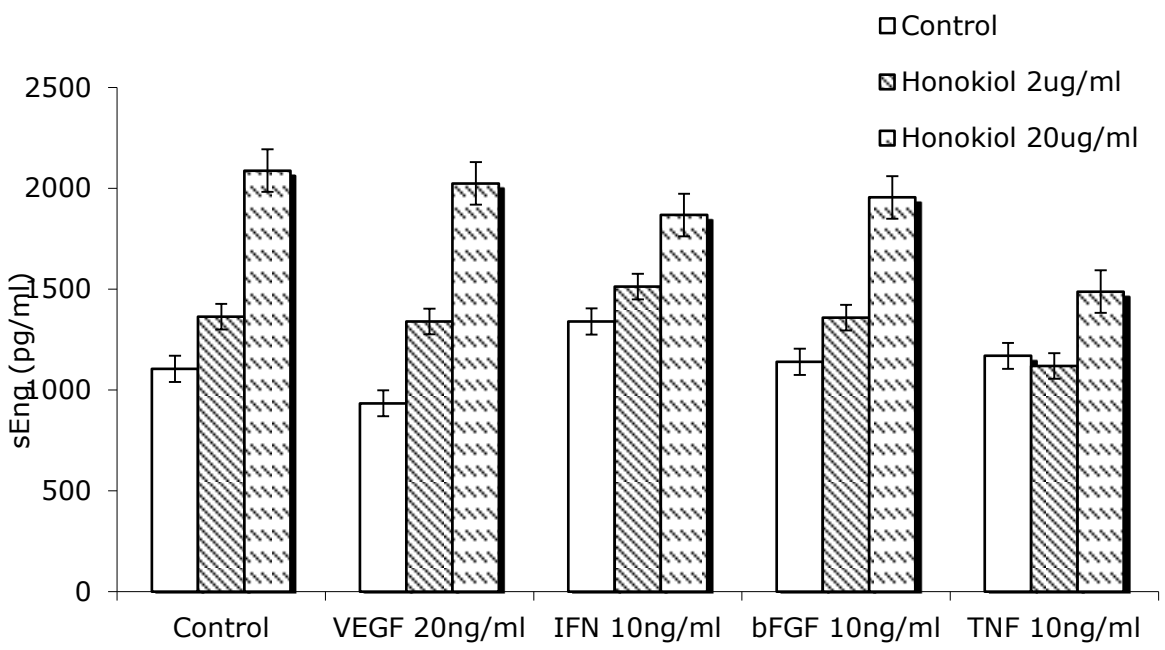

Figure 19. Bar chart showing the dose dependant increase in sEng release from HUVECs following 24 hour treatment with honokiol and then a one hour treatment with various growth factors.

Levels of sEng increase the higher the concentration of honokiol particularly in the presence of VEGF, Interferon γ, basic FGF and TNFα (statistical analysis was carried out using t-test n=3).

Different growth factors were also used to see if these also had any effect on sEng release. The HUVECS were pretreated with VEGF(VEGF), Interferon γ (IFN), basic fibroblast growth factor (bFGF) and tumour necrosis factor α (TNF) since these have previously been shown to have an effect on sEng release (control 1104.3 ± 152.6, Honokiol 2 1363.2 ± 163.4, Honokiol 20 2087 ± 120.4, VEGF 934 ± 153.9, VEGF + Honokiol 2 1339.8 ± 177.3, VEGF + Honokiol 20 2023 ± 116.8, IFN 1339.3 ± 177.3, IFN + Honokiol 2 1512.7 ± 127.3, IFN + Honokiol 20 1867.5 ± 107.8, bFGF 1139.3 ± 165.7, bFGF + Honokiol 2 1358.6 ± 178.4, bFGF + Honokiol 20 1954.5 ± 112.8,

TNF 1168.8 ± 167.4, TNF + Honokiol 2 1118.3 ± 164.5, TNF + Honokiol 20 1487.6 ± 185.9, n=4). The results varied significantly but demonstrated a dose dependant increase in the sEng release which was attenuated in the presence of the growth factors.

Conclusion

Conclusion

Various angiogenic growth factor levels were measured in human plasma collected at time of delivery from both maternal and fetal patients to examine a possible relationship between the concentrations and the pregnancy specific disorders of preeclampsia IUGR and SGA and were compared to their gestationally age matched control. The findings confirmed as found in previous studies that concentrations of sVEGFR-1, which were previously reported to be increased in women with established preeclampsia (Maynard et al., 2003) begin to show an increase with increased severity of the clinical disease from preeclampsia through to eclampsia and eventually to HELLP syndrome. With the increase in the sVEGFR-1 level, there was an overall excess of sVEGFR-1 in the preeclamptic states which was not present in the gestationally age matched control suggesting that the increase in these factors may be attributable in part to binding by sVEGFR-1 to VEGF and PlGF causing an anti-angiogenic effect. Women with preterm preeclampsia and a small-for-gestational-age infant had higher sVEGFR-1 levels, which is consistent with previous studies. (Taylor et al., 2003, Levine et al., 2004).

sVEGFR-1

Normal placental conditioned media promoted angiogenesis and pre-incubation with media containing exogenous sVEGFR-1 significantly attenuated cell migration and tube formation whereas this addition to preeclamptic conditioned media had no

further effect on angiogenesis due to saturating concentrations of sVEGFR-1 (Ahmad and Ahmed, 2004).

An imbalance in circulating angiogenic factors is emerging as a prominent mechanism that mediates the endothelial dysfunction and the clinical signs and symptoms of preeclampsia. sVEGFR-1, an endogenous anti-angiogenic factor that is a potent VEGF antagonist, is elevated 4-fold in the maternal circulation of preeclamptic patients and increased with severity of the disorder. Although there was a trend to show an increase with severity, it should be highlighted that due to the low number of patients in the eclamptic and HELLP syndrome categories, it would not be appropriate to make a definite conclusion on this basis until a further insight is made into this trend. The fetal circulation remained stable suggesting the sVEGFR-1 is released on the maternal side of the placenta and doesn't pass through the maternal:fetal barrier.

Soluble Endoglin

We did not see any significant increase from the preterm control although it did show an increasing trend and as the Stepan *et al* paper found, the level of sEng found in IUGR patients was marginally lower than the PE patients (Stepan et al., 2008). IUGR and PE both occur due to an impaired placental function including poor trophoblast invasion. This study clearly defined IUGR patients as a fetus below a certain weight percentile due to a placental insufficiency and clearly distinguished between SGA fetuses which include healthy fetuses with a low genetic growth potential. The

slightly elevated levels of sEng in this study may suggest that it is partially involved in the pathology of IUGR this would concur with the idea that sEng induces *in vivo* vascular permeability and PE typical symptoms, including hypertension. It is not possible to conclude whether sEng is produced in response to an increase in sVEGFR-1, vice versa or independent of each other via a different mechanism. The data supports previous findings that there is not an increase in sVEGFR-1 concentrations in IUGR (Shibata et al., 2005, Wathen et al., 2006). Although some studies have shown an increase in sVEGFR-1 in IUGR patients (Tsatsaris et al., 2003, Wallner et al., 2007, Stepan et al., 2004), they do not exclude that some of the IUGR patients would have developed PE later on and confirm that some IUGR pregnancies were close to the threshold of PE symptomatic criteria whereas our samples were collected upon delivery.

With regards to the PE + SGA samples, unfortunately SGA alone samples were not available at the time of the study and so comparisons can't be drawn against it. The only comparisons that can be made are in comparison to the PE alone states and PE + IUGR allowing an insight into whether PE itself was causing a difference or the fetus seeing as the two disorders differ little in terms of the fetal effect. This allows us to make a more critical conclusion on the placenta alone and eliminate the possibility of a "fetal burden" causing an overload effect on the maternal circulation or upon the placenta for nutrition.

VEGF

VEGF is not only important in angiogenesis, but also in maintaining endothelial health including the formation of endothelial fenestrae (a hallmark of the glomerular vascular endothelium). sVEGFR-1 overexpression in animals induces glomerular endotheliosis with the loss of endothelial fenestrae that resembles the renal histological lesions of preeclampsia. More severe forms of preeclampsia, including the HELLP syndrome, may be explained by a concomitant elevation in both sVEGFR-1 and soluble endoglin, another anti-angiogenic factor. Angiotensin II plays multiple roles in the pathology of pre

eclampsia, including contributing to hypertension, increasing oxidative stress via production of superoxide anions, and activating platelets (Shah, 2005). The study showed that the VEGF levels only increased in the maternal circulation of the PE alone states and not in the presence of IUGR or SGA. It may be that the IUGR and SGA conditions are causing VEGF levels to return to normal via a mechanism that is hindered by PE alone but it is not possible to conclude such a theory without further investigation. It could be that VEGF is produced in excess to flatten the imbalance of VEGFR-1:VEGF in the maternal circulation but again, it is not clear enough to conclude. Previous studies have also found that VEGF is elevated in PE states (Lam et al., 2005) but since VEGF is bound with high affinity to sVEGFR-1 in PE, the overall result is still an excess of sVEGFR-1. serum VEGF is unlikely to serve as a useful screening marker until ELISA kits that are sensitive enough to detect single-digit picogram concentrations with high reliability become available (Thadhani et al., 2004, Taylor et al., 2003). VEGF loss must be due to a loss of AKT since a loss of

AKT results in an increase of sVEGFR-1 and sEng (Cudmore et al., 2011) and it must be a maternal loss of AKT since fetal levels remain stable.

PlGF

Many studies have shown PlGF levels to be low in PE serum (Maynard et al., 2003, Tsatsaris et al., 2003, Levine et al., 2004) probably due to it being bound to sVEGFR-1 rather than a decreased production by the placenta, PlGF has been shown to slowly increase in the first stages of pregnancy and then steadily decline (Reuvekamp et al., 1999). Seeing as PE symptoms are prevalent in the latter stages of pregnancies, it may be that the PlGF levels are decreasing due to the increase presence of sVEGFR-1. Decreased levels of urinary PlGF during mid-gestation predict subsequent development of clinical preeclampsia (Levine et al., 2005).

Physiologically, the antagonism of VEGF and PlGF as a mechanism involved in the pathogenesis of preeclampsia is consistent with what is known about the disease. The placenta produces sVEGFR-1 in high amounts in preeclampsia; free VEGF and PlGF are respectively low. In fact, sVEGFR-1 concentrations are high 5-6 weeks prior to the onset of clinical disease and concentrations of free VEGF and PlGF are also found to be low prior to clinical preeclampsia (Chen et al., 2009).

Free VEGFR-1

Previous studies have suggested that there is an increase in sVEGFR-1 in PE patients most notably Rajakumar *et al* who looked at peripheral blood mononuclear cells and an increase in sVEGFR-1 production under hypoxia (Rajakumar et al., 2005). This study calculated directly that there is an excess of sVEGFR-1 in all the PE states independent of IUGR and SGA further supporting the idea that sVEGFR-1 is the underlying factor with this disorder. It also provides a definite concentration of how much excess is produced and how much of a fold increase there is compared to the preterm control. The data calculations were ensured to be done per patient before any comparable means were interpreted in order to ensure a clearer result to demonstrate the excess is across the scale and not dependent on outliers assisting the calculation to provide a clearer spread of data.

While it is not clear what is causing the excess sVEGFR-1 production the data does leave us with several unanswered questions including what is causing the maternal pro-inflammatory state considering the same is not happening in the paired fetal circulations? PE is a placental disorder and so the maternal inflammatory response must be by the placenta, and more on the maternal side of the placenta. This study excludes the fetal involvement seeing as it is not happening in IUGR and SGA doesn't affect the excess increase of sVEGFR-1 in the maternal circulation.

Our study had limitations. We found the sVEGFR-1 levels in women with established preeclampsia to be higher than others have reported them to be. Longer freezer-storage times may have resulted in decreased sVEGFR-1 levels in their studies

(Levine et al., 2004, Maynard et al., 2003). A more likely explanation is that the women in our study may have had more severe preeclampsia. The mean gestational age of the fetus at the time when specimens were obtained from women with clinical preeclampsia was 30 weeks in this study, as compared with 34-38 weeks or less in other studies (Maynard et al., 2003)

Heme-oxygenase

We saw an elevation of sVEGFR-1 by VEGF following knockdown by HO-1 siRNA and not by HO-2 in HUVECs. This complies with previous studies that have seen HO-1 decreased and sVEGFR-1 levels increased in PE placenta (Ahmad and Ahmed, 2004, Zhou et al., 2002). Our group has also previously showed that adenoviral over-expression of HO-1 in endothelial cells inhibited VEGF mediated sVEGFR-1 release (Cudmore et al., 2007) and inhibition of HO-1 caused sVEGFR-1 levels to increase. Although the same effect was not seen with HO-2, Knockdown of HO-2 has been shown to reduce Akt phosphorylation *in vivo* (Turkseven et al., 2007)and an inter-dependency between Akt-1 and HO-1 has been shown by our group recently (Cudmore et al., 2011).

Honokiol

The experimental data showed that sVEGFR-1 levels decreased with increasing concentration of Honokiol past 0.1µg/ml. No current studies have been done to show the effect of Honokiol on sVEGFR-1 levels providing it a potential therapeutic agent

for PE. Although honokiol is an inhibitor of the phosphorylation of Akt and so we hypothesised that it would increase the release of SVEGFR-1, it decreased it. It is likely that honokiol is exerting its effect by some other signalling pathway, which has not yet been fully investigated. Interestingly sEng levels were elevated with increasing concentrations of honokiol as we expected suggesting maybe sVEGFR-1 and sEng work by different mechanism and although this agent is yet to be fully investigated there may still be some positive progress with this drug.

Unfortunately due to time restrictions the experiments with heme oxygenase and Honokiol were not further investigated to see if they would have any further effects particularly on PE patient samples, possibly as a quencher of sVEGFR-1.

Clinical Application

Once a predictive biomarker has been established and is commercially and available for the diagnosis of early-onset preeclampsia, pregnant woman at risk of developing PE could be stabilised with statins. Statins may alleviate symptoms of PE since they inhibit cytokine – mediated sVEGFR1 release in placental explants (Cudmore, 2007). Low dose aspirin only mildly reduces the risk of future PE in high risk mothers (Ofori 2007). New methods of treatment either by inducing HO-1 activity or reducing sVEGFR-1 and sEng may have therapeutic potential and in turn prolong pregnancy in early onset PE, resulting in a decreased burden upon both mother and fetus (Ahmed 2011). The world's first randomised placebo-controlled trial, StAmP (**St**atins to **Am**eliorate Early Onset **P**reeclampsia) will inform medical practitioners whether statins are viable in PE.

This would seriously improve outcomes for mothers and foetuses globally and decrease the negative impact PE has on both.

Future Work

With more time and resources it would have been interesting to test the samples for a number of other possible markers including the possible implication of Ang II as a possible release mechanism for sVEGFR-1 in pregnancy. Ang II stimulates sVEGFR-1 production in a dose- and time-dependent manner from human villous explants and cultured trophoblasts but not from endothelial cells, suggesting that trophoblasts are the primary source of sVEGFR-1 during pregnancy (Zhou et al., 2007). This has not been investigated in actual serum from patients and could be a potential diagnostic marker.

Since this research was carried out, a number of research groups have carried out similar studies and so a lot of research has gone into this disorder leading to many advances and insight into the complications involved in preeclampsia and IUGR.

Selenium was found to be lower in patients with preeclampsia than controls. Since selenium is an antioxidant it raises the interesting the question of whether a small increase in selenium intake might help prevent preeclampsia in susceptible women (Rayman et al., 2003)

Most notably the involvement of HO-1 and its metabolite carbon monoxide (CO) in preeclampsia, inhibiting the release of sVEGFR-1 and sEng. Although animal models have been made to show the positive effects of HO-1 and CO, due to the risk of it being moved forward to clinical trial, none has been done yet until further research is carried out. Women with preeclampsia exhale less CO than normal pregnant women

suggesting preeclampsia may be due to a loss of HO-1 activity (Ahmed, 2011). Following on from this in April 2012 our group started a Phase II clinical trial (StAmP) into the potential role of pravastatin ameliorating early onset preeclampsia due to pravastatin increasing HO-1 expression and down-regulating tissue factor expression in monocytes and trophoblasts, preventing placental damage (Ahmed, 2011).

It would be beneficial to understand the intracellular signalling pathway involved with HO-1 to cause the decrease in sVEGFR-1 as possible drug targets in the future.

Unfortunately at the time of the completion of this thesis, Uterine artery Doppler results were not available. It may be a method that could be adopted by physician in a clinical setting if they see any significant measurable differences between PE or IUGR groups versus controls (see appendix ii).

A reliable animal model of IUGR does not exist at the moment without surgical intervention but could be developed to provide a better understanding of it since our current data shows there may be a different mechanism involved.

It is my opinion that preeclampsia could possibly be a genetic condition or have a genetic link triggered by environmental factors. A gene array of preeclamptic patients versus IUGR SGA and controls would allow us to observe any differences. Dietary deficiencies may be another cause and so could be observed. Wallis *et al* found that preeclampsia rates increased by nearly 25 % in between 1987 and 2004 in the USA (Wallis et al., 2008).

Further research on the role and mechanisms regarding the early diagnosis and treatment of PE and the reduction of sVEGFR-1 would help in the development of clinical benefits both during PE and in the future for both mother and fetus after birth.

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Appendix

Clinical Test	Gestationally Age Matched Control	PE	Eclampsia	HELLP syndrome	IUGR	PE + IUGR	PE + SGA
Aspartate aminotransferase U/L	25	66	112	193	21	48	29
Alanine aminotransferase U/L	25	76	78	188	21	46	37
Lactate dehydrogenase U/L	165	573	84	1025	275	566	459
1 minute Apgar score	8	8	6	7	8	6	8
5 minute Apgar score	9	9	8	9	9	9	10
Umbilical artery pH	7.26	7.24	N/A	7.25	7.18	7.20	7.20
Umbilical vein pH	7.33	7.29	7.20	7.29	7.26	7.26	7.27
Z Umbilical artery PI	-0.26	1.37	1.50	5.58	12.87	11.96	0.28
Z Uterine artery mean PI	0.40	4.25	N/A	5.80	4.47	5.41	4.07
Z Middle cerebral artery PI	-0.11	-1.05	N/A	-1.89	-2.76	-2.03	-1.47
Z Cerebro placental ratio	0.15	-1.75	N/A	-2.45	-3.66	-3.17	-0.41

Appendix ii. Clinical data collected during pregnancy of the different states of expectant mothers.

Preeclampsia; PE. Hemolysis, elevated liver enzymes, low platelets; HELLP. Intrauterine growth restriction; IUGR. Small for gestational age; SGA.