5.4 Discussion

To the best of our knowledge, this is the first study designed to examine the role of activated human primary peripheral blood monocytes from PE patients, on endothelial cell function in this condition. A model of human primary peripheral blood monocytes and HUVECs co-culture (n=9 each) was established, and then inflammatory responses to TLR2 and TLR4 ligands were investigated. The main finding in this experiment is that, compared to NP monocytes-HUVECs co-cultures, the inflammatory cytokine IL-6 and chemokines IL-18 and MCP-1 were amplified in PE monocytes-HUVECs co-cultures, whereas the anti-inflammatory cytokine IL-10 was attenuated.

Monocytes were chosen to study their effect on endothelial dysfunction seen in PE, because of the evidence of their key role in the regulation of endothelial cell proliferation, angiogenesis, and ECs survival (Schubert *et al.*, 2011, Schubert *et al.*, 2008). Also, they are activated in PE, as shown by this study (in Chapters 3 and 4) and others (Gervasi *et al.*, 2001, Luppi and Deloia, 2006). For example, there is up-regulation of CD11 and reactive oxygen species from PE monocytes. In addition to LPS (TLR4 ligand) and PDG (TLR2 ligand), fibrinogen and HMGB1 (endogenous ligands of TLR4) were selected as TLR agonists for this experimental model, owing to the results from our previous experiments (reported in Chapters 4 and 7): fibrinogen was the only endogenous TLR4 ligand that induced inflammatory cytokine release from PE monocyte cultures, and the plasma levels of both fibrinogen and HMGB1 are known to be deranged in PE.

Several studies have shown that IL-6 levels are elevated in the plasma of pre-eclamptic women and is involved in the pathogenesis of the condition (Conrad *et al.*, 1998, Freeman *et al.*, 2004, Greer *et al.*, 1994, Jonsson *et al.*, 2006, Kupferminc *et al.*, 1996, Vince *et al.*, 1995, Xiao *et al.*, 2012). Our observation buttresses a functional role for this cytokine in this condition. IL-6 is a multifunctional cytokine that modulates both pro- and anti-inflammatory functions in different sites, and under different conditions (Jones, 2005), an acute-phase response in the liver (Castell *et al.*, 1990, Heinrich *et al.*, 1990) and stimulates thrombopoiesis (Kaser *et al.*, 2001). It has been reported that IL-6 induces endothelial dysfunction through increasing the permeability of endothelial cells,

disorganising the morphological changes and altering the distribution of ultra-structural tight junctions (Desai *et al.*, 2002). One study reported that IL-6, released from ovarian carcinoma cells, induces aberrant angiogenesis (Nilsson *et al.*, 2005). Another showed that angiogenesis can be inhibited by IL-6 (Hatzi *et al.*, 2002). All these studies indicate that IL-6 could be a key regulatory factor in the modulation of physiological or pathological angiogenesis in normal pregnancy or one with pregnancy-induced hypertension. The results of this experiment revealed the elevation of IL-6 levels in PE monocytes-HUVECs co-cultures, suggesting that IL-6 might promote vascular endothelial dysfunction in PE. Furthermore, the bacterial TLR ligands-treated PE monocytes-HUVECs co-cultures induced extremely high levels of IL-6, suggesting that endothelial vascular damage in PE may involve TLR-mediated mechanisms. IL-6 participates in fibrinogen synthesis (Vasse *et al.*, 1996), which also induces the release of IL-6 (Jensen *et al.*, 2007).

One recent study has demonstrated an interaction between IL-6 and fibrinogen genetic variants in the pathogenesis of cardiovascular diseases. They found a potential gene-gene association which could influence fibrinogen levels, but did not appear to influence cardiovascular disease (Carty *et al.*, 2010). As compared to NP, fibrinogen induced the IL-6 production from PE monocytes monoculture, but reduced its release from PE monocytes-HUVECs co-culture. The mechanism of this observation is unclear but it seems plausible that monocyte release of IL-6 in the presence of fibrinogen may be altered by the presence of ECs in the microvasculature. It may also be that fibrinogen at this interface regulates monocyte activation and release of cytokines in order to protect endothelial cells from inflammatory damage.

It has been shown that IL-1β is a critical mediator of vascular inflammation in porcine and murine vascular diseases and co-cultures of primary human monocytes, and endothelial cell models (Chamberlain *et al.*, 2006, Morton *et al.*, 2005, Ward *et al.*, 2009). Interestingly, these studies found that IL-6 amplification from LPS-treated monocytes-HUVEC co-culture is inhibited by IL-1-dependent mechanisms, adding an important vascular role for IL-1β. Data from the current study demonstrate that IL-1β did not differ between PE mono- and co-cultures at basal levels. However, fibrinogen significantly induced IL-1β in PE compared to NP mono- or co-cultures model. One study reported that IL-1β, but not IL-1α, binds to fibrinogen and augments endothelial

cell activity in the bound form (Sahni *et al.*, 2004). It is plausible that impairment of such binding in PE may lead to an increase of free IL-1β that modulates further vascular inflammation.

IL-8 is a chemo-attractant for neutrophils and T cells (Baggiolini and Clark-Lewis, 1992), and is also defined as a potent angiogenic marker (Koch *et al.*, 1992) , whilst MCP-1 is a chemo-attractant for mononuclear leukocytes (Leonard and Yoshimura, 1990), and acts via its CCR2 receptor. Previous studies have shown that IL-8 and MCP-1 trigger firm adhesion of monocytes to the E-selectin, expressed by monolayers of vascular endothelium (Gerszten *et al.*, 1999), are highly expressed during monocytes-endothelial cells interaction (Lukacs *et al.*, 1995), and may enhance monocyte adherence to vascular endothelium (Beekhuizen and van Furth, 1993, Gerszten *et al.*, 1999, van Gils *et al.*, 2009) (also, see chapter 1 section 1.3.4). Kauma *et al*., demonstrated that plasma of severely pre-eclamptic women expressed higher levels of IL-8 and MCP-1, and induced HUVEC to release IL-8 and MCP-1(Kauma *et al.*, 2002). Thus, results of our study demonstrate elevation of chemokine production from NP monocytes-HUVECs co-cultures, indicating their functional role in promoting normal angiogenesis dysregulation of which may explain their amplification from PE monocytes-HUVECs co-cultures. Despite the fact that bacterial ligands induced production of IL-8 from PE monocytes-HUVECs co-cultures, fibrinogen appeared to suppress its production. The implication of this finding is unclear, but suggests some role for fibrinogen during angiogenesis. MCP-1 secretion was enhanced by LPS-treatment of endothelial cells in culture, whereas NP or PE monocytes inhibited MCP-1 production by endothelial cells in the co-cultures, after LPS stimulation. On the other hand, MCP-1 was amplified from PDG-treated PE monocyte-HUVECs co-cultures, whilst fibrinogen and HMGB1-treated PE monocyte-HUVECs co-cultures reduced its release. Taken together our data suggests a complex role for the TLR-2 and -4 receptors and their ligands in mediating vascular damage and repair in PE, which merits further investigation.

Various studies have reported that IL-10, an anti-inflammatory cytokine, was reduced in PE sera and placenta (Bowen *et al.*, 2005, Hennessy *et al.*, 1999, Makris *et al.*, 2006). Kalkunte *et al*. reported that IL-10 has immuno-modulatory activities and plays a protective role against blood pressure elevations, and inflammation-mediated vascular

dysfunction in the rat maternal-fetal interface (Kalkunte *et al.*, 2011, Lai *et al.*, 2011, Tinsley *et al.*, 2010). One study in a mouse model of pregnancy-induced hypertension demonstrated that the administration of exogenous IL-10 improved the hypertension and the endothelial function (Tinsley *et al.*, 2010). It is therefore plausible that the reduced secretion of IL-10 in PE monocytes-HUVECs co-cultures may reflect defective protection of vasculo-genesis from impairment mediated by other pro-inflammatory cytokines.

The activation of endothelial cells and leukocytes is a hallmark of the pathogenesis of PE (Ahn *et al.*, 2011, Austgulen *et al.*, 1997, Budak *et al.*, 1998, Gervasi *et al.*, 2001, Heimrath *et al.*, 2004, Higgins *et al.*, 1998, Holthe *et al.*, 2004, Kim *et al.*, 2004, Krauss *et al.*, 1997, Luppi and Deloia, 2006, Redman *et al.*, 1999, Roberts *et al.*, 1989, Sacks *et al.*, 1998, Sibai, 2004, Veas *et al.*, 2011, Yinon *et al.*, 2010). Whether the activation of endothelial cells is a consequence of the release of pro-inflammatory cytokines from activated leukocytes, is unclear. On the other hand, endothelial cell dysfunction caused by other placental-derived circulating factors, could exacerbate the inflammatory response in PE. Further studies are required to clarify these interactions.

Our co-culture experiments attempted to explore some of the inflammatory interactions between PE monocytes and normal endothelial cells. A drawback of the primary monocyte-HUVECs co-culture model is the inability to identify the cellular source of the inflammatory cytokines. However endothelial cells, which poorly secrete cytokines at basal levels, produce increased amounts of IL-6, IL-8 and MCP-1 when stimulated with LPS (TLR4 ligands); consistent with other reports (Zeuke *et al.*, 2002, Zhao *et al.*, 2001), and observations also made when HUVECs were co-incubated with PE monocytes. Moreover, our observations that endothelial cell monocultures produced IL-6, IL-8 and MCP-1 in response to LPS but not to PDG, is consistent with previous reports that endothelial cells lack the capacity to respond to TLR2 ligand (Elson *et al.*, 2007). To determine the cellular source of IL-6 and IL-1β in co-cultures stimulated by LPS, Ward *et al*. simply applied the supernatants from the LPS-treated monocytes on fresh HUVECs, or from LPS-treated HUVECs on fresh monocytes. This was followed by determining the activated levels of cytokines from cells (Ward *et al.*, 2009). He found that HUVECs were able to produce IL-6 but not IL-1β; and that amounts of IL-6 produced from endothelial cells were higher than observed from the HUVECs with

direct LPS stimulation. Also, the monocytes were able to produce both cytokines, and these levels were greater than observed from monocytes with direct LPS stimulation. Thus, they concluded that the endothelial cells are not a passive producer of IL-1β, but they can regulate their secretion from monocytes. However, these studies used non-validated methods to determine cellular origin of the inflammatory cytokines, which can be achieved with intracellular staining. Results of the current study demonstrated that endothelial cell type does not appear to be able to release IL-1β and IL-10 at basal levels and following stimulation with LPS, supporting the results of previous work (Ward *et al.*, 2009).

Our findings suggest that PE monocytes may play a role in the modulation of endothelial cell dysfunction in PE by altering the inflammatory processes mediated by TLR -2 and -4. Further work is needed to study the effect of monocyte on endothelial cell function. Such work may include cell migration assays (Murohara *et al.*, 1999), tubule formation assay (Arnaoutova *et al.*, 2009), viability and endothelial cell proliferation studies. In the next chapter we investigated angiogenic and vascular adhesion markers during normotensive and pe-eclamptic pregnancy using the HUVEC-monocyte co-culture model. Also, apoptosis and endothelial cell proliferation studies were attempted but could not be completed successfully (Appendix 4).

Chapter 6: The expression levels of angiogenic, anti-angiogenic and vascular adhesion molecule markers in a co-culture model of primary human endothelial cells and peripheral blood monocytes of women with pre-eclampsia and normotensive pregnant controls.

6.1 Introduction

Vascular endothelial cell dysfunction characterises PE (PE) and is associated with disturbance of angiogenic markers, vascular adhesion molecules and haemostasis (Austgulen *et al.*, 1997, Budak *et al.*, 1998, Heimrath *et al.*, 2004, Higgins *et al.*, 1998, Kim *et al.*, 2004, Krauss *et al.*, 1997, Roberts *et al.*, 1989, Veas *et al.*, 2011, Yinon *et al.*, 2010). Vascular endothelial growth factor (VEGF) is an angiogenic peptide responsible for increased vascular permeability, endothelial cell (EC) proliferation and migration (Tammela *et al.*, 2005, Yano *et al.*, 2006). VEGF acts through its receptors expressed by ECs: VEGF receptor -1 and -2 also referred as Flt-1 and Flk-1/KDR respectively. The soluble fms-like tyrosine kinase 1 (sFlt-1; also known as sVEGFR-1) is an unbound circulating splice variant of membrane-bound Flt-1. The molecule sFlt-1 is a powerful antagonist for VEGF and placenta growth factor (PIGF) (Kendall and Thomas, 1993). The elevated amount of sFlt-1 in PE is associated with reduced circulating levels of free VEGF and PIGF (Levine *et al.*, 2004, Powe *et al.*, 2011). The imbalance between the angiogenic factors, including VEGF and PIGF, and the anti-angiogenic factor (sFlt-1) released by the placenta, may play an important role in mediating severe endothelial cell dysfunction, impaired angiogenesis, hypertension, and proteinuria in females who develop PE (Levine *et al.*, 2004, Maynard *et al.*, 2003).

In the previous chapter, we demonstrated that PE monocytes activated human ECs and induced expression of inflammatory cytokines and chemokines compared to normal pregnant (NP) women. Additionally, the inflammatory responses to TLR ligands (lipopolysaccharides, peptidoglycan, fibrinogen and HMGB1) were investigated by comparing PE monocytes co-cultured with ECs to NP monocytes co-cultured with ECs.

In the observations reported in the current chapter we employed the same experimental model to determine the role of activated PE monocytes in dys-angiogenesis, and the effects of TLR ligands on the expression of angiogenic (VEGF), anti-angiogenic (sFlt-1), and cell adhesion molecule promoting (VCAM-1) factors in PE.

6.2 Materials and methods

(For more details, please refer to chapter 2.)

Subjects and samples

Please see Chapter 5 Section 5.2 for further details about subjects and samples used.

Stimulation of monocytes and HUVEC cultures

For more details, please refer to Chapter 5 Section 5.2.

Enzyme-linked Immunosorbent Assay (ELISA)

Human sVEGFR-1 / sFlt-1 Immunoassay (Quantikine ELISA kit catalogue number: DVR100B) was provided by R&D Systems, Europe, Ltd., and was used for the quantitative determination of human sFlt-1 concentrations in cell culture supernatants. All measurements were made in triplicate on 1:5 dilutions of the culture media.

Cytometric bead array

The cytometric bead array technique was used to measure the soluble vascular cell adhesion molecule-1 (VCAM-1) and VEGF in the supernatant medium collected from monocytes-HUVECs mono- or co-cultures.

Statistical analysis

Results are illustrated as mean ± standard error of the mean (SEM). The Mann Whitney test was used to compare differences between NP monocyte-HUVEC and PE monocyte-HUVEC co-cultures, using GraphPad Prism software version 5.0. The one way anova (Bonferroni multiple comparison test) was used to compare differences between responses to various ligands (bacterial and endogenous ligands of TLRs versus untreated samples). P values < 0.05 were considered significant.

6.3 Results

6.3.1 Clinical characteristics

Patient characteristics are shown in the table below.

Table 6.1 Patient characteristics

	Normal pregnant (n = 9)	Pre-eclampsia (n = 9)	*P* value
Age (years)	31.6 ± 6.4	31.5 ± 3.6	0.91
Gestational age (weeks)	33.15 ± 4.0	33.18 ± 5.8	0.67
Gravidity	1-4	1-3	
SBP (mm Hg)	114.0 ± 11.0	154 ± 5.8	0.0001
DBP (mm Hg)	70.0 ± 10.0	90.8 ± 2.4	0.0001
24 hour urine collection (g/24hrs)	0	1.6 ± 1.7	
Urine Dipstick Protein Test	0	(1+ - 4+)	

6.3.2 Fibrinogen attenuates sFlt-1-induced dys-angiogenesis

Human vascular endothelial cells monocultures produced high expression basal levels of sFlt-1. However, the expression levels of sFlt-1 from HUVECs was down-regulated by co-culture with monocytes from pregnant women ($P<0.001$); down-regulation of sFlt-1 by NP monocytes was significantly more than by PE monocytes ($P<0.05$) (**Figure 6.1**).

LPS or PDG-treatment of HUVECs monocultures reduced sFlt-1 production, but this observation did not attain statistical significance. On the other hand, stimulation of monocytes-HUVEC co-cultures by bacterial ligands, significantly induced sFlt-1 production in both NP and PE women ($P<0.05$).

Fibrinogen treatment of HUVECs monocultures significantly reduced sFlt-1 production ($P<0.05$). Furthermore, exposure of HUVEC-PE monocyte co-cultures to fibrinogen resulted in down-regulation of sFlt-1 production whilst this was unchanged for HUVEC-NP monocytes co-culture ($P<0.05$).

HMGB1-treatment of HUVECs monocultures appeared to reduce sFlt-1 production, but this did not attain statistical significance. HMGB1-treatment of HUVEC-NP monocytes co-cultures, increased sFlt-1 production, but not HMGB1-treatment of HUVEC-PE monocytes co-culture.

6.3.3 The angiogenic factor VEGF is reduced in HUVEC-PE monocytes co-cultures

Vascular endothelial growth factor was measured by cytometric bead array in collected supernatants from HUVEC monocultures; HUVEC with NP or PE monocytes co-cultures. Although the results obtained from the cytometric bead array were below the standard levels for VEGF, the differences between the variance groups were prominent as shown in (**Figure 6.2**).

HUVEC monocultures produced higher expression basal levels of VEGF than HUVEC with NP or PE monocyte co-cultures. Moreover, the VEGF production was significantly

lower for PE monocyte-HUVEC co-cultures compared to NP monocyte-HUVEC co-cultures ($P<0.05$).

After stimulation of mono or co-cultures with TLR ligands, there were no significant changes except for HMGB1. HMGB1-treatment of PE monocyte monocultures induced VEGF production ($P<0.05$).

6.3.4 Fibrinogen enhanced vascular adhesion in PE

This study also aimed to determine the interaction between monocytes of PE women and vascular endothelial cells by measuring the soluble vascular cell adhesion molecule, as depicted in (**Figure 6.3**).

HUVECs and NP/PE monocytes in monocultures produced very low levels of VCAM-1. Conversely, when the cell-to-cell interaction occurred during co-cultures experiments, there was a significant increase in VCAM-1 production, remarkably from HUVEC-PE monocytes co-cultures ($P<0.001$).

Non-stimulated EC alone produced extremely low levels of VCAM-1. LPS stimulation, but not other TLR ligands, was associated with higher levels of VCAM-1 ($P<0.05$) expression.

Compared to HUVEC-NP monocyte, HUVEC-PE monocyte co-culture was associated with higher basal expression levels of VCAM-1 ($P<0.05$), and their exposure to TLR bacterial ligands was also associated with an exaggerated VCAM-1 response ($P<0.05$).

Furthermore, HUVEC-PE monocyte co-culture exhibited an amplified VCAM-1 secretion in response to fibrinogen compared to HUVEC-NP monocytes co-culture ($P<0.05$). Additionally, HMGB1-treatment of HUVEC-PE monocyte induced slightly higher levels of VCAM-1 than NP co-cultures.

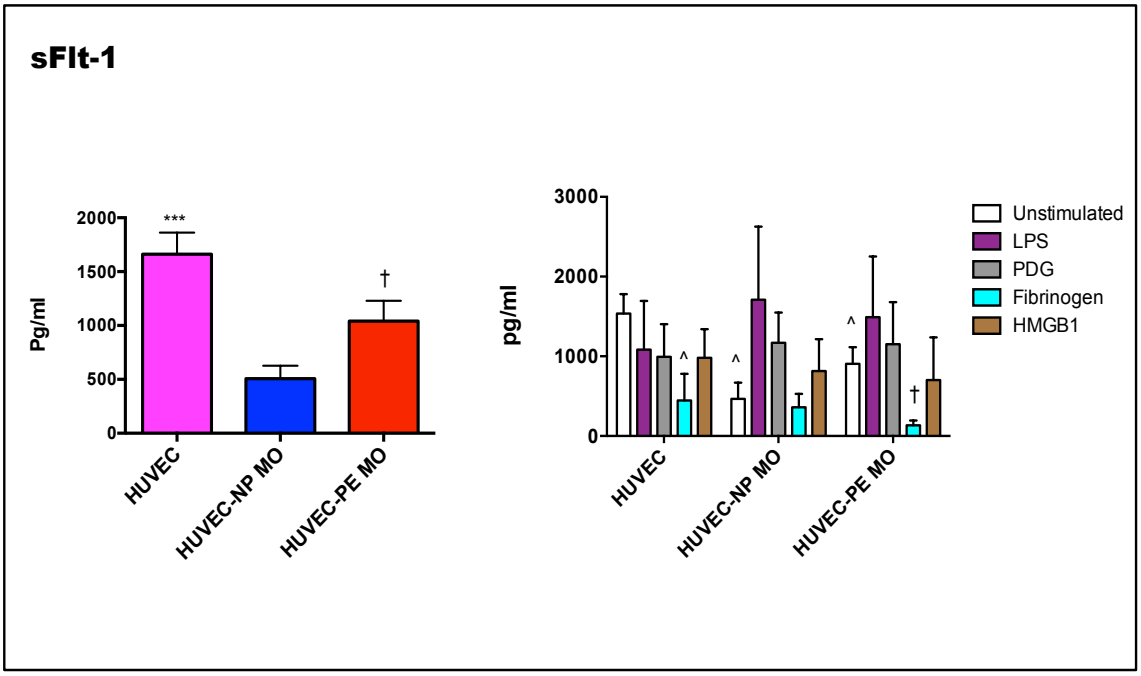

Figure 6.1 Fibrinogen attenuates sFlt-1-expression

Left panel: Basal levels of sFlt-1 production by HUVEC monoculture, HUVEC-NP MO co-culture or HUVEC-PE MO co-culture ***Right panel:*** Graphic representation of the response of HUVEC monoculture and HUVEC- NP or PE MO co-cultures to LPS, PDG, fibrinogen and HMGB1. Comparisons were made between HUVECs monoculture versus NP and PE co-cultures; NP versus PE co-cultures; and non-stimulated versus stimulated cultures. Statistical significance was determined by the Mann Whitney U test or one-way anova (n=5-7). $^{***}p < 0.001$ HUVEC as compared with HUVEC-NP/PE MO, $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ HUVEC-PE MO as compared with HUVEC- NP MO, and $^{\wedge}p < 0.05$ non-stimulated control compared with stimulated cultures.

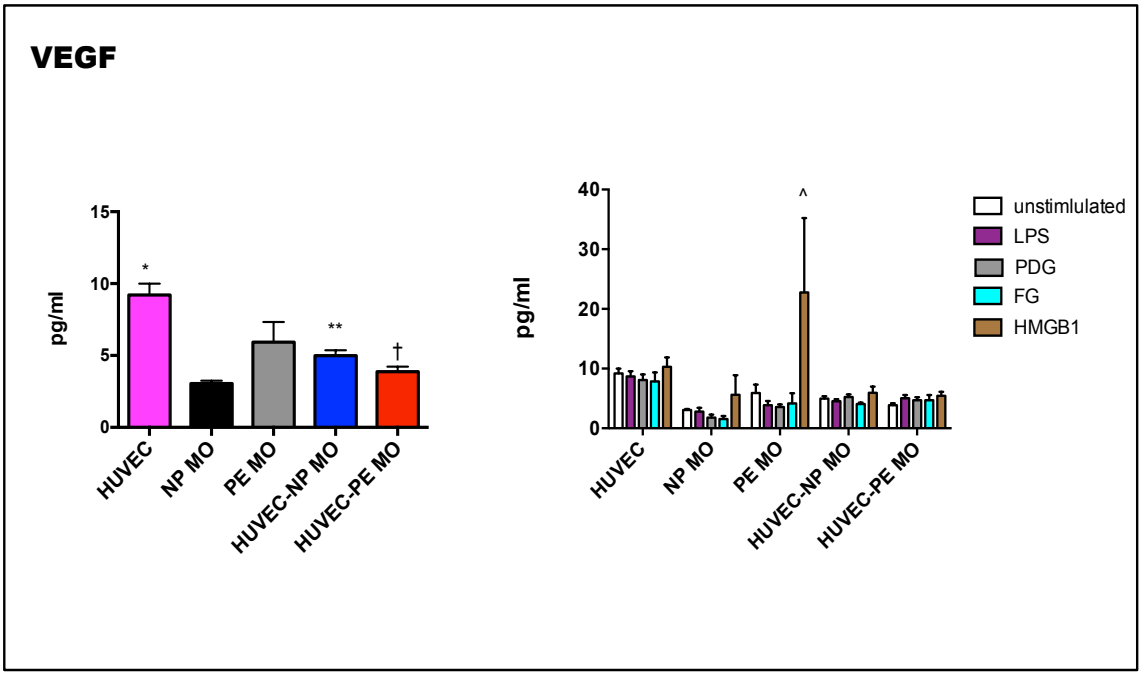

Figure 6.2 The angiogenic factor VEGF is reduced in HUVEC-PE monocytes co-cultures

Left panel: Basal levels of VEGF production by HUVEC monoculture, NP/PE MO monocultures, HUVEC-NP MO co-culture or HUVEC-PE MO co-culture ***Right panel:*** Graphic representation of the response of HUVEC monoculture, NP/PE MO monocultures and HUVEC- NP or PE MO co-cultures to LPS, PDG, fibrinogen and HMGB1. Comparisons were made between HUVECs monoculture versus NP and PE co-cultures; NP versus PE co-cultures; and non-stimulated versus stimulated cultures. Statistical significance was determined by the Mann Whitney U test or one-way anova (n=7-9). $^{*}P < 0.05$ HUVEC as compared with HUVEC-NP/PE MO, $^{**}P < 0.001$ NP monocytes monoculture as compared with HUVEC-NP MO co-culture, $^{\dagger}p < 0.05$ HUVEC-PE MO as compared with HUVEC-NP MO and $^{\wedge}p < 0.05$ stimulated cultures compared with non-stimulated control.

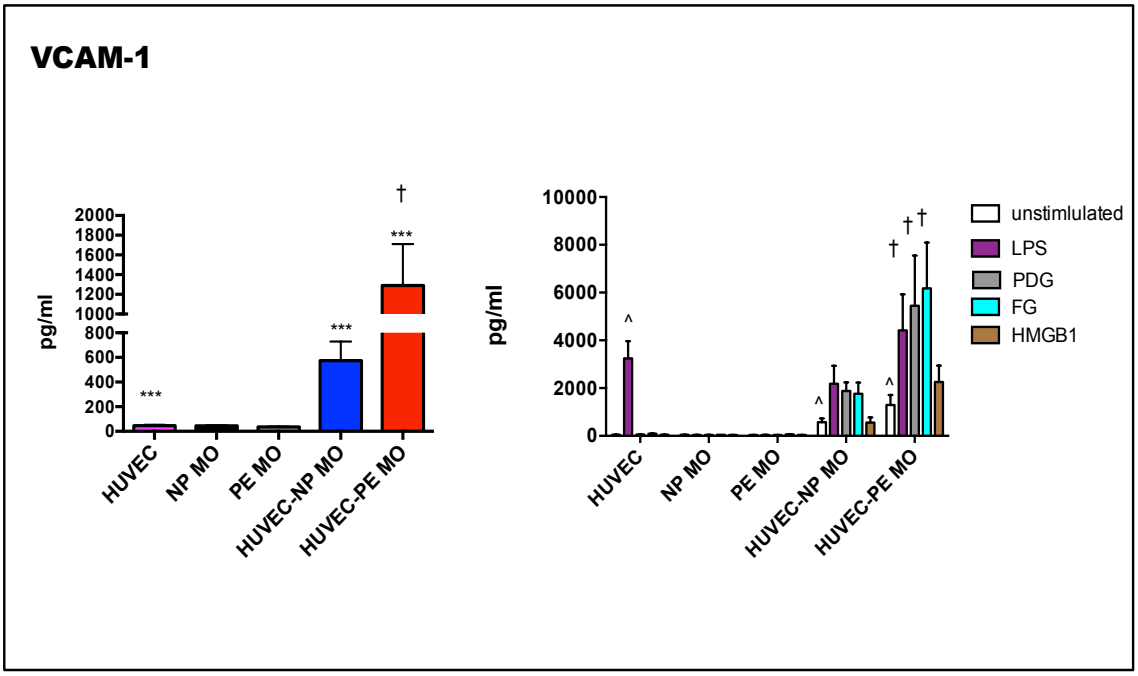

Figure 6.3 Fibrinogen enhanced vascular adhesion molecule expression in PE

Left panel: Basal levels of VCAM production by HUVEC monoculture, NP/PE MO monocultures, HUVEC-NP MO co-culture or HUVEC-PE MO co-culture ***Right panel:*** Graphic representation of the response of HUVEC monoculture, NP/PE MO monocultures and HUVEC- NP or PE MO co-cultures to LPS, PDG, fibrinogen and HMGB1. Comparisons were made between HUVECs monoculture versus NP and PE co-cultures; NP versus PE co-cultures; and non-stimulated versus stimulated cultures. Statistical significance was determined by the Mann Whitney U test or one-way anova (n=7-9). $^{***}P < 0.0001$ HUVEC or NP/PE MO monocultures as compared with HUVEC-NP/PE MO, $^{\dagger}p < 0.05$ HUVEC-PE MO as compared with HUVEC-NP MO and $^{\wedge}p < 0.05$ non-stimulated control compared with stimulated cultures.

6.4 Discussion

It is now generally accepted that circulating sFlt-1 anti-angiogenic protein plays an important role in dysangiogenesis in PE by suppressing circulating PIGF and VEGF, (Hertig *et al.*, 2004, Koga *et al.*, 2003, Levine *et al.*, 2004, Maynard *et al.*, 2003). Prior studies have shown that sFlt-1 is elevated in the sera of PE women, while PIGF is reduced even before the onset of the clinical disease (Hertig *et al.*, 2004, Lam *et al.*, 2005). Various cell types produce the sFlt-1, placental cells, endothelial cells and monocytes, but the exact physiological role of sFlt-1 remains unknown (Barleon *et al.*, 2001, Cho *et al.*, 2003, Clark *et al.*, 1998a, Kendall *et al.*, 1996). In the current study, we sought to determine if sFlt-1 could be produced by endothelial cells, by monocytes of PE or NP women, or even by the two cell types interacting together. Moreover, we sought to answer the question, 'is TLR4 likely to play a role in angiogenesis through the activity of any of its endogenous ligands?' In a previous experiment (Chapter 5), we demonstrated that the inflammatory responses to TLR ligands were altered in the PE co-culture model in comparison to normal pregnancy. In the current experiments, employing the same model in which co-cultures were treated with LPS, PDG, fibrinogen and HMGB1, we investigated key angiogenic (VEGF), anti-angiogenic (sFlt-1/sVEGFR-1), and cell adhesion molecule (VCAM-1) markers.

In contrast to the previous study demonstrating that peripheral blood monocytes were capable of producing sFlt-1, and that this production was enhanced by LPS stimulation (Barleon *et al.*, 2001), our results showed undetectable sFlt-1 release from PE or NP monocytes cultures, even after TLR stimulations. The differences between these studies could be explained by either the incubation period of monocytes monocultures (in this experiment, monocytes were incubated for 24 hours, but in the previous experiment, a longer incubation time of 72 hours was used) or the variation of blood donors (NP or PE monocytes cultures were used in the current study, but in the previous study, monocytes cultures isolated from buffy coats of normal volunteers were used). Thus, it is plausible that monocytes from pregnant women may demonstrate attenuated capacity to release sFlt-1 in order to promote angiogenesis. We have demonstrated that endothelial cells produced higher basal levels of sFlt-1, consistent with results of previous studies (Barleon *et al.*, 2001, Kendall *et al.*, 1996). For the first time however, we demonstrate

that sFlt-1 production by HUVECs can be inhibited by fibrinogen. Furthermore, the monocytes from both pregnant groups attenuated the sFlt-1 production from endothelial cells, but PE monocytes did not appear to suppress its release to the same degree as NP monocytes. Although the LPS- or PDG-treated NP/PE co-cultures induced the sFlt-1 production, the more surprising result is that the fibrinogen suppressed sFlt-1 production in the HUVEC-PE monocyte co-culture but not in the HUVEC-NP monocyte co-culture.

Fibrinogen is implicated in the regulation of coagulation and haemostasis, immunomodulation, inflammatory responses, and extracellular matrix (ECM) formation (Davalos and Akassoglou, 2012, Lominadze *et al.*, 2010, Mosesson, 2005). Numerous reports exist describing the influence of fibrinogen on such vascular endothelial cell functions as adhesion formation, spreading, microfilaments arrangements, migration, proliferation and angiogenesis (Bunce *et al.*, 1992, Chalupowicz *et al.*, 1995, Dejana *et al.*, 1987, Dejana *et al.*, 1985, Languino *et al.*, 1993, Sporn *et al.*, 1995), but its vascular role in PE is not understood. Sahni demonstrated that fibrinogen has two independent high affinity binding sites for VEGF, and fibroblast growth factor (FGF) angiogenic factors, which are able to stimulate ECs proliferations (Sahni and Francis, 2000). Interactions between ECM and integrins of ECs lead to stimulation of proliferation of endothelial cells and vascular morphogenesis (Davis and Bayless, 2003, Davis *et al.*, 2002, Davis and Senger, 2005, Senger, 1996). Fibrinogen, fibronectin and collagen are the most important ECM molecules responsible for these interactions, and bind to their integrin receptors on ECs (i.e. $\alpha_v\beta_3$ or $\alpha_5\beta_1$ receptors for fibrin matrices and $\alpha_2\beta_1$ or $\alpha_2\beta_1$ receptors for collagen matrices) (Davis and Camarillo, 1996, Senger *et al.*, 2002). Also, fibrinogen activates signalling mechanisms through $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins which lead to vasoactive effects, including decrease in endothelial cell-layer integrity, increase in vascular permeability and blood viscosity (Lominadze *et al.*, 2010). These altered physiological characteristics of the endothelial vascular architecture are considered to be a critical stage for development of hypertension, which is the fundamental clinical feature of PE. Nevertheless, the vascular endothelial VE-cadherin receptor (CD144) expressed on ECs binds to fibrinogen at $B\beta^{15-42}$ sequence, stimulating capillary tube formation and angiogenesis (Bach *et al.*, 1998). A study investigated the role of the ECM environment, including fibrin, vitronectin, fibronectin, laminin and collagen IV, on the growth factor receptors on ECs (FGFR-1, FGFR-2, VEGFR-1, and VEGFR-2)

(Tsou and Isik, 2001). They found that endothelial cells cultured on fibrin matrices had markedly reduced growth factor receptors; particularly FGFR-1, FGFR-2 and VEGFR-1, whilst vitronectin (it is a glycoprotein present in serum and ECM that participates in cell adhesion and haemostasis (Preissner and Seiffert, 1998)) induced all growth factor receptors. The reduction of FGFR-1, FGFR-2 and VEGFR-1 factors by fibrin was accompanied by diminished phosphorylation of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Tsou and Isik, 2001). Additionally, our findings of reduced soluble VEGFR-1 (sFlt-1) from ECs or ECs-PE monocytes is in agreement with a previous study demonstrating that ECs express lower levels of growth factor receptors (including VEGFR-1), when grown in fibrin matrices (Tsou and Isik, 2001). Taken together, in addition to its well-established role in vascular haemostasis, our observations suggest that fibrinogen may contribute to endothelial repair in PE by enhancing angiogenesis through mechanisms that may include, suppression of sFlt-1 production.

Integrins and ECM also play a role in trophoblast cell migration during placental implantation (Burrows *et al.*, 1996). The villous trophoblast cells express high levels of laminin receptor ($\alpha_6\beta_4$) however they become fibronectin receptor ($\alpha_5\beta_1$) and laminin/collagen receptors ($\alpha_5\beta_1$) upon migrating out from the villous basement membrane (Burrows *et al.*, 1996). In PE, trophoblast cells are unable to make this switch of receptor expression, and as a result alter their capacity for migration toward maternal decidua and spiral arteries, leading to inadequate neovascularisation and placental ischemia (Burrows *et al.*, 1996, Khong *et al.*, 1986). During angiogenesis, ECs penetrate the basement membrane, a process that requires the balanced synthesis and removal of ECM proteins (Lu *et al.*, 2011), and involves proteolytic enzymes including plasminogen activator (PA) and matrix metalloproteinases (MMPs) (Lu *et al.*, 2011). It has been suggested that the excretion of particular MMPs by extra-villous trophoblast would be critical for the degradation of specific matrix proteins in the decidua that allows trophoblast migration into stromal decidual vasculature (Burrows *et al.*, 1996). Trophoblastic cells (during implantation and placental development) and neoplastic cells (during cancer invasion) are similar in their proliferation, migration, invasiveness of ECM and angiogenesis (Zygmunt *et al.*, 2003). However, they differ as early pregnancy angiogenesis is self-limiting whilst neoplastic invasion and neovascularisation during oncogenesis, is unregulated.

A unifying hypothesis of the physiological processes may be summarised as follows. The angiogenic factors, VEGF, PIGF and FGF and TNF-α initiate angiogenesis by increasing vascular permeability. Proteolytic enzymes induce degradation of ECM; and stimulate proliferation of ECs, chemotactic migration and invasion of ECM to complete the formation of the lumen of mature blood vessels (neovascularisation) (Zygmunt *et al.*, 2003). The angiogenic phenotype of these proliferative ECs express integrins especially α_v, which mediates cell adhesion, spreading and proliferation of ECM containing fibrinogen and vitronectin (Zygmunt *et al.*, 2003). The interactions between ECM and integrins thus promote vascular growth and trophoblast invasion of the endometrium, a process controlled by specific MMPs and macrophages. Further studies will clarify the interaction between ECM proteins in placental development and PE.

Furthermore, our result demonstrated reduced levels of VEGF in PE monocytes-HUVECs co-culture compared to NP, confirming previous reports of increased sFlt-1 and reduced VEGF in PE (Hertig *et al.*, 2004, Lam *et al.*, 2005). One unanticipated finding that HMGB-1-treatment of PE monocytes monoculture induced VEGF production whilst other TLR ligands did not. The explanation for this finding that HMGB-1 stimulates VEGF production from PE monocyte through different signalling pathway for example, advanced glycation end product (RAGE) (Huang *et al.*, 2010). This suggestion is supported by a recent study demonstrating that HMGB1 promotes VEGF-C expression through RAGE signalling pathway in oesophageal squamous cell carcinoma (Chen *et al.*, 2012).

During acute inflammation, endothelial cells regulate monocytes adhesion and migration from the vasculature, to sites of inflammation through expression of endothelial surface adhesion molecules (Beekhuizen and van Furth, 1993), more details on Chapter 1 Section 1.3.4. A considerable amount of literature has been published on endothelial cells and monocytes interaction (Barleon *et al.*, 1996, Beekhuizen and van Furth, 1993, Faruqi and DiCorleto, 1993, Lukacs *et al.*, 1995, Takahashi *et al.*, 1996a). These studies described the molecular basis of cell-cell interactions, including adhesions molecules expressed on either ECs or monocytes (Beekhuizen and van Furth, 1993, Takahashi *et al.*, 1996a). In-vitro studies revealed that stimulation of cultured endothelial cells by inflammatory cytokines, such as IL-1 and TNF-α, increased their expression for adhesion molecules and adhesiveness to leukocytes (i.e monocytes)

(Beekhuizen *et al.*, 1991, Beekhuizen and van Furth, 1993). Monocytes are able to adhere to cytokines-stimulated ECs by at least three adhesion mechanisms: a) binding of VLA4 (CD49D/CD29) expressed on monocytes to VCAM-1 on ECs; b) interaction of LFA-1 (CD11a/CD18) expressed on monocytes with intercellular adhesion molecule-1 (ICAM-1) on endothelial cells; and c) binding of E-selectin expressed on ECs to as an yet unidentified molecule on monocytes (Beekhuizen and van Furth, 1993). Moreover, numerous studies demonstrated that monocytes co-cultured with ECs augmented their surface expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, which are partially inhibited by anti-IL-1 and anti-TNF monoclonal antibodies (Combe *et al.*, 1995, Noble *et al.*, 1996, Takahashi *et al.*, 1996a, Tsouknos *et al.*, 2003). The soluble adhesion molecules include VCAM-1, ICAM-1 and E-selectin and are reported to be elevated in the sera of PE patients (Higgins *et al.*, 1998, Krauss *et al.*, 1997). Additionally, a study demonstrated that sera of PE women activated the ECs to express ICAM-1 but only in the presence of monocytes (Faas *et al.*, 2010).

In the present study we investigated adhesion molecule expression changes during the interaction of ECs and monocytes in NP and PE, and to determine the effects, if any of TLR ligands on this interaction. Consistent with previous studies (Faas *et al.*, 2010, Higgins *et al.*, 1998, Krauss *et al.*, 1997), the PE monocyte-EC interaction increased expression levels of VCAM-1, and that induction was enhanced by bacterial TLR ligands (LPS and PDG) and fibrinogen. It has been shown that the inflammatory cytokines (IL-1 and TNF-α), or microbial LPS stimulation induced expression of adhesion molecules on ECs (Beekhuizen and van Furth, 1993, Swerlick *et al.*, 1992, Zeuke *et al.*, 2002). My data demonstrates that LPS-treated HUVECs induce expression of VCAM similar to the inflammatory cytokines: IL-8, IL-6 and MCP-1. Also, the PE monocytes-HUVECs co-culture, expresses extremely high levels of adhesion molecules and inflammatory cytokines at basal level and more so following treatment with LPS (see Chapter 5). Taken together, it is plausible that the TLR ligands which we have studied, stimulate adhesive molecule formation either directly or indirectly through the induced expression of inflammatory cytokines as reported in Chapter 5.

As a response to injury and inflammation the attachment of monocytes to vascular endothelium is amplified (Issekutz *et al.*, 1981). Moreover, monocyte-ECs interaction play an important role in the pathogenesis of atherosclerosis in which monocytes bind

inappropriately to damaged endothelial cells of large and medium sized arteries, and then migrate to the sub-endothelial space where they mature into lipid-containing foam cells (macrophages) (Gerrity, 1981, Moore and Tabas, 2011). There are similarities between PE and atherosclerosis: their risk factors and their pathophysiology, including oxidative stress and endothelial dysfunction, are similar (Belo *et al.*, 2008). Acute atherosis alterations in the uterine spiral artery of PE resemble an early stage of atherosclerotic lesion which is featured by focal endothelial distraction, vascular wall fibrinoid necrosis, accumulation of sub-endothelial lipid-laden foam cells, perivascular mononuclear cells infiltration, lipid and lipoprotein deposition (Staff *et al.*, 2010). Both diseases are characterised by hyperlipidemia and involve monocytes and macrophages (Staff *et al.*, 2010). The risk of subsequent development of cardiovascular diseases (i.e hypertension) in later life is increased for both mother and their offspring after PE (Anderson, 2007). Additionally, fibrinogen is considered as an independent risk factor for cardiovascular diseases such as hypertension, and may play a crucial role in the initiation and progression of atherosclerotic plaques by increasing blood viscosity, activating ECs and smooth muscle cells, inducing platelet aggregation and by recruiting immune cells (Levenson *et al.*, 1995, Lominadze *et al.*, 2010). Languino *et al*. revealed in their studies that fibrinogen acts as molecular bridge between endothelial cells and leukocytes, enhancing the adhesion of leukocytes to vascular endothelium and facilitating leukocyte trans-endothelial migration (Languino *et al.*, 1995, Languino *et al.*, 1993). Also, they found that ICAM-1 is a fibrinogen receptor on ECs and mediates bridging pathways with leukocytes; anti-ICAM-1 monoclonal antibodies block the interaction of leukocytes with endothelium usually facilitated by fibrinogen (Languino *et al.*, 1995). The study showed that fibrinogen-ICAM-1 recognition is structurally different from what was implicated in previous studies on the ECs-monocytes interaction via CD11a/CD18 (Languino *et al.*, 1995). The fibrinogen-ICAM-1 interaction is associated with activation of MMPs and the digestion of junction proteins, thus disrupting endothelial layer integrity and increasing vascular permeability (Lominadze *et al.*, 2010). Fibrinogen seems to accumulate in the sub-endothelium at the site of vascular injury and may then act as matrices over which endothelial cells could attach, proliferate and spread (Dejana *et al.*, 1987, Lominadze *et al.*, 2010). It has been shown that monocytes incubated with conditioned medium of HUVECs or indirect contact with HUVECs stimulate expression of MMP-9 that is implicated in atherogenic progression (Amorino and Hoover, 1998). Additionally, Kaneider *et al.* showed that

fibrinogen induces pro-coagulant activation by monocytes; and increases MMP-9 release (Kaneider *et al.*, 2010). Moreover, fibrinogen stimulates TLR4 signalling in macrophages to release MMP-1 and MMP-9 (Hodgkinson *et al.*, 2008). All of these findings suggest some similarities in the pathophysiology of atherosclerosis and PE. Fibrinogen may bind to ICAM-1 in ECs and/or stimulate TLR4 to induce MMP release, increased vascular permeability, leading to its deposition in the sub-endothelial spaces. Adhesion of PE monocytes to vascular endothelium may interfere with its trans-migration across the feto-maternal interface, thereby impeding the vascular remodelling associated with normal placental development (Gomez-Lopez *et al.*, 2010). The accumulation of activated monocytes and other inflammatory cellular elements in the vascular sub-endothelium may also lead to the local vessel inflammation and placental ischemia seen in PE.

In conclusion, fibrinogen is a large molecule with multiple binding sites for platelets, leukocytes and ECs. Such sites promote its overlapping functions in haemostasis and immunomodulation (See Section 1.3.6.2.E) (Davalos and Akassoglou, 2012, Mosesson, 2005). During normal pregnancy, plasma fibrinogen is increased and fibrinolysis activity is suppressed ostensibly to promote coagulability of blood (Brenner, 2004, Thornton and Douglas, 2010). There are conflicting data regarding fibronoegn levels in PE, with some studies, including ours demonstrating increased circulating levels (Karehed *et al.*, 2010, Williams *et al.*, 2007), whilst others demonstrate reduced levels (Schjetlein *et al.*, 1997). Moreover, immunofluorescence microscopy has revealed fibrin deposition in the vascular compartments of the placenta, kidney and liver in severe PE (Arias and Mancilla-Jimenez, 1976, Bonnar *et al.*, 1971, Petrucco *et al.*, 1974, Roberts and Post, 2008, Stillman and Karumanchi, 2007). Therefore, fibrinogen appears to be required for placental angiogenesis in early pregnancy. Perturbation of the equilibrium of fibrinogenesis and fibrinolysis may lead to its deposition in vascular sub-endothelium, induce inflammation and lead to micro-vascular dysfunction. Further studies are required to explore this thesis in order to clarify the pathophysiology of PE. Our findings from the monocytes-ECs co-culture experiments indicate that fibrinogen induced extremely high levels of VCAM-1 from PE versus NP co-cultures, which is similar to the LPS-induced PE co-culture VCAM-1 expression (**Figure 6.3**). However, it suppressed sFlt-1 production in the HUVEC-PE monocyte co-culture, which contrasts with LPS-induced PE co-culture sFlt-1 levels

(**Figure 6.1**). Therefore, it seems that fibrinogen may act through TLR4-dependent mechanisms to induce adhesion molecule expression; but that a mechanism of sFlt-1 suppression may involve TLR4-independent mechanism. We did not employ a specific TLR4 antagonist such as Eritoran (Chapter 1 Section 1.3.6.3) to demonstrate blocking of TLR-mediated activities. Furthermore we did not set out to study inhibitors of the fibrinogen receptors for leukocytes and integrins. It is recommended that such further research be undertaken to: a) explore novel molecular mechanisms involving fibrinogen in the regulation of ECs function (adhesion, viability, migration and proliferation); and b) to determine whether angiogenesis in normal pregnancy and pregnancy complicated by PE involves TLR4 and integrin receptors.

Chapter 7: Placental localisation of TLR4 endogenous ligands and their expression in plasma.

7.1 Introduction

The innate immune system recognises pathogens via pattern recognition receptors called toll-like receptors (TLRs) (Akira *et al.*, 2001, Medzhitov *et al.*, 1997). Toll-like receptor 4 (TLR4) is known for its ability to recognise lipopolysaccharide (LPS). In addition to recognizing LPS, TLR4 interacts with a host of endogenous molecules and induces inflammatory and immune responses in the absence of infection (Tsan and Gao, 2004b). These endogenous ligands contain proteins, such as heat shock proteins, heparan sulfate proteoglycan, fibrinogen, fibronectin, extracellular matrix hyaluronan and high mobility group box 1 protein (HMGB1), all released from damaged cells during stress.

Recent studies have demonstrated up-regulation of TLR4 in trophoblast, placenta and leukocytes in PE, but the exact role of TLR4 in the pathogenesis of PE remains unclear (Kim *et al.*, 2005, Xie *et al.*, 2009). Additionally, the data from this thesis (chapter 3) demonstrate high expression levels of TLR4 in PE monocytes and its non-classical subpopulation.

The main hypothesis of this research is that TLR4 activity plays a key role in the pathogenesis of PE through one or more of its endogenous ligands, which may be produced by the ischaemic placenta. This study also attempted to determine the plasma levels of endogenous ligands, such as fibrinogen, fibronectin, heparan sulphate, HMGB1 and hyaluronan, in pre-eclamptic women; these levels were compared to the levels of both non-pregnant (Non-p) and normal pregnant (NP) women. Furthermore, the results from chapters 4, 5 and 6 demonstrate that fibrinogen can induce inflammatory cytokines and adhesion molecules in the PE monocytes monocultures, or in the PE monocytes-endothelial cells co-cultures. As a result of these findings, the fibrinogen expression level was investigated in the placenta of PE and NP women.

7.2 Materials and methods

(For more details, please refer to chapter 2.)

Subjects and samples

The study group consisted of 18 PE, 17 NP and 5 non-pregnant women for measuring plasma levels of fibrinogen. For the purpose of measuring plasma levels of fibronectin, heparan sulphate, HMGB1 and hyaluronan, the study group consisted of 10 PE, 10 NP and 5 non-pregnant women. Women with established PE, that was diagnosed using the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (2004), were recruited for the study. Once their diagnosis was confirmed, the participants were approached in the Day Assessment Unit of Jessop Wing Hospital. Healthy NP women attending routine antenatal clinics at Jessop Wing were recruited to be a part of the control group. All the participants in the study were negative for systemic infection, or urinary tract infection. Consent was obtained from the study groups and, during a scheduled blood collection, an additional 12 ml of fresh blood was collected from each subject into a tube containing EDTA to prevent clotting. Plasma was collected from the top layer of the blood, centrifuged on top of Ficoll-Paque and immediately frozen at -80°C.

Immediately, following a normal vaginal delivery or a caesarean section, the placenta was collected from participants (6 PE and 5 NP women) who had previously given consent. When compared to a healthy placenta, the placenta obtained from a woman with severe PE with foetal growth restriction may be smaller, and may demonstrate areas of calcification and infarction. Two or three pieces were removed from the umbilical cord and immediately soaked and fixed for 24–48 hours in 10% formalin. The samples were wax embedded for immunohistochemistry staining.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA kits were used to measure the plasma levels of endogenous ligands of TLR4 heparan sulphate (Uscn Life Science Inc. Wuhan, China), fibrinogen (Abnova, Germany), fibronectin (Bender MedSystems, Vienna, Austria), HMGB1 (Shino-Test Corporation, Japan) and hyaluronan (Echelon Biosciences Inc. Salt Lake City, UT)]

from the three groups of subjects we studied. The manufacturer's instructions were followed in the triplicate measurements.

Immunohistochemistry

Wax-embedded placental tissues were immunostained for fibrinogen antibody (Dako UK Ltd) as described in the immunohistochemistry protocol (Chapter 2).

Analysis of staining intensity

The immunostained slides were examined using an Olympus CKX41SF (Olympus Corporation, Tokyo, Japan) microscope connected to an Olympus digital camera at the following magnifications: X100, X250 and X400. The appearance of brown staining indicated a positive fibrinogen reaction. Based on previous work (Karehed *et al.*, 2010), the intensity of staining was scored on a scale from one to four. One indicated no staining and four indicated strong staining. The percentage of cells stained in each score was estimated. The intensities of fibrinogen staining in the endothelial layer of foetal blood vessels and villous stromal cells were evaluated separately. The analysis was performed in five randomly selected areas on each slide (i.e., three immunostained slides for each patient) with a double blind producer. Negative control experiments were performed for each patient using rabbit immunoglobulin fraction (Dako, catalogue number: X0936), and non-specific staining was not noticed. In order to identify the histology, hematoxylin and eosin (H&E) staining was performed for each placenta **(Figure 7.1)**.

Statistical analysis

This was performed using GraphPad Prism software version 5.0. The results from the plasma levels of endogenous ligands are illustrated as mean ± standard error of the mean (SEM). The results from placental fibrinogen are illustrated as median and interquartile range. Mann-Whitney *U*-test was used to compare the differences between Non-P and NP or NP and PE. Values < 0.05 were considered significantly different.

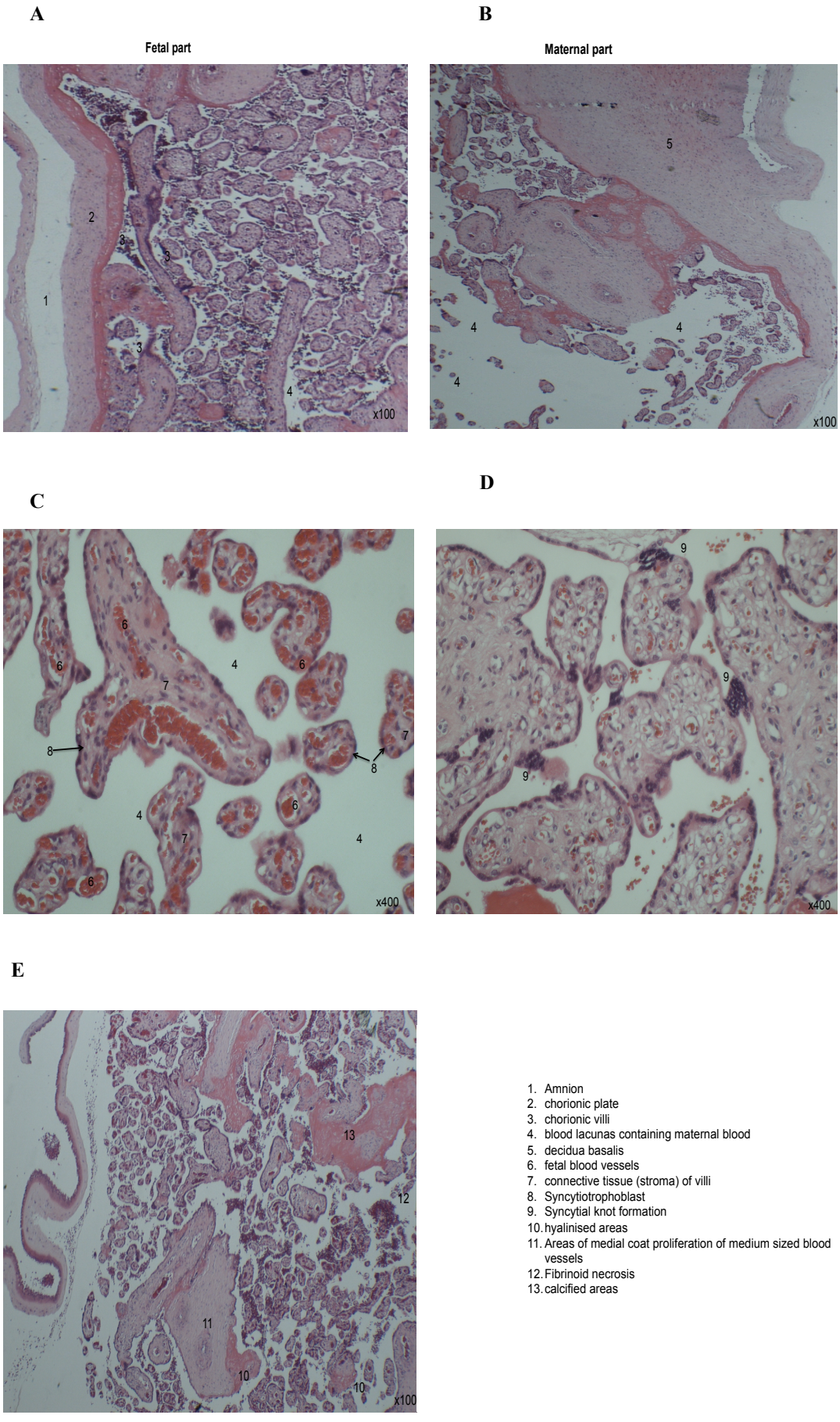

Figure 7.1 Microscopic features of normal pregnant (NP) and PE (PE) placenta (H and E stain)

A, B and C represent the histological anatomy of NP placenta delivered by SVD. **D and E** represent abnormal histological features of PE placenta delivered by SVD.

7.3 Results

7.3.1 Clinical characteristics

Patient characteristics are shown in table below.

Table 7. 1 Patient characteristics

	Non-pregnant	Normal pregnant	PE	P value
Plasma fibrinogen Plasma endogenous ligands Placenta fibrinogen	(n=11) (n=5)	(n=17) (n=10) (n=5)	(n=18) (n=10) (n=6)	
Age (years)	31.0 ± 5.4	32.5 ± 6.1	32.06 ± 5.9	0.81
Gestational age (weeks)		31.4 ± 4.2	32.03 ± 4.3	0.68
Gestational length (weeks)		39.5 ± 0.84	36.6 ± 3.0	
Gravidity		1-4	1-4	
SBP (mm Hg)	110.0 ± 10.0	114.6 ± 10.6	151 ± 7.5	0.0001
DBP (mm Hg)	70.0 ± 10.0	74.7 ± 8.6	96 ± 4.5	0.0001
24 hour urine collection (g/24hrs)	0	0	0.9 ± 1.2	
Urine Dipstick Protein Test	0	0	(1+ - 3+)	

7.3.2 Fibrinogen, fibronectin and HMGB1 were elevated in the plasma of PE

Fibrinogen levels in plasma from the pregnancy groups were higher than those in non-pregnant group ($P<0.01$). Also, when compared to NP women, the fibrinogen levels were higher in the PE women; however, the data was not statistically significant. The levels of fibronectin did not differ between the Non-P and NP women, but it was significantly higher in the PE women than in NP and Non-P ($P<0.01$) (**Figure 7.2)**.

The HMGB1 levels were significantly lower in the plasma from the NP than in the Non-P group ($P<0.01$). Women with PE had higher levels of HMGB1 than in NP women, but the data were not statistically significant. Heparan sulphate levels did not differ between the three study groups (**Figure 7.2)**.

7.3.3 Fibrinogen expression levels in the placental tissue

As depicted in the Figures 7.3 and 7.4, the fibrinogen expression levels were lower in the endothelial layer of the foetal blood vessels from the placental tissues of PE women when compared to NP controls; however, the data did not achieve statistical significance ($P = 0.4103$). Fibrinogen expression levels in the villous stromal cells did not differ significantly between placentae from normal pregnancy, and those from women with PE placental ($P = 0.5368$) (**Figure 7.3 and 7.4**).

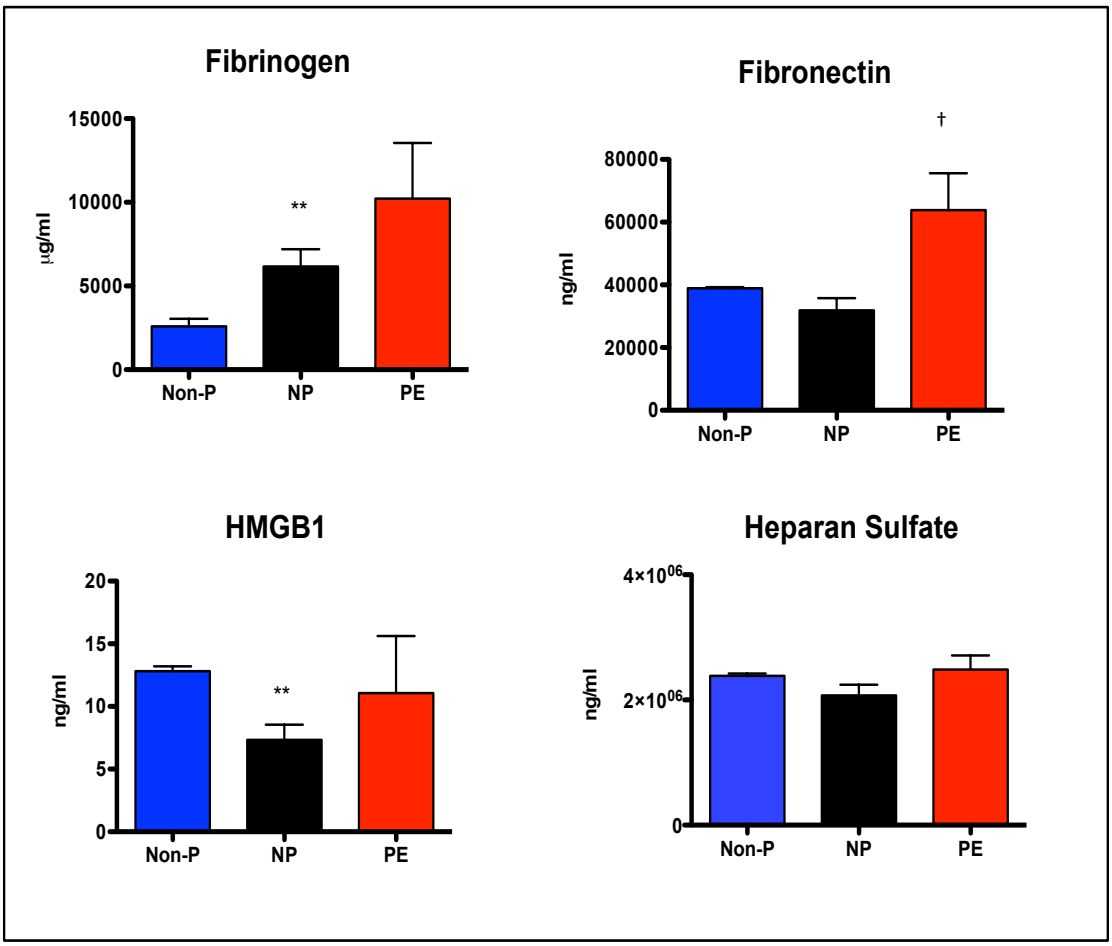

Figure 7.2 Plasma levels of endogenous ligands measured in three study groups; non-pregnant (Non-p), normal pregnant (NP), PE (PE) women

Values are illustrated as mean ± SEM. Statistical significance was determined using the Mann-Whitney *U* test (n = 5-18); $^{**}p < 0.01$ as compared with Non-P; $^{\dagger}p < 0.05$ as compared with NP.

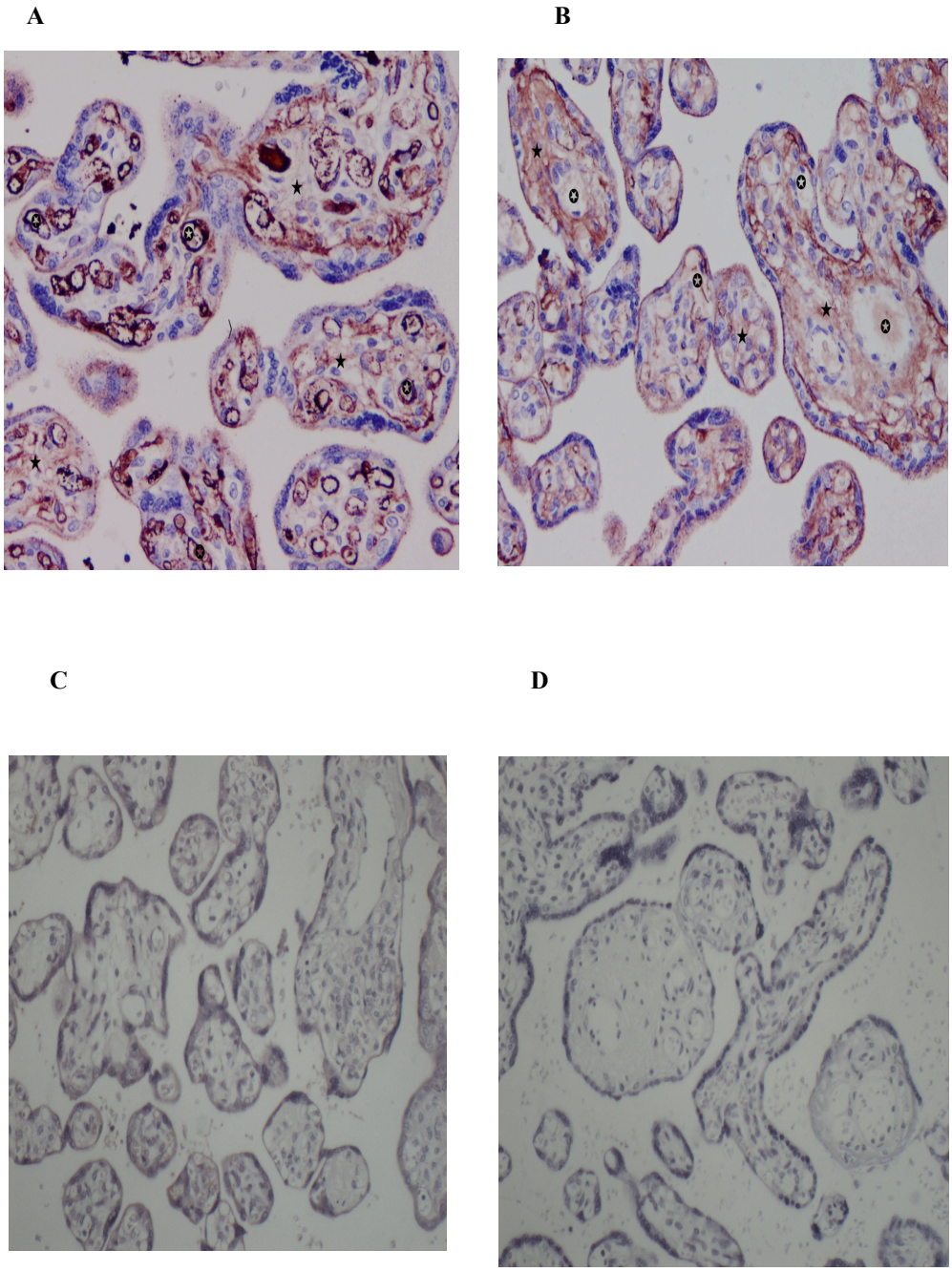

Figure 7.3 Photomicrographs of immunostaining of fibrinogen in the human placenta

A. Fibrinogen (brown colour) in normal pregnant placenta **B.** Fibrinogen (brown colour) in PE placenta **C.** Negative control using rabbit immunoglobulin fraction **D.** Negative control by omitting the primary antibody. ✪ Represents the endothelial layer of foetal blood vessels; ⋆ Represents the villous stromal cells.

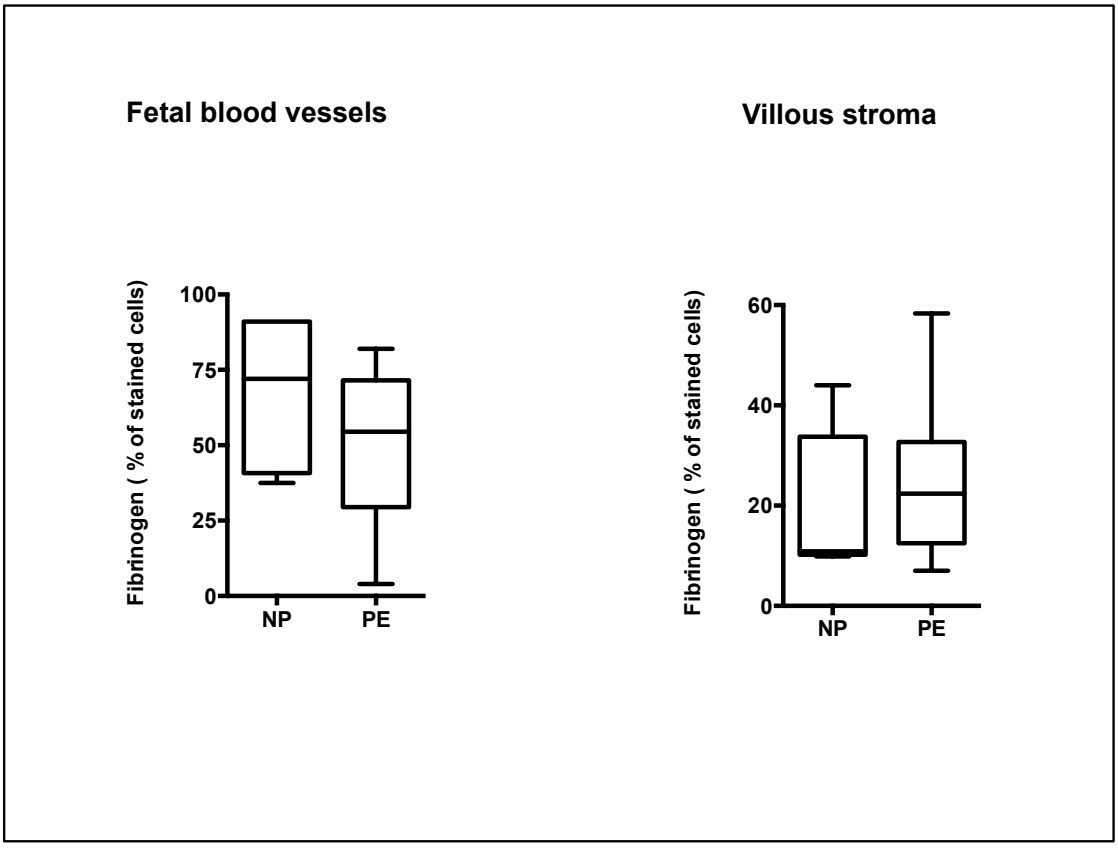

Figure 7.4 Fibrinogen expression in placentas of normal pregnant (NP) and PE women

Quantification of fibrinogen expression in the endothelial layer and villous stromal cells. The box length represents the interquartile range; the line inside the box represents the median; and the bars on top of the box represent the maximum and on the bottom represent the minimum values (n=5-6).

7.4 Discussion

The results from this chapter are consistent with previous work showing that plasma fibrinogen is increased in the plasma of PE women, but decreased in the endothelial layer of the foetal blood vessels of the placenta (Karehed *et al.*, 2010). The latter study also showed that early onset PE, but not late onset disease, results in higher plasma fibrinogen than in gestational age-matched pregnant control women (Karehed *et al.*, 2010). Additionally, compared to normotensive pregnancy, plasma fibrinogen is elevated in both mild and severe PE women (Ustun *et al.*, 2005). In contrast, other studies found lower plasma fibrinogen levels in moderate and severe PE women as compared to healthy pregnant controls (Schjetlein *et al.*, 1997, Thorburn *et al.*, 1982), yet others found no significant difference (Bonnar *et al.*, 1971, Heilmann *et al.*, 2007). Moreover, women with severe PE have been shown to demonstrate elevated levels of dimeric fibrin fragment D-dimer, suggesting increased fibrinogen degradation and fibrin deposition in multiple organs (Heilmann *et al.*, 2007, Schjetlein *et al.*, 1997). This could explain the reduction of fibrinogen in the endothelium of PE placenta, as shown by our study and by Karehed (Karehed *et al.*, 2010). Unfortunately, due to the limited number of participant, fibrinogen levels in the present studies did not differ significantly and could not be correlated with either the onset, or the severity of the disease.

Altogether, altered plasma fibrinogen levels in PE may reflect either an increase of the acute phase reaction (releasing proteins include fibrinogen) in response to the inflammation, or an increase in fibrinolytic activity: fibrin deposits reported in these women may be related to the onset or the severity of the disease (Kanfer *et al.*, 1996). TLR4 signalling is reported to induce fibrinogenesis during hepatic injury (Guo and Friedman, 2010), suggesting that TLR4 may play a role in inducing fibrinogen formation in PE to limit inflammation in PE.

Fibronectin binds fibrin and is deposited at the site of injury to promote blood clot formation. It is part of the ECM that is involved in cell attachment, tissue repair and, more recently, TLR4 signalling (Okamura *et al.*, 2001, Pankov and Yamada, 2002). Our results show that fibronectin does not differ between normal pregnant women and non-pregnant women, but is significantly increased in women with PE, consistent with several studies that have reported elevated fibronectin levels in pregnancy-induced

hypertension. (Brubaker *et al.*, 1992, Chen *et al.*, 1994, Eriksen *et al.*, 1987, Thurnau *et al.*, 1987). One study demonstrated that fibronectin expression decreased in the foetal villous vessels of PE placenta compared to placental tissue from normotensive women (Anunciado *et al.*, 1987).

High mobility group box 1 protein (HMGB1) is a DNA-binding nuclear protein that also releases extranuclear by injured-necrotic cells and acts as a pro-inflammatory cytokine in sepsis, and acute inflammation by inducing signalling via TLR2, TLR4 and receptor for advanced glycation end product (RAGE) (Huang *et al.*, 2010, Yu *et al.*, 2006). Recent studies have demonstrated non-significant increases in expression of HMGB1 in placental tissue from PE women (Holmlund *et al.*, 2007, Wang *et al.*, 2011). Moreover, Bo Wang and other investigated the HMGB1 in the sera of PE women and NP women but did not include a Non-P control; their results show no significant difference between the two study groups (Wang *et al.*, 2011). In our study, we have shown that there is a significant reduction of plasma HMGB1 during pregnancy. However, the levels were higher in PE subjects, than in NP controls. A possible explanation for this might be that HMGB1 is produced from necrotic trophoblasts in PE: one study demonstrated that the TLR2 bacterial ligand induces necrosis in murine trophoblastic stem cells and is associated with the release of HMGB1 (Rose *et al.*, 2011). Another possible explanation is that in normal pregnancy the necrotic trophoblasts are cleared by phagocytic decidual macrophages, which clear apoptotic debris (Abrahams *et al.*, 2004b), a process that may be defective in PE, causing overexpression of HMGB1 in plasma and placenta.

Hyaluronan is one of the ECM components implicated in cell proliferation, migration, tissue repair, embryogenesis, angiogenesis and inflammatory processes (Nangia-Makker *et al.*, 2000, Taylor *et al.*, 2004, Termeer *et al.*, 2000). Several studies have shown increased hyaluronan in the plasma and the placenta of PE women (Matejevic *et al.*, 1999, Matejevic *et al.*, 2001, Osmers *et al.*, 1998). Unfortunately, the hyaluronan ELISA kit did not work in our study, because the concentration of hyaluronan in the diluted plasma samples was below detection.

To the best of our knowledge, no previous study has investigated heparan sulphate in the plasma of PE. We have found that plasma heparan sulphate levels did not differ in women with PE, NP and Non-P women.

In conclusion, we have confirmed that plasma levels of some TLR4 endogenous ligands, including fibrinogen, fibronectin and HMGB1 are raised in PE women. Whether any of them plays a role in the pathogenesis of PE, directly or indirectly activating TLR4 signalling is unknown and warrants further study. Our observations suggest a role for fibrinogen in the processes of inflammation, cell adhesion and angiogenesis in PE. Further studies should explore these observations, as well as determine whether endogenous ligands, not included in the current study, such as heat shock proteins, may play a role in the pathophysiology of pregnancy-induced hypertension.

Chapter 8: Summarising discussion and future directions

8.1 Summary of study findings

Four key findings emanate from this work. The first outcome (Chapter 3) is that PE (PE) monocytes have distinct phenotypes, with a predominance of the non-classical ($CD14^{low}CD16^{+}$) monocyte subpopulation, compared to normotensive pregnant (NP) and non-pregnant women. Also, PE patients displayed a reduced number of $HLA\text{-}DR^{+}$ (a MHC class II cell surface receptor) and $TIE2^{+}$ (a receptor of angiopoietin) monocytes, whereas $CCR5^{+}$ (a receptor of the macrophage inflammatory protein) and $TLR4^{+}$ monocytes were dramatically increased. Secondly, of the endogenous ligands (namely fibrinogen, fibronectin, heparan sulfate and hyaluronan) employed to stimulate primary monocyte cultures, fibrinogen induced inflammatory (IL-6, TNF-α) and anti-inflammatory cytokine (IL-10) release from PE monocytes to an extent comparable to bacterial ligands (Chapter 4). Another key finding (Chapter 7) is that fibrinogen, fibronectin and high mobility group box protein (HMGB1) were increased in the plasma of PE patients compared to controls, whilst, fibrinogen was reduced in the endothelial layer of the foetal blood vessels from PE compared to NP placentae. The fourth outcome, from this work, emphasises the interaction between PE monocytes and human umbilical vein endothelial cells (HUVECs) (Chapters 5 and 6). Firstly, as compared to NP monocytes-HUVECs co-cultures, inflammatory cytokine IL-6, chemokines IL-18 and MCP-1, and adhesion molecule VCAM-1 were amplified in PE monocytes-HUVECs co-cultures, whereas, the anti-inflammatory cytokine IL-10 was attenuated. Moreover, evidence of increased angiogenesis during pregnancy was provided by our observation that monocytes from both pregnant groups down-regulated sFlt-1 production from endothelial cells, and PE monocytes less so than NP monocytes. Secondly, TLR2 and TLR4 bacterial ligands and fibrinogen altered the expression of these inflammation, adhesion and angiogenic markers in PE monocyte-HUVECs co-cultures as compared to NP monocyte-HUVECs co-cultures. Fibrinogen was able to induce greater amounts of IL-1β and VCAM-1 from PE co-culture than from NP-co-culture, similar to the TLR4 bacterial ligand effect on PE co-culture. On the other hand, fibrinogen suppressed IL-6, IL-8, MCP-1 and sFlt-1 productions in the HUVEC-PE monocyte co-culture as compared to HUVEC-NP monocyte co-culture, which differs from the TLR4 bacterial ligand effect on PE co-culture.

The results of this study did not show any significant increase in the secretion of inflammatory cytokines from the PE monocytes cultures after the treatment with fibronectin, heparan sulfate and hyaluronan. Additionally, there was no significant difference in the plasma heparan sulphate levels between the three study groups. In the current study, there were no significant differences between fibrinogen expression levels in the villous stromal cells from the placental tissues of PE women when compared to, NP women. The sFlt-1 and VCAM-1 levels did not change significantly in the HMGB1-treated PE monocytes-HUVECs co-cultures when compared to non-stimulated controls. The study of Annexin V/PI apoptosis, and of BrdU labelling proliferation on endothelial cells (ECs) from monocytes-HUVECs co-cultures could not be successfully completed (appendix 4).

8.2 Implications and future research directions

8.2.1 Monocyte subpopulations and their TLR4 expression in PE

Prior studies have shown that PE is characterised by increased production of inflammatory cytokines and abnormal activation of inflammatory cells such as monocytes (Gervasi *et al.*, 2001, Redman *et al.*, 1999). Although, there is strong evidence that monocytes are activated in PE, this work is a first published study that has investigated human peripheral blood monocyte subpopulations from PE patients (Al-ofi *et al.*, 2012). Another, a more recent study has investigated the effects of pregnancy and PE on monocyte subsets in both humans and rats (Melgert *et al.*, 2012). In the current study, the proportion of non-classical $CD14^{low}CD16^{+}$ monocyte was significantly increased in PE as compared to NP ($P<0.001$). Melgert *et al*. demonstrated that the combined non-classical/intermediate monocyte percentage is high in normal human pregnancy and is even higher with PE, however the separate monocyte subsets do not differ significantly. This observation partially supports our observation of increased proportion of non-classical monocytes in PE, consistent with the exaggerated systemic inflammatory response observed in previous studies (Redman *et al.*, 1999). The current study and Melgert *et al*. are consistent in showing that the proportion of classical

$CD14^{high}CD16^{-}$ monocytes was significantly decreased in PE patients compared to NP women. But they differ to the extent that whilst we have reported that NP exhibited a greater proportion of classical $CD14^{high}CD16^{-}$ monocytes compared to Non-P women, Melgert *el al*. demonstrated a lower proportion of classical $CD14^{high}CD16^{-}$ monocytes in NP versus Non-P women. However, both are pilot studies with small subject groups.

To the best of our knowledge, present work is the only published study that investigates the expression of TLR -2 and -4 in human peripheral blood monocytes, and subpopulations from normal and PE-complicated pregnancies (Al-ofi *et al.*, 2012). The results of the current study show that TLR4 expression in peripheral blood monocytes was significantly increased in PE compared to NP. Also, our data show that TLR4 expression was increased in the $CD14^{low}CD16^{+}$ monocytes of PE women. Up-regulation of TLR4 expression in peripheral blood monocytes and its non-classical subset from PE women suggest a role for this pattern-recognition receptor, in the exaggerated systemic inflammatory response seen in this condition, but it is unclear whether this occurs as a cause or consequence of PE.

Altogether, this initial study has aimed to highlight observations that may be explored by definitive functional experiments. Larger sample sizes studied across various gestational durations (including first-trimester, mid-trimester, third-trimester and post-delivery subjects to determine any temporal effects) will be informative. It would also be interesting to determine whether monocytes subtypes differ between women with severe early onset PE and those with later onset disease, both of which are recognised to be aetiologically distinct subtypes of PE (Huppertz, 2008, von Dadelszen *et al.*, 2003). Longitudinal studies throughout pregnancy aimed to determine at what stage a switch occurs from a normotensive to a pre-eclamptic monocyte "phenotype" could potentially enable the prediction of the risk of development, and adverse outcomes in PE. Moreover, the severity of the disease could contribute to the excessive inflammatory response seen in pre-eclampsia (Redman *et al.*, 1999, Sibai, 2004). Thus, in the future it is recommended that longitudinal studies, including those investigating mild, moderate and severe PE women, be conducted.

8.2.2 Stimulation of PE Monocytes with TLR4 ligands

Studies reported in Chapter 4 show that stimulation of PE monocytes with peptidoglycan (TLR2 bacterial ligand), *E. coli* LPS (TLR4 bacterial ligand) and fibrinogen (TLR4 endogenous ligand) was associated with an exaggerated release of TNF-α, IL-6 and IL-10 cytokines compared to NP monocytes, consistent with an exaggerated systemic response of PE monocytes to factors that induce inflammation. Data from Chapter 3 showed that TLR4 is upregulated in non-classical $CD14^{low}CD16^{+}$ monocytes of PE women, and is very likely to be responsible for this hyper-response to TLR ligands. Furthermore, this may contribute to exacerbation of the disease. One reviewer of this paper, now published (Al-ofi *et al.*, 2012), suggested methodological studies to unravel our observations and resulting hypothesis: this included isolating three monocyte subsets from PE women by using available, well-advanced magnetic separation kits, sub-culturing these subpopulations and then treating them with TLR2 and 4 ligands. However, addressing all these methods will be expensive and was also not feasible within the time frame of this PhD project.

8.2.3 The interaction between PE monocytes and HUVECs

Human peripheral monocytes have been suggested as regulating endothelial cell proliferation and angiogenesis functions (Schubert *et al.*, 2008); and protecting ECs against apoptosis by enhancing their survival (Noble *et al.*, 1999, Schubert *et al.*, 2011). In chapters 5 and 6, it was postulated that activated "inflammatory" monocytes in PE may impair vascular endothelial functions seen in the condition evidenced as altered chemo-attractant, angiogenic, adhesiveness and exaggerated inflammatory responses. Currently, multiple methods are available to detect endothelial cell function, including endothelial cell proliferation (BrdU labelling), migration assay, tube formation assay and apoptosis assay. Apoptosis and proliferation studies with flow cytometer were carried out but unfortunately failed (see Appendix 4). Because of the time that is needed to optimise these studies and the cost of flow cytometry materials, it has been decided to postpone these experiments until a post-doctoral fellowship.

Further work will be required to explore the above hypothesis. Such lines of investigation may include the following work:

- Isolate total primary human peripheral blood monocyte and its three subpopulations from both NP and PE women by using magnetic separation kits.
- Fractionate the isolated monocyte cells on top of cultured primary human umbilical cord endothelial cells to see the effect of co-culturing total monocyte, non-classical $CD14^{low}CD16^{+}$ monocytes, intermediate $CD14^{high}CD16^{+}$ monocytes and classical $CD14^{high}CD16^{-}$ monocytes with HUVECs under different co-culture conditions, including media seeded with plasma from women with PE and NP women.
- Investigate the effects of total monocyte and its subsets on the endothelial functions by the tube-formation, migration, proliferation, and apoptosis assays.

8.2.4 Impact of TLR4 endogenous ligand(s) in PE

The main hypothesis of the current thesis is that the TLR4 activity may play a key role in pathogenesis of PE through one or more of its endogenous ligands (e.g. heparan sulphate, fibrinogen, fibronectin, HMGB1 and hyaluronan), produced by the ischaemic placenta. We found that of all the endogenous ligands studied, fibrinogen plays a critical role in the pathophysiology of PE (**Figure 8.1**) (promotes cell adhesion and angiogenesis and suppresses the expression of inflammatory markers in co-culture experiments) by ill-understood mechanisms that may or may not involve the TLR4 receptor. It has been demonstrated that fibrinogen is able to induce PE monocytes to release inflammatory cytokines, induce adhesion and inhibit dysangiogenesis between PE monocyte and endothelial cells. Also, results of the current study (see Chapter 7) demonstrated that fibrinogen is increased from plasma whilst it is reduced from placental tissues of PE women, consistent with other research findings (Karehed *et al.*, 2010). However, HMGB1 did not alter cell adhesion and angiogenesis processes from PE co-cultures. Despite our results demonstrating increase of HMGB1 from plasma of PE women as compared to normotensive pregnancy, Bo Wang did not show any differences between the sera of two study groups, however HMGB1 was increased from placental tissues of PE women (Wang *et al.*, 2011). Consequently, HMGB1 may not be involved in the vascular remodelling, which might be a result of TLR4-independent mechanisms, but could be overproduced from PE necrotic trophoblasts, that had not been cleared by phagocytic macrophage (Abrahams *et al.*, 2004b). Overall, this raises the possibility that fibrinogen interacts with other integrins expressed by monocytes and

ECs rather than TLR4 only. However, there are limits in this thesis as to how far the concept of TLR4 antagonist can be taken. Such study may uncover potential therapeutic role for drugs that may antagonise TLR4 activity, in this condition.

In conclusion, the elevation of some plasma levels of TLR4 endogenous ligands, including fibrinogen, fibronectin and HMGB1, in PE women (see Chapter 7) supports our hypothesis that one or possibly a combination of endogenous ligands activates TLR4 signalling, and may be implicated in the pathophysiology of PE. An objective of our study was to identify whether any endogenous ligands of TLR4, implicated in PE, may serve as a useful biomarker of disease production, or severity. Our findings do not suggest clinical utility for production of disease progression. However, the sample sizes in this study were small, and therefore, more definitive studies in larger sizes stratified for disease severity, may clarify this question.

8.2.5 Fibrinogen mechanisms that may be involved in the pathophysiology of PE

To understand how fibrinogen could be implicated in the pathophysiology of PE, we need to understand its functional roles in coagulation and haemostasis, inflammation and immune system and micro-vascular functions. Interaction of fibrinogen with integrins expressed by monocytes and endothelial cells may be linked with inflammation, coagulation and endothelial dysfunction in PE (Bach *et al.*, 1998, Davalos and Akassoglou, 2012, Fan and Edgington, 1993, Lishko *et al.*, 2004, Lominadze *et al.*, 2010, Mosesson, 2005) (Chapter 1 Section1.3.6.2.E). Usually, these three processes have been studied independently in women with PE. Fibrinogen is considered an essential constituent of the extracellular matrix (ECM) which interacts with several integrins of ECs (Davis and Senger, 2005), trophoblasts (Burrows *et al.*, 1996, Zygmunt *et al.*, 2003), and leukocytes (Ugarova and Yakubenko, 2001). These ECM-cellular interactions may allow the normal embryogenic/placental angiogenesis in early pregnancy. Acute atherosis lesions in the spiral arteries from PE women (Staff *et al.*, 2010), similar to atherosclerosis, may occur due to fibrinogen deposition in the vascular sub-endothelium. As a result it causes an increase in monocyte adhesion to endothelial cells of injured blood vessels, in turn leading to accumulation of sub-endothelial lipid-laden foam cells (Dejana *et al.*, 1987, Lominadze *et al.*, 2010). Figure

8.1 summarises a unifying hypothesis of the processes in which fibrinogen seems to play a role in pivotal-leukocyte-mediated inflammatory processes, coagulation and haemostasis, vascular endothelial function and abnormal placentogenesis. Altered metabolism of fibrinogen may be pivotal to the pathophysiology of PE (**Figure 8.1**).

Fibrinogen has binding sites for plasma fibronectin in its C-terminal region by which fibrin(ogen) may be incorporated into the ECM (Mosesson, 2005). The binding of fibronectin with fibrin is suggested to be important for cell adhesion and migration, and may be involved with macrophage clearance of fibrin clots from peripheral blood after tissue injury or inflammation (Pankov and Yamada, 2002). Moreover, the heparin binds fibrin β-15-42 sequence, facilitating fibrin assembly and contributing to the cellular-ECM interaction which induces platelet spreading, fibroblast proliferation, EC proliferation, spreading and angiogenesis (Mosesson, 2005). A programmed process of embryogenesis/placentogenesis requires collaboration from cellular (*i.e.,* trophoblasts, ECs and leukocytes) and ECM molecules that are regulated by several mediators including angiogenic factors, inflammatory cytokines, adhesion molecules and matrix metalloproteinases (MMPs) (Burrows *et al.*, 1996, Zygmunt *et al.*, 2003). Therefore, any disturbance in vascular remodelling, such as defects of MMPs (Lalu *et al.*, 2007, Palei *et al.*, 2013, Plaks *et al.*, 2013) or macrophage dysfunction during apoptotic cell clearance (Abrahams *et al.*, 2004b), may lead to overproduction of endogenous ligands such as fibrinogen, fibronectin and HMGB1 (fragments of ECM or cellular debris (Erridge, 2010)) in turn stimulating TLR4 in PE. These assumptions are supported by the most recent study which demonstrated that MMP9 deficiency in pregnant mice leads to pathophysiological and placental abnormalities similar to PE (Plaks *et al.*, 2013). Further studies should explore those endogenous ligands which are most likely to play a role in combination (such as fibrinogen and fibronectin fragments), and other molecules yet-to-be explored, such as heat-shock proteins. These studies should focus on the pathophysiology of pregnancy-induced hypertension.

Further work is needed to clarify the molecular mechanisms linking these processes and to target therapeutic intervention in PE. Additionally, future work should involve some inhibitory molecules that interact with binding sites of fibrinogen in either in-vitro models or in-vivo animal models of PE. In this respect the following represent some of

the experiments that may help to clarify further pathophysiology of PE, given our observations:

- Antagonising the inflammatory cytokines released from PE monocyte cultures in response to fibrinogen, by inhibiting specific molecular mechanisms such as TLR4 antagonists, NF-κB or antibodies against leukocyte receptors (CD11b) blockers.
- Antagonising the angiogenesis and adhesion functions of fibrinogen in monocyte-endothelial cell co-cultures, by using antibodies against endothelial cell receptors (VE cadherin), leukocyte receptors (CD11b) or ICAM-1 linking both cell types.

TLR4 antagonists

Now that the structure and function of TLR4 has been clarified, there has been an increase in research interest regarding TLR4 receptor antagonism. The rationale for these developments is the derivation of drugs, which would combat the multi-systemic consequences of Gram-negative sepsis and endotoxic shock. Several novel compounds have been synthesised that antagonise LPS – the product of Gram-negative bacteria, which plays a key role in the multi-organ manifestation of sepsis, via its receptor, TLR4. The aim of these drugs is to fight sepsis and other immune diseases caused by ligands of TLR4. These synthetic molecules include E5531, E5564, and CRX-526. These drugs are lipid A analogues as it is known that lipid A is the unique fatty-acylated diphosphorylated diglucosamine portion of LPS that is a common element of LPS from most pathogenic bacteria (Leon *et al.*, 2008).

E5564 (**Eritoran™**): [α-D-glucopyranose,3-O-decyl-2-deoxy-6-O-[2-deoxy-3-O-[(3R)-3 methoxydecyl]-6-O-methyl-2-[[(11Z)-1-oxo-11-octadecenyl]amino]-4-O-phosphono-ß-D-glucopyranosyl]-2-[(1,3-dioxotetradecyl)amino]-1-(dihydrogen phosphate), tetrasodium salt], is a synthetic molecule derived from the lipid A structure of the non-pathogenic *Rhodobacter sphaeroides,* that was synthesised by the Eisai Research Institute of Boston (Andover, MA, USA). In vitro, E5564 dose-dependently blocked lipopolysaccharide-mediated stimulation of human or animal whole blood, primary cultures of human myeloid cells, and mouse tissue culture macrophage cell lines as

measured by the release of inflammatory cytokines. In primed mice, in vivo studies have shown that Eritoran inhibited production of lipopolysaccharide-induced cytokines and lipopolysaccharide- or bacterial-induced lethality. Eritoran blocked LPS-mediated activation of NF-κB in TLR4/MD-2-transfected cells. E5564 seems to be a specific antagonist for the TLR4 receptor and is inactive against TLR2-directed agonists compared with E5531, another analogue of lipid A from *Rhodobacter capsulatus,* which demonstrates inhibitory activity against TLR2 as well. E5564 is synthetically and structurally less complex, yet seems to possess superior pharmacological properties than E5531 (Mullarkey *et al.*, 2003). Recently, the crystal structure of the TLR4/MD-2 complex with Eritoran has been described suggesting that the mechanism of action of E5564 is binding through a large internal pocket in MD-2, as shown in **Figure 8.2**. Due to this strong ability to antagonise TLR4 signalling, Eritoran is currently in phase III clinical trials for the treatment of severe sepsis (Kim *et al.*, 2007a).

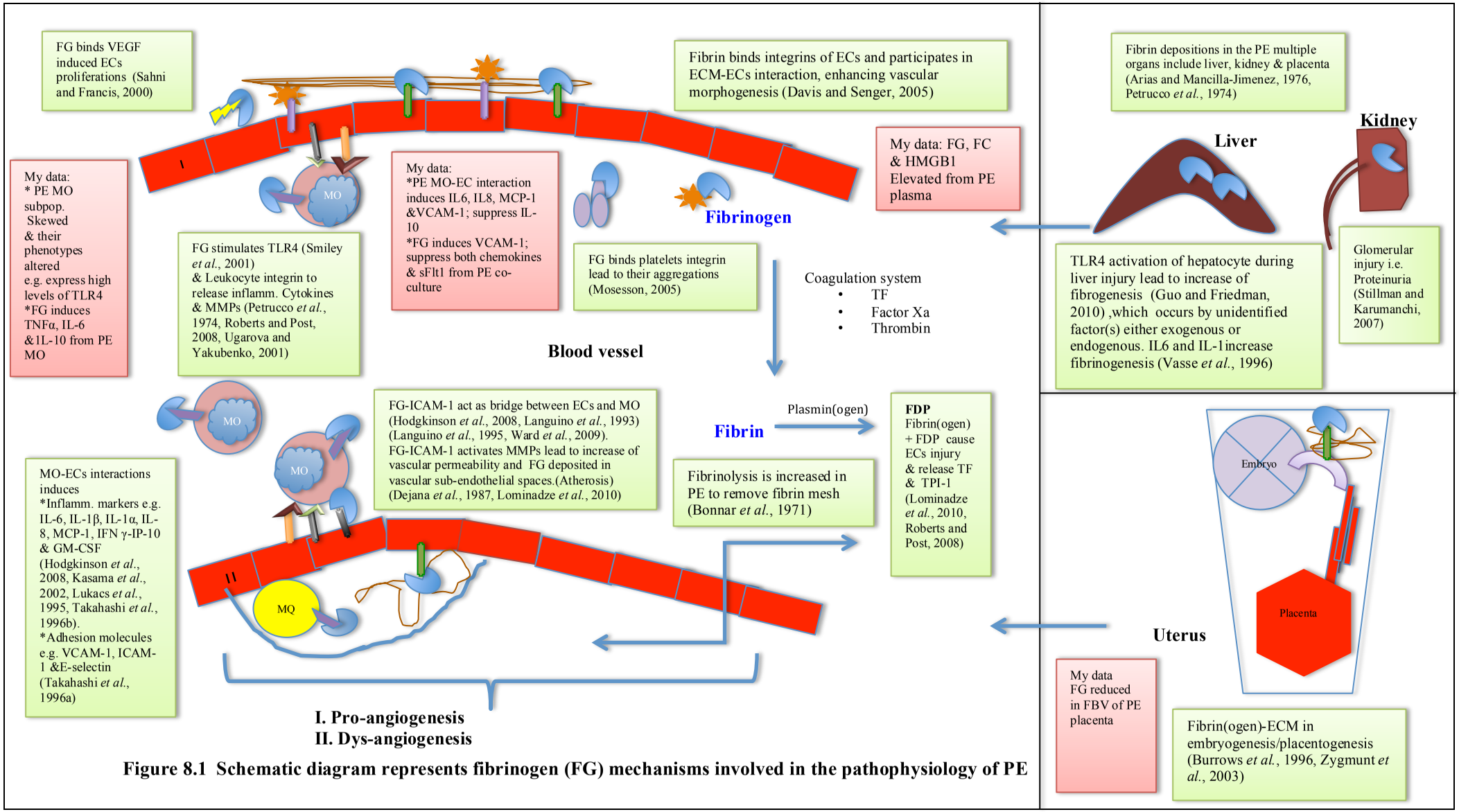

Figure 8.1 Schematic diagram represents fibrinogen (FG) mechanisms involved in the pathophysiology of PE

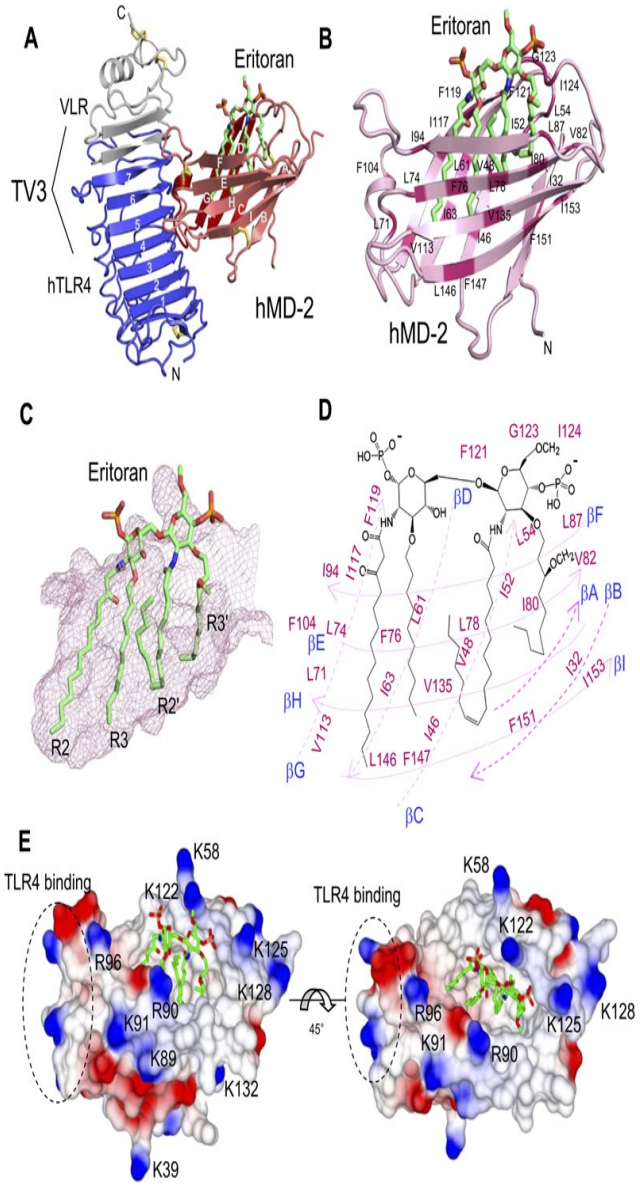

Figure 8.2 Structure of the TLR4-MD-2-Eritoran Complex

(A) Overall structure of the TV3-hMD-2-Eritoran complex. The TLR4 part of TV3 is coloured blue and the VLR part grey.

(B) Enhanced view of the human MD-2 and Eritoran complex. The bound TV3 hybrid is omitted for clarity. The carbon, oxygen, and phosphorous atoms of Eritoran are green, red, and orange, respectively. MD-2 residue interacting with the hydrophobic acyl chains of Eritoran are colored magenta and labelled.

(C) Structure of the Eritoran-binding pocket. The surface of MD-2 is drawn in purple mesh. The four acyl chains of Eritoran are labeled.

(D) Chemical structure of Eritoran. MD-2 residues interacting with Eritoran are labelled. The ß strands are shown schematically as broken arrows.

(E) Surface representation of MD-2. Positively and negatively charged surfaces are coloured blue and red, respectively. Adapted with permission from the publisher (Elsevier) (Kim *et al.*, 2007a)

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Appendices

Appendix 1 Consent and information sheets

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

CONSENT FORM HEALTHY NON-PREGNANT WOMEN

The role of the Toll 4 receptor in the vascular inflammation seen in pregnant women who develop pre-eclampsia.

Researchers:

Dr Dilly Anumba MD, MRCOG Obstetrician	Senior Clinical Lecturer/Consultant
Dr Ebtisam Alofi MBBS	PhD Student
Dr Neil Chapman PhD	Non-clinical Lecturer

Please initial box

1. I confirm that I have read and understand the information sheet dated 29/03/2009 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I agree to take part in the above study and would consent to a blood sample being taken from me. ☐
4. I wish/do not wish to be provided with a summary of the results of the study ☐
5. I understand that data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust where it is relevant to my taking in research. I give permission for these individuals to have access to my data.

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Patient Information Sheet – Non-pregnant Women

The role of the Toll 4 receptor in the vascular inflammation seen in pregnant women who develop pre-eclampsia.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You can seek further advice from the Patient Advice & Liaison Service (PALS), 722 Prince of Wales Road, Sheffield S9 4EU Tel: 0800 085 7539, Email: pals.manager@sheffieldpct.nhs.uk.

1. What is the purpose of the study?
Women who develop the condition called pre-eclampsia (toxaemia of pregnancy) are known to show inflammation of the small blood vessels in several organs and tissues of their body. The cause and mechanism of this inflammatory disorder is not well described or understood but there is some research evidence that this inflammation may be regulated by the function of a protein receptor found on the cells lining the blood vessel called Toll 4. The purpose of this study is to understand how this protein receptor works and how it may be deranged in pre-eclampsia. This study might also help us understand how this condition may be prevented or treated.

2. Why have I been chosen?
We are looking for normal, young, healthy women to take part in a research study. We would like to establish how Toll 4 is regulated outside of pregnancy, and to then compare our findings to those from a) normal pregnant women and b)those pregnant who develop pre-eclampsia.

3. Do I have to take part?
No. It is up to you to decide whether or not to take part. You are free to decline to take part, or to withdraw at any time after you have agreed to take part, without your decision affecting your care. You can simply tell us by telephone or in writing that you no longer wish to take part.

4. What will happen to me if I take part?
You will be given this information sheet to keep, and asked to sign a consent form. The study will involve taking a sample of blood from your vein.

5. What do I have to do?
If you decide to take part in the study you simply need to sign the consent form. We will obtain 12ml of blood on each of two occasions about 2 weeks apart, similar to the timing of blood collections from women who have developed pre-eclampsia.

6. What are the side effects of any treatment received when taking part?

You will receive no medication as part of the study. There are no side effects. The extra blood taken poses no risks to you. There is slight pain and inconvenience associated with any blood test.

7. What are the benefits of taking part?
You will not derive any benefits from taking part in the study. The study will not be able to prevent pre-eclampsia. However there is the possibility that the findings may enable future treatments and preventive measures to be identified for this condition.

8. What will happen when the research study stops?
We hope to determine whether any of our observations during this study may predict problems or assist carers look after women whose pregnancies may be at risk of, or complicated by, pre-eclampsia. This study will in part be presented as a thesis for an educational qualification. We will also publish our results in medical journals and present our findings at conferences. You will not be identified in any report/publication. We will not send you the results of the study unless you ask us to.

9. Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The data that we obtain from you in relation to this study is kept anonymised so that no one can trace the information to any individual study participant.

10. What will happen if I do not want to carry on with the study?
If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal. Any stored blood samples that can still be identified as yours will be destroyed if you wish. You will receive the same quality of clinical care even if you withdrew from the study.

11. What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will arrange for your care to continue. On receiving new information, your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is discontinued for any other reason, you will be told why and your continuing care will be arranged.

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12. Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by the South Yorkshire Research Ethics Committee.

13. What if I wish to complain about the way in which this study has been conducted?
If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.
If you have any complaints, queries or concerns please contact either the project co-ordinator: Mr Dilly Anumba Consultant Obstetrician and Gynaecologist on 0114 226 1075

OR

Otherwise you can use the normal hospital complaints procedure and contact: Dr Mike Richmond, Medical Director, STH NHS Trust on (0114) 2712786.

PLEASE FEEL FREE TO CONTACT Mr Dilly Anumba (0114 2268317) from the Academic Unit of Reproductive and Developmental Medicine if you have any questions.

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Study Number:
Patient Identification Number for this trial:

CONSENT FORM PREGNANT WOMEN

The role of the Toll 4 receptor in the vascular inflammation seen in pregnant women who develop pre-eclampsia.

Researchers:

Dr Dilly Anumba MD, MRCOG Obstetrician	Senior Clinical Lecturer/Consultant
Dr Ebtisam Alofi MBBS	PhD Student
Dr Neil Chapman PhD	Non-clinical Lecturer

Please initial box

6. I confirm that I have read and understand the information sheet dated 29/03/2009 for the above study and have had the opportunity to ask questions. ☐

7. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

8. I agree to take part in the above study and would consent to **(circle as appropriate)**:
   Two blood samples being taken from me during my pregnancy Yes / No
   A piece of the placenta and umbilical cord being taken after the baby is born and the placenta delivered. Yes / No

9. I wish/do not wish to be provided with a summary of the results of the study ☐

10. I understand that data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust where it is relevant to my taking in research. I give permission for these individuals to have access to my data.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Patient Information Sheet – Normal Pregnant Women

The role of the Toll 4 receptor in the vascular inflammation seen in pregnant women who develop pre-eclampsia.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You can seek further advice from the Patient Advice & Liaison Service (PALS), 722 Prince of Wales Road, Sheffield S9 4EU Tel: 0800 085 7539, Email: pals.manager@sheffieldpct.nhs.uk.

1. What is the purpose of the study?
Women who develop the condition called pre-eclampsia (toxaemia of pregnancy) are known to show inflammation of the small blood vessels in several organs and tissues of their body. The cause and mechanism of this inflammatory disorder is not well described or understood but there is some research evidence that this inflammation may be regulated by the function of a protein receptor found on the cells lining the blood vessel called Toll 4. The purpose of this study is to understand how this protein receptor works and how it may be deranged in pre-eclampsia. This study might also help us understand how this condition may be prevented or treated.

2. Why have I been chosen?
You have been chosen because you are pregnant but **do not have pre-eclampsia**. We would like to compare women without pre-eclampsia such as you to women who are pregnant and have developed pre-eclampsia.

3. Do I have to take part?
No. It is up to you to decide whether or not to take part. You are free to decline to take part, or to withdraw at any time after you have agreed to take part, without your decision affecting your care or that of your baby in any way. You can simply tell us by telephone or in writing that you no longer wish to take part.

4. What will happen to me if I take part?
You will be given this information sheet to keep, and asked to sign a consent form. The study will involve taking a sample of blood from your vein on two occasions during the later half of your pregnancy. We will also request your permission to allow us take a small piece of the placenta and the attached cord after your baby is born. These tissues would usually be discarded after you have given birth so there are no risks to you or your child. We will try to take this blood at the same time that blood is collected for your clinical investigation and therefore this should cause no additional discomfort.

5. What do I have to do?

If you decide to take part in the study you simply need to sign the consent form. We will obtain 12ml of blood on each of two occasions about 2 weeks apart, similar to the timing of blood collections from women who have developed pre-eclampsia.

6. What are the side effects of any treatment received when taking part?
You will receive no medication as part of the study. There are no side effects. The extra blood taken poses no risks to you. There is slight pain and inconvenience associated with any blood test.

7. What are the benefits of taking part?
You will not derive any benefits from taking part in the study. The study will not be able to prevent pre-eclampsia. However there is the possibility that the findings may enable future treatments and preventive measures to be identified for this condition.

8. What will happen when the research study stops?
We hope to determine whether any of our observations during this study may predict problems or assist carers look after women whose pregnancies may be at risk of, or complicated by, pre-eclampsia. This study will in part be presented as a thesis for an educational qualification. We will also publish our results in medical journals and present our findings at conferences. You will not be identified in any report/publication. We will not send you the results of the study unless you ask us to.

9. Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The data that we obtain from you in relation to this study is kept anonymised so that no one can trace the information to any individual study participant.

10. What will happen if I do not want to carry on with the study?
If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal. Any stored blood samples that can still be identified as yours will be destroyed if you wish. You will receive the same quality of clinical care even if you withdrew from the study.

11. What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will arrange for your care to continue. On receiving new information, your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is discontinued for any other reason, you will be told why and your continuing care will be arranged.

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12. Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by the South Yorkshire Research Ethics Committee.

13. What if I wish to complain about the way in which this study has been conducted?
If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

If you have any complaints, queries or concerns please contact either the project co-ordinator: Mr Dilly Anumba Consultant Obstetrician and Gynaecologist on 0114 226 1075

OR

Otherwise you can use the normal hospital complaints procedure and contact: Dr Mike Richmond, Medical Director, STH NHS Trust on (0114) 2712786.

PLEASE FEEL FREE TO CONTACT Mr Dilly Anumba (0114 2268317) from the Academic Unit of Reproductive and Developmental Medicine if you have any questions.

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Patient Information Sheet – Pregnant Women with Preeclampsia

The role of the Toll 4 receptor in the vascular inflammation seen in pregnant women who develop pre-eclampsia.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You can seek further advice from the Patient Advice & Liaison Service (PALS), 722 Prince of Wales Road, Sheffield S9 4EU Tel: 0800 085 7539, Email: pals.manager@sheffieldpct.nhs.uk.

1. What is the purpose of the study?
Women who develop the condition called pre-eclampsia (toxaemia of pregnancy) are known to show inflammation of the small blood vessels in several organs and tissues of their body. The cause and mechanism of this inflammatory disorder is not well described or understood but there is some research evidence that this inflammation may be regulated by the function of a protein receptor found on the cells lining the blood vessel called Toll 4. The purpose of this study is to understand how this protein receptor works and how it may be deranged in pre-eclampsia. This study might also help us understand how this condition may be prevented or treated.

2. Why have I been chosen?
You have been chosen because you are pregnant and have been found to have the condition called pre-eclampsia. We would like to compare women with pre-eclampsia such as yourself to women who are pregnant but do not develop pre-eclampsia.

3. Do I have to take part?
No. It is up to you to decide whether or not to take part. You are free to decline to take part, or to withdraw at any time after you have agreed to take part, without your decision affecting your care or that of your baby in any way. You can simply tell us by telephone or in writing that you no longer wish to take part.

4. What will happen to me if I take part?
You will be given this information sheet to keep, and asked to sign a consent form. The study will involve taking a sample of blood from your vein on two occasions during your pregnancy. We will also request your permission to allow us take a small piece of the placenta and the attached cord after your baby is born. These tissues would usually be discarded after you have given birth so there are no risks to you or your child.. The blood sample will be obtained at the same time that blood is collected for your clinical investigation and therefore this causes no additional discomfort.

5. What do I have to do?
If you decide to take part in the study you simply need to sign the consent form. We will then obtain an extra 12ml of blood at the time of your blood test to investigate

toxaemia. The priority regarding the blood taken will be for your clinical investigation: no blood will be used for research if the sample obtained suffices only for your clinical tests. If you require further blood tests in a further 2 weeks we will take a further blood sample at the time of your blood test to monitor your condition in order to determine any changes in Toll function as pregnancy advances further.

6. What are the side effects of any treatment received when taking part?
You will receive no medication as part of the study. There are no side effects. The extra blood taken when you are having your required blood test poses no risks to you. There is slight pain and inconvenience associated with any blood test.

7. What are the benefits of taking part?
You will not derive any benefits from taking part in the study. The study will not be able to prevent pre-eclampsia. However there is the possibility that the findings may enable future treatments and preventive measures to be identified.

8. What will happen when the research study stops?
We hope to determine whether any of our observations during this study may predict problems or assist carers look after women whose pregnancies may be at risk of, or complicated by, pre-eclampsia. This study will in part be presented as a thesis for an educational qualification. We will also publish our results in medical journals and present our findings at conferences. You will not be identified in any report/publication. We will not send you the results of the study unless you ask us to.

9. Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The data that we obtain from you in relation to this study is kept anonymised so that no one can trace the information to any individual study participant.

10. What will happen if I do not want to carry on with the study?
If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal. Any stored blood samples that can still be identified as yours will be destroyed if you wish. You will receive the same quality of clinical care even if you withdrew from the study.

11. What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will arrange for your care to continue. On receiving new information, your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is discontinued for any other reason, you will be told why and your continuing care will be arranged.

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12. Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by the South Yorkshire Research Ethics Committee.

13. What if I wish to complain about the way in which this study has been conducted?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.
If you have any complaints, queries or concerns please contact either the project co-ordinator: Mr Dilly Anumba Consultant Obstetrician and Gynaecologist on 0114 226 1075

OR

Otherwise you can use the normal hospital complaints procedure and contact: Professor Chris Welsh, Medical Director, STH NHS Trust on (0114) 2712786.

PLEASE FEEL FREE TO CONTACT Mr Dilly Anumba (0114 2268317) from the Academic Unit of Reproductive and Developmental Medicine if you have any questions.

Appendix 2 Ethical approval

South Yorkshire Research Ethics Committee
1st Floor Vickers Corridor
Northern General Hospital
Herries Road
Sheffield
S5 7AU

Telephone: 0114 226 9153
Facsimile: 0114 256 2469
Email: joan.brown@sth.nhs.uk

14 April 2009

Dr D O C Anumba
Senior Clinical Lecturer
The University of Sheffield
Jessop Wing
Sheffield
S10 2SF

Dear Dr Anumba

Full title of study: **Functional expression of endogenous ligands of TLR4 and associated inflammatory markers in normotensive pregnancy and pregnancy complicated by pre-eclampsia.**
REC reference number: **09/H1310/12**

Thank you for your letter of 29 March 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS sites only, management permission for research ("R&D approval") should be obtained

from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*	
Key Collaborator CV - Dr Ebtisam Aziz Al Ofi			
Covering Letter		21 January 2009	
Investigator CV			
Application		23 January 2009	
Advertisement	1	16 January 2009	
Peer Review			
Clarifications to queries in provisional opinion letter		29 March 2009	
Response to Request for Further Information		29 March 2009	
Participant Consent Form: Healthy Non-Pregnant Women	3	9 April 2009	
Participant Consent Form: Normal Pregnant Women	2	29 March 2009	
Participant Consent Form: Pregnant Women with Pre-eclampsia	2	29 March 2009	
Participant Information Sheet: Healthy Non Pregnant Women	3	9 April 2009	
Participant Information Sheet: Normal Pregnant Women	2	29 March 2009	
Participant Information Sheet: Pregnant Women with Pre-eclampsia	2	29 March 2009	
Covering Letter		29 March 2009	
Protocol	2	29 March 2009	

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review –guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1310/12	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Jo Abbott
Chair

Enclosures: "After ethical review – guidance for researchers" SL-AR2

Copy to: STH R & D Department

Appendix 3 ELISA protocols

Fibrinogen ELISA assay (Abbnova ELISA kit)

Assay principle

The Human Fibrinogen ELISA kit is designed for detection of human FBG in plasma. This assay employs a quantitative competitive enzyme immunoassay technique that measures FBG in less than 3 hours. A murine antibody specific for FBG has been pre-coated onto a 96-well microplate with removable strips. FBG in standards and samples is competed by a biotinylated FBG sandwiched by the an immobilized antibody and a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Assay protocol

1. Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30°C).
2. Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
3. Add 25 µl of standard or sample per well and immediately add 25 µl of biotinylated FBG to each well (on top of the standard or sample). Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
4. Wash five times with 200 µl of wash buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of wash buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
5. Add 50 µl of streptavidin-peroxidase conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
6. Wash a microplate as described above.
7. Add 50 µl of chromogen substrate per well and incubate for about 8 minutes or till

the optimal color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

8. Add 50 µl of stop solution to each well. The color will change from blue to yellow.

9. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Fibronectin ELISA assay (eBioscience ELISA kit)

Assay principle

An anti-human fibronectin coating antibody is adsorbed onto microwells. Human fibronectin present in the sample or the standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human fibronectin antibody is added and binds to human fibronectin captured by the first antibody. Following incubation unbound biotin-conjugated anti-human fibronectin antibody is removed during the wash step. Streptavidin- HRP is added and binds to the biotin- conjugated anti-human fibronectin antibody. Following incubation unbound Streptavidin- HRP is removed during the wash step, and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of human fibronectin present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human fibronectin standard dilutions and human fibronectin sample concentration determined.

Assay protocol

1. Predilute your samples before starting with the test procedure. Dilute serum and plasma samples 1:10.000 with assay buffer (1x).

2. Wash the microwell strips twice with approximately 400µl wash buffer per well with thorough aspiration of microwell contents between washes. Allow the wash buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess wash buffer. Use the microwell strips immediately after washing. Alternatively, microwell strips can be placed upside down

on a wet absorbent paper for no longer than 15 minutes. Do not allow wells to dry.

3. Standard dilution on the microwell plate**:** Add 100 µl of assay buffer (1x) in duplicate to all standard wells. Pipette 100 µl of prepared standard in duplicate into well A1 and A2. Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 20.00 ng/ml), and transfer 100 µl to wells B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human fibronectin standard dilutions ranging from 20.00 to 0.31 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

4. Add 100 µl of assay buffer (1x) in duplicate to the blank wells.

5. Add 50 µl of assay buffer (1x) to the sample wells.

6. Add 50 µl of each sample.

7. Add 50 µl of biotin-conjugate to all wells.

8. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 100 rpm.

9. Prepare streptavidin-HRP

10. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point c. of the test protocol. Proceed immediately to the next step.

11. Add 100 µl of diluted streptavidin-HRP to all wells, including the blank wells.

12. Cover with an adhesive film and incubate at room temperature (18°to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.

13. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 2 of the test protocol. Proceed immediately to the next step.

14. Pipette 100 µl of TMB substrate solution to all wells.

15. Incubate the microwell strips at room temperature (18°to 25°C) for bout 10 min. Avoid direct exposure to intense light.

16. Stop the enzyme reaction by quickly pipetting 100 µl of stop solution into each well. It is important that the stop solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the stop solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

17. Read absorbance of each microwell on aspectro-photometerusing 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by

using the blank wells. Determine the absorbance of both the samples and the standards.

HMGB1 ELISA assay (IBL International HMGB1 ELISA kit II)

Assay principle

HMGB1 ELISA is a sandwich-enzyme immunoassay for the quantitative determination of HMGB1 in serum and plasma. The wells of the microtiter strips are coated with purified anti-HMGB1 antibody. HMGB1 in the sample binds specifically to the immobilized antibody and is recognized by a second enzyme marked antibody. After substrate reaction the HMGB1 concentration is determined by the colour intensity.

Assay protocol

1. Add 100 µL of *sample siluent* to each well.
2. Add 10 µL of *sample siluent* to zero well.
3. Add 10 µL of standards and samples to the wells.
4. After shaking the plate with a plate mixer, cover all wells tightly using *plate seal* and incubate for 20 - 24 hours at 37^{o}C.
5. Wash the wells 5 times with diluted wash buffer (400 µL / well). After the final wash, turn over the plate and gently tap 4 or 5 times on a lint-free paper towel to remove any remaining wash buffer.
6. Add 100 µL of enzyme conjugate solution to each well.
7. Cover all wells tightly with plate seal and incubate for 2 hours at 25^{o}C.
8. Wash the wells 5 times with wash solution (400 µL / well). After the final wash, turn over the plate and gently tap 4 or 5 times on a lint-free paper towel to remove any remaining wash buffer.
9. Add 100 µL of colour solution to each well at a regular time interval. Incubate for 30 minutes at room temperature. During the reaction, cover the plate with a plate cover or plastic wrap to avoid contamination during the reaction.
10. Add 100 µL of stop solution to each well in the same sequence and at the same time intervals to the additions of substrate solution.
11. Clean the back of the wells. Be careful not to scratch the wells as this may interfere with measurements.
12. Read the absorbance of each well at 450 nm using a microplate reader within 60 minutes after adding stop solution.

Heparan Sulfate (HS) ELISA assay (Uscn Life Science ELISA kit)

Assay principle

The microtiter plate provided in this kit has been pre-coated with an antibody specific to HS. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for HS. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain HS, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm 2 nm. The concentration of HS in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Assay protocol

1. Add 100 each of dilutions of standard (see reagent preparation), blank and samples into the appropriate wells. Cover with the plate sealer. Incubate for 2 hours at 37 ^{o}C.
2. Remove the liquid of each well, don't wash.
3. Add 100 of detection reagent A working solution to each well. Incubate for 1 hour at 37 ^{o}C after covering it with the plate sealer.
4. Aspirate the solution and wash with 400 of 1X wash solution to each well using a multi-channel pipette. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100 of detection reagent B working solution to each well. Incubate for 30 minutes at 37 ^{o}C after covering it with the plate sealer.
6. Repeat the aspiration/wash process for five times as conducted in step 4.
7. Add 90 of substrate solution to each well. Cover with a new plate sealer. Incubate for 15 - 25 minutes at 37 ^{o}C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of substrate solution.
8. Add 50 of stop solution to each well. The liquid will turn yellow by the addition of stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Hyaluronan (HA) ELISA assay (echelon ELISA kit)

Assay principle

The HA-ELISA is a quantitative enzyme-linked immunoassay designed for the in vitro measurement of HA levels in human or animal biological fluids (blood, serum, urine, diffusate, synovial fluid). This simple protocol is a standard competitive ELISA format and requires 3 hours. The HA-ELISA is a competitive ELISA assay in which the colorimetric signal is inversely proportional to the amount of HA present in the sample. Samples to be assayed are first mixed with the HA Detector, and then added to the HA ELISA plate for competitive binding. An enzyme-linked antibody and colorimetric detection is used to detect the HA detector bound to the plate. The concentration of HA in the sample is determined using a standard curve of known amounts of HA. The enzyme / substrate system is a colorometric assay comprised of alkaline phosphatase / pNPP phosphatase substrate. It should be read at 405 nm.

Assay protocol

1. Set up the incubation plate (yellow U-bottom plate) as illustrated. (Each well should contain 150 μL)
    - Add 100 μL of standards and samples into corresponding wells.
    - Add 150 μL of diluent to the blank control wells and 100 μL of diluent to the Zero HA control wells.
    - Add 50 μL of working detector to all wells except the blank wells.
2. Mix the plate gently, cover with plate seal and incubate for one hour at 37 ^{o}C.
3. Following the incubation step, transfer 100 μL of controls and samples from the incubation plate to the corresponding wells of the detection plate.
4. Once the transfer is complete, mix the detection plate by gently tapping. Cover with a plate seal and incubate at 4 ^{o} C for 30 minutes.
5. Shake out the solution from the detection plate. Wash the plate four times with 300 μL of 1X wash concentrate. Ensure all wash buffer is removed from the plate by inverting the plate and blotting it out on absorbent paper.

6. Add 100 µL of working enzyme to each well of the detection plate.

7. Mix the detection plate gently, cover with plate seal and incubate at 37 °C for 30 minutes.

8. Repeat wash step 5.

9. Add 100 µL working substrate solution to each well of the detection plate.

10. Incubate the detection plate in the dark at room temperature.

11. Measure the absorbance of each well at 405 nm beginning at T = 15 min.

- The appropriate incubation time should be determined based on the ratio of the Zero HA standard control to the 1,600 ng/ml HA standard control. When the OD0 / OD1600 ratio is > 3.0 the incubation is complete and can be stopped with the Stop Solution (K-1209). This is achieved by reading at 15 min., 30 min. or 45 min. Generally the best results are obtained after 30 minutes of development.
- The Blank should have an absorbance of < 0.20 and the ratio of the Zero HA Control to the 1,600 ng/mL HA Standard should be > 3.0.

12. Stop the reaction by adding 50 µL stop solution to each well.

13. Generate a best fit curve for the standards in order to extrapolate relative sample values.

Human soluble VEGFR1/Flt-1 ELISA assay (R and D Systems ELISA kit)

Assay principle

The Quantikine Human VEGFR1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human soluble VEGFR1 in cell culture supernates, serum, plasma, saliva, and follicular fluid. It contains Sf 21-expressed recombinant human VEGFR1/Fc chimera and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human VEGFR1 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring soluble VEGFR1.

Assay protocol

1. Prepare all reagents, standard dilutions, and samples as directed in the product insert.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of assay diluent to each well.
4. Add 100 µL of standard, control, or sample to each well. Cover with a plate sealer, and incubate at room temperature for 2 hours on a horizontal orbital microplate shaker.
5. Aspirate each well and wash, repeating the process 3 times for a total of 4 washes.
6. Add 200 µL of conjugate to each well. Cover with a new plate sealer, and incubate at room temperature for 2 hours on the shaker.
7. Aspirate and wash 4 times.
8. Add 200 µL substrate solution to each well. Incubate at room temperature for 30 minutes on the benchtop. Protect from light.
9. Add 100 µL of Stop Solution to each well. Read at 450 nm within 30 minutes. Set wavelength correction to 540 nm or 570 nm.

Appendix 4 Endothelial cell apoptosis and proliferation studies (preliminary work)

Introduction

PE (PE) is associated with placental oxidative stress and elevated lipid peroxidation markers such as 4-hydroxynonenal and nitrotyrosine staining, oxidative protein damage and oxidizing potential (Raijmakers *et al.*, 2004). An amplified systemic inflammatory response with increased circulatory inflammatory cytokines and leukocyte activation is an essential feature of PE, as shown in Chapters 3 and 4, and by other research. For example, neutrophils from women with PE produced more superoxide and reactive oxygen species (ROS) than from normal pregnant (NP) women (Lee *et al.*, 2003, Tsukimori *et al.*, 1993), and PE monocytes demonstrate up-regulated CD11b and ROS (Gervasi *et al.*, 2001, Holthe *et al.*, 2004, Luppi and Deloia, 2006, Sacks *et al.*, 1998).

Circulating inflammatory cytokines and oxygen free radicals are thought to be responsible for vascular endothelial dysfunction in PE women. Molecules and conditions that are known to induce vascular endothelial cell apoptosis under physiological or pathological conditions, include ROS, NO in high concentration, mechanical forces or inflammatory cytokines TNF α, IL-I and IFN γ (Geng, 2001).

Therefore, we hypothesised that inflammatory PE monocytes may induce the apoptosis of endothelial cells (ECs) in our monocyte-HUVEC co-culture model by elaborating some of these inflammatory factors. It has been evidenced that human primary monocytes promote ECs survival and protect them against apoptosis even under TNFα cytokine stress and low nutrients environments, in direct cellular contact-dependent mechanism (Noble *et al.*, 1999, Schubert *et al.*, 2011). In this work, we report preliminary work investigating the effect of NP monocytes on endothelial cell viability, and particularly, whether NP monocytes prolong ECs viability and reduce apoptosis, and whether PE monocytes reduce ECs survival and induce apoptosis.

It has been demonstrated that peripheral blood monocytes can regulate endothelial cell proliferation and promote normal angiogenesis (Schubert *et al.*, 2008). Therefore, we

sought to investigate how PE and NP monocytes affect endothelial cell proliferation and apoptosis.

Materials and methods

Subjects

For experimental optimisation, monocytes were isolated from peripheral blood of eight consenting NP women who were recruited from the routine antenatal clinics at Jessop Wing Hospital. No study participants had any systemic infection or urinary tract infection. Following consent, during scheduled blood collections for clinical indications, an additional 10 ml of fresh blood was obtained from each subject into a tube containing EDTA to prevent clotting. The blood specimen was then immediately taken to research laboratories at the Academic Unit of Obstetrics and Gynaecology at Jessop Wing for the isolation of monocytes for the co-culture experiment. Consent was obtained from NP women in the first stage of labour in the delivery ward at Jessop Wing, and umbilical cords were collected for isolation of HUVECs.

Monoclonal antibodies used for flow cytometry

Anti-human CD14 conjugated with Alexa Flour 700

Supplier: BioLegend, UK

Catalogue number: 325614

Clone number: HCD14

Format: Alexa Flour 700

Size: 100μg

Isotype: Mouse IgG1, κ

Storage: 2–8 °C in the dark

Anti-human CD146 conjugated with Phycoerythrin/Cy7

Supplier: BioLegend, UK

Catalogue number: 342010

Clone number: SHM-57

Format: Phycoerythrin/Cy7

Size: 100 tests

Isotype: Mouse IgG2a, κ

Storage: 2–8 °C in the dark

Anti-human TIE2 conjugated with Allophycocyanin (APC)

Supplier: R & D Systems, UK

Catalogue number: FAB3131A

Clone number: 83715

Format: APC

Size: 100 tests

Isotype: Mouse IgG1

Storage: 2–8 °C in the dark

Anti-human CD106 (VCAM-1) Phycoerythrin

Supplier: eBioscience, UK

Catalogue number: 12-1069

Clone number: STA

Format: Phycoerythrin

Size: 20 µl/test (0.125 µg/test)

Isotype: Mouse IgG1, κ

Storage: 2–8 °C in the dark

Anti-human CD54 (ICAM-1) Pacific Blue

Supplier: BioLegend, UK

Catalogue number: 322715

Clone number: HCD54

Format: Pacific Blue

Size: 0.5 mg/ml

Isotype: Mouse IgG1, κ

Storage: 2–8 °C in the dark

Anti-human BrdU Biotin

Supplier: BioLegend, UK

Catalogue number: 317904

Clone number: MoBU-1

Format: Biotin

Size: 100 μg

Isotype: Mouse IgG1, κ

Storage: 2–8 °C in the dark

Anti-human streptavidin, Alexa Fluor 750

Supplier: Invitrogen, UK

Catalogue number: S-21384

Format: Alexa Fluor 750

Size: 1 mg

Storage: ≤ -20 ^{o}C in the dark

Mouse IgG1, κ isotype control conjugated with Allophycocyanin (APC)

Supplier: BD Bioscience, UK

Catalogue number: 555751

Clone number: MOPC-21

Format: APC

Size: 100 tests

Isotype: Mouse IgG1, κ

Storage: 2–8 °C in the dark

Mouse IgG1, κ isotype control conjugated with Phycoerythrin

Supplier: e Bioscience, UK

Catalogue number: 12-4714

Clone number: P3.6.2.8.1

Format: Phycoerythrin

Size: 0.2 mg/ml

Isotype: Mouse IgG1, κ

Storage: 2–8 °C in the dark

Annexin V-FITC apoptosis detection kit

Supplier: e Bioscience,UK

Catalogue number: BMS500FI/100

Principle of the test: Annexin V exhibits anti-phospholipase activity and binds to phosphatidylserine. FITC labelling allows simple direct detection by FACS analysis. Counterstaining by propidium iodide allows the discrimination of apoptotic cells.
Components:

- Annexin V FITC
- Binding buffer
- Propidium iodide (20 μg/ml)

Size: 100 tests
Storage: 2–8 °C in the dark

Monocytes and HUVEC co-cultures

(For more details see Chapter 2 Section 2.2.2.2)

Human vascular endothelial cells were isolated from umbilical cords, cultured and passaged, and used on the second through fifth passage. HUVECs were seeded onto gelatin-coated, 12-well tissue-culture plates at a density of 10^5 cells per well and incubated with 1 ml of medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-Glutamine and penicillin-streptomycin) at 37^{o}C with 5% CO_2 until they reached 70–90% of confluence, usually within 2 to 3 days. The day before the experiment, the cells were washed with 1ml of pre-warmed PBS and cultured with low serum media (DMEM supplemented with 5% heat inactivated FBS without antibiotics) for serum starvation.

Then monocytes, freshly isolated from peripheral blood of NP women by using the negative magnetic selection method (Pan monocyte isolation kit, Miltenyi Biotic, Germany, catalogue number: 130-096-537), were plated on top of the HUVEC monolayer at the fractional rate of 1 monocyte to 5 HUVECs. Co-culture assays were performed in DMEM with 5% heat-inactivated FBS without antibiotic media.

Annexin V detection assay

Apoptosis is cell death, programed to occur, to remove damaged cells by using phagocytic leukocytes such as macrophages. These phagocytic cells can recognize apoptotic cells, which express phosphatidylserine (PS) on the outer cell surface membrane, but cannot recognize the PS usually present in the inner surface membrane of live, normal cells (Li *et al.*, 2003). Annexin V is used as a probe to identify cells with exposed PS on their surfaces during apoptosis (Vermes *et al.*, 1995). Annexin V detection assay uses a fluorescent together with Annexin V, which binds to the PS of apoptotic cells in a suitable binding buffer (Ca^{2+} dependent). Assay is accompanied by nuclei staining with propidium iodide (PI), or live/dead staining to detect dead cells, in either of which the membrane becomes more permeable. Analysis is then performed by flow cytometry and FACS (Vermes *et al.*, 1995). The section below describes methods used to perform Annexin V detection assay on endothelial cells.

In the first experiment:

- The cells from endothelial monocultures and monocyte-HUVECs co-cultures were harvested after 24 hours of incubation at 37^{O}C with 5% CO_2 with cell dissociation solution, as described previously in the methodology chapter 2.
- Complete growth media was added to cell pellets and pipetted repeatedly to dissociate clumps. The cell suspension was washed 3 times by centrifugation at 5000 RPM for 5 minutes at 4^{O}C, and the supernatants were discarded. Cell counting was carried out by the haemocytometer method.
- Cells were distributed in 11 micro-centrifuge eppendorf tubes for monoclonal antibody staining. Initially, cells were blocked with 5µl of anti-human Fc receptor blocking reagent (MACS-Miltenyi Biotic/catalogue number: 1 30-059-901) and refrigerated for 10 minutes. Then, cells were mixed with monoclonal antibodies as follows. The first eppendorf tube had unstained cells only, the second had cells stained with 5 µl Alexa Fluor 700 conjugated antibody against CD14, the third had cells stained with 20 µl PE-cy7 conjugated antibody against CD146. In the fourth eppendorf tube, cells were stained with 5 µl FITC conjugated antibody against Annexin V; the fifth tube had cells stained with 5 µl PE conjugated antibody against VCAM-1. The sixth tube had cells stained with 5 µl pacific blue conjugated antibody against ICAM-1; the seventh tube had

cells stained with 20 µl APC conjugated antibody against TIE2. The eighth tube had cells stained with 1 µl of live/dead blue fixable cell stain kits (UV) 450 (Invitrogen catalogue number: L23105). The ninth tube had cells stained with isotype controls for ICAM, VCAM and TIE2. The tenth tube had cells stained with monoclonal antibodies and their isotypes (minus-one cells). The last tube had cells stained with all the above monoclonal antibodies. All eppendorf tubes were incubated in a refrigerator for 30 minutes. Cells were then washed twice with 1ml of cold BD flow cytometry stain buffer (FSB) and centrifuged at 5000 RPM for 2 minutes each time. Cells were aliquot in 500 µl of FBS and proceeded to flow cytometry. Analysis was done using a multi-laser and detector analyser (LSR II Flow Cytometer from BD Bioscience) at the Flow Cytometry Department in the core facility of the University of Sheffield Medical School.

The next experimental step focused on apoptosis studies with the Annexin V-FITC apoptosis detection kit only, as follows:

- HUVECs with or without monocyte cultures were stimulated with 100ng/ml Lipopolysaccharides from *Escherichia coli* (rough strains) for 24 hours at 37^{O}C with 5% CO_2 (to induce apoptosis).
- Un-stimulated and LPS-stimulated cells were harvested with cell dissociation solution, as explained above.
- Complete growth media was added to cell pellets and pipetted repeatedly to dissociate clumps. The cell suspension was washed 3 times by centrifugation at 5000 RPM for 5 minutes at 4 ^{O}C, and the supernatants were discarded. Cell counting was carried out by the haemocytometer method.
- Cells were equally disbursed to five micro-centrifuge eppendorf tubes for monoclonal antibody staining (**Figure A4.1**). The first tube had unstained cells. In the second tube, cells were stained with 5 µl Alexa Fluor 700 conjugated antibody against CD14. The third tube had cells stained with 20 µl PE-cy7 conjugated antibody against CD146. The fourth tube had cells stained with both monoclonal antibodies. And the last tube had cells for Annexin V-FITC staining.

- Cells were incubated in a refrigerator for 30 minutes. They were then washed twice with 1ml of cold BD flow cytometry stain buffer and centrifuged at 5000 RPM for 2 minutes each time.
- Cells stained with Alexa Fluor 700-CD14 and PE-Cy7-CD146 monoclonal antibodies (fourth tube) were used for detection of apoptosis. The fifth tube had cells stained only with the Annexin V-FITC apoptosis detection kit, as described in the manual sheet. In summary, cells in the fourth and fifth tubes were resuspended in 195 µl of binding buffer supplied with the kit, and then 5 µl FITC conjugated antibody against Annexin V was added, mixed and incubated for 10 minutes at room temperature.
- Cells were washed and resuspended in 195 µl of binding buffer, and 10 µl of propidium iodide, obtainable with the kit, was added. FACS analysis was done immediately by LSR II machine.

For the final experiment, we decided to use trypsin-EDTA for faster harvesting of cells and to reduce frequency of washing to once and increase centrifuging velocity and timing for depositing cells, as described below.

- The LPS-stimulated and un-stimulated monocyte-HUVECs co-cultures (incubated at 37°C with 5% CO_2 for 18 hours) were washed twice with warm PBS and 750 µl/well of trypsin-EDTA (Sigma catalogue number: T4299) was added. Cells were incubated for 5 minutes at 37 °C with 5% CO_2.
- Complete growth media was added to cell pellets and pipetted repeatedly to dissociate clumps. The cell suspension was washed once by centrifugation at 1000 RPM for 10 minutes at 4 ^{o}C, and the supernatants were discarded.
- Cells were stained for FACS analysis as described above for a previous experiment (page number 249) (**Figure A4.1**).

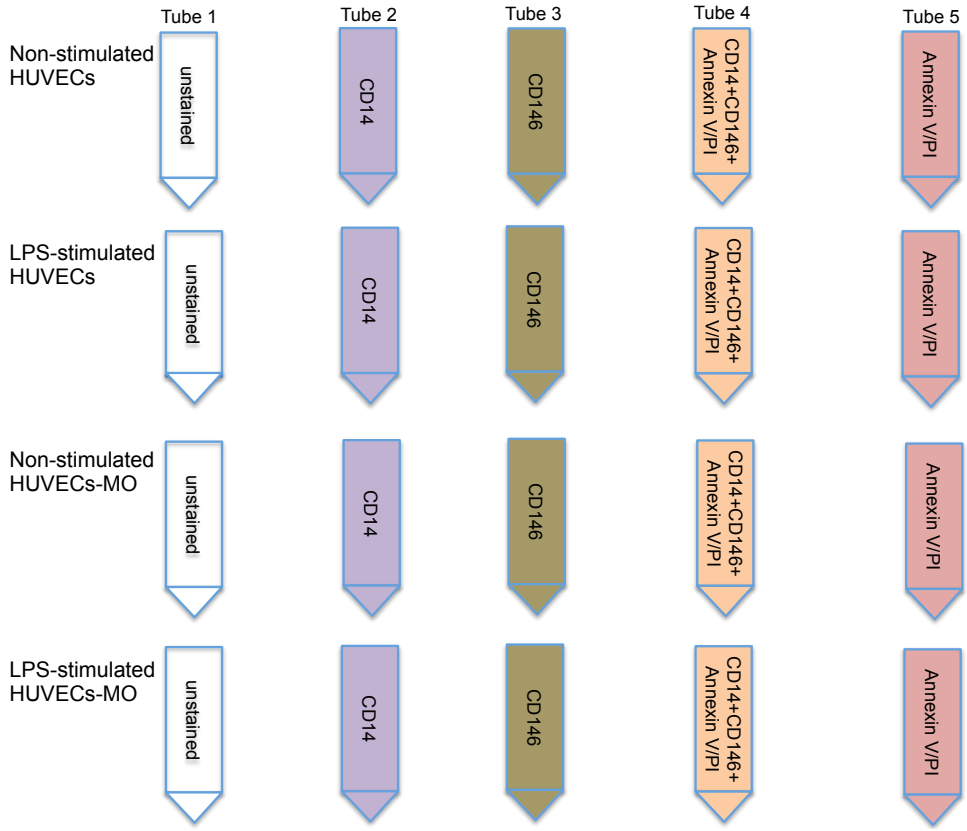


Figure A4.1 Diagram illustrate Annexin V-FITC/PI and monoclonal antibody staining of monocyte (Mo) and HUVECs mono- or co-cultures

The mono- and co-cultures were harvested and stained as follow the first tube had unstained cells; the second tube, cells were stained with anti-CD14-Alexa Fluor 700; the third tube had cells stained with anti-CD146-PE Cye7; the fourth tube had cells stained with both monoclonal antibodies plus Annexin V-FITC /PI; and the last tube had cells for only Annexin V-FITC/PI staining.

BrdU labelling

Bromodeoxyuridine (BrdU) is a uridine derivative and thymidine structural analogue that can be incorporated into DNA as a substitute to thymidine during the S-phase of the cell cycle. It is commonly used as an indicator for cell proliferation (Dolbeare *et al.*, 1983, Lehner *et al.*, 2011). The proportion of cells in the S-phase of the cell cycle can be determined by pulse-labelling with BrdU, staining with fluorescent antibodies against BrdU and analysing by flow cytometry. This section describes the steps of BrdU labelling, as performed in previous work (Schubert *et al.*, 2008).

- Endothelial monocultures and monocyte-HUVECs co-cultures were incubated for 20 hours at 37 ^{o}C with 5% CO_2.
- Culture media were removed and replaced with BrdU labelling solution (Invitrogen catalogue number: 00-0103), and tissue culture plats were incubated overnight at 37 ^{o}C with 5% CO_2.
- BrdU solution was removed and cells were washed twice with pre-warmed PBS. Cells were harvested with 5 mmol/L EDTA/PBS for 15 minutes and centrifuged at 5000 RPM for 2 minutes. Supernatants were discarded, and cell counting was carried out by the haemocytometer method.
- Cells of mono- or co- cultures were distributed equally in five micro-centrifuge eppendorf tubes for flow cytometry staining. The first tube had unstained cells only; the second tube had cells stained with 5 µl Alexa Fluor 700 conjugated antibody against CD14, and the third tube had cells stained with 20 µl PE-cy7 conjugated antibody against CD146. The fourth tube had cells stained with both monoclonal antibodies. And the last tube had cells for fixation/permeabilization and BrdU staining only.
- Cells were incubated in a refrigerator for 30 minutes. They were then washed twice with 1ml of cold BD flow cytometry stain buffer and centrifuged at 5000 RPM for 2 minutes each time.
- Cells from the fourth and fifth tubes were fixed with pre-warmed BD Bioscience fixation buffer (catalogue number: 554655) for 10 minutes. The fixation buffer was removed and cells washed with PBS and centrifuged at 5000 RPM for 2 minutes. Supernatants were discarded.
- Cells in the fourth and fifth tubes were then permeabilized with ice-cold BD Bioscience perm buffer III (catalogue number: 558052) for 10 minutes. The

perm buffer was removed and cells washed with PBS and centrifuged at 5000 RPM for 2 minutes. Supernatants were discarded.

- The cells' DNA was denatured with DNase I solution (Invitrogen catalogue number: 18047-019) for 1 hour at 37^{O}C. The DNase I solution was removed and cells washed with PBS and centrifuged at 5000 RPM for 2 minutes. Supernatants were discarded.
- Cells in the fourth and fifth tubes were stained with 0.5µg/100µl anti-BrdU Biotin antibody and incubated in a refrigerator for 30 minutes.
- The anti-BrdU Biotin labelled cells were stained with 5µl streptavidin Alexa Fluor 750 and incubated in a refrigerator for 30 minutes.
- Cells were then washed twice in 1ml of cold BD flow cytometry stain buffer and centrifuged at 5000 RPM for 2 minutes each time. Cells were aliquot in 500 µl of FBS and proceeded to flow cytometry.
- Analysis was done using a multi-laser and detector analyser (LSR II Flow Cytometer from BD Bioscience) at the Flow Cytometry Department in the core facility of University of Sheffield Medical School.

Results

Annexin V

In the first experiment, there was such a huge spectral overlap between multi-fluorescence colours that it was difficult to compensate for flow cytometry. Therefore, we decided to study apoptosis and adhesion separately with angiogenesis.

The second experiment focused on Annexin V staining only. Viable endothelial cells and monocytes were identified on SSC and FSC, but cells did not appear to take the Annexin V stain (**Figure A4.2**). Possible explanations are: all cells were viable but excessive washing was done after isolation of cells and apoptotic cells were lost with discarded supernatants; or damage of Annexin V molecule on cell membranes occurred due to longer incubation with the cell dissociation solution (≥ 15 minutes) and

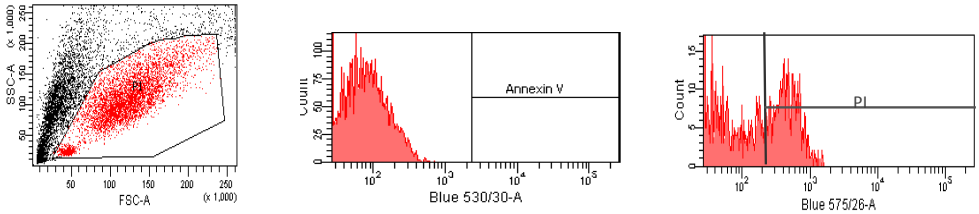
harvesting of adhesive endothelial cells that grew in gelatine-coated plats more than 48 hours (Mukhopadhyay *et al.*, 2007).

Therefore, in the last attempt, we allowed endothelial cells to grew in gelatin-coated plates for less than 48 hours (Mukhopadhyay *et al.*, 2007), harvested cells more quickly (≤ 5 minutes), reduced the frequency of washing and increased centrifuging velocity and timing before staining with Annexin V. We confirmed the viability of endothelial cells and monocytes but, unfortunately, still could not demonstrate any staining for Annexin V.

BrdU Labelling

Unfortunately, the results of the BrdU labelling experiment were negative, as seen in Figure A4.3. This result was due to the low cell count used in this experiment and to an excess of debris, mainly cells lost during washing.

A. Un-stimulated MO-HUVECs



B. LPS-stimulated MO-HUVECs

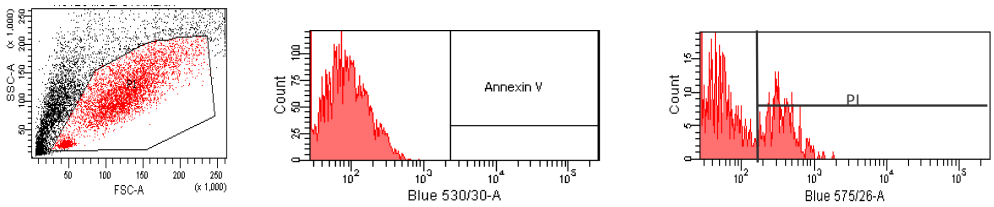
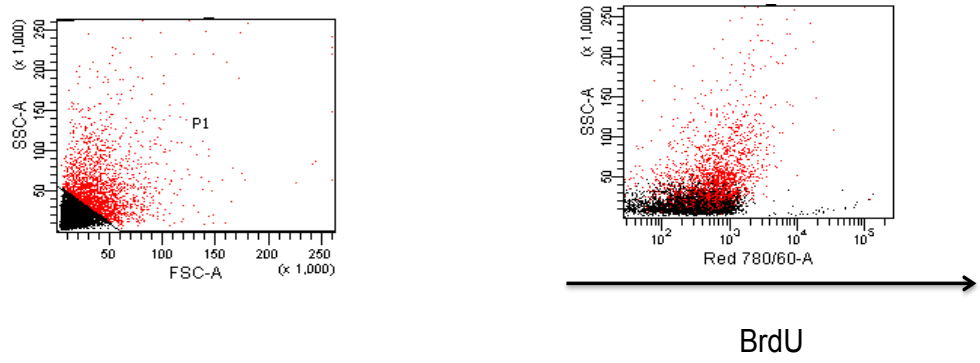


Figure A4.2 Flow cytometry graphs representing Annexin V staining of monocyte and HUVECs co-culture

A. Flow cytometry SSC/FSC dot plot graph of unstimulated monocyte-HUVECs co-culture (***left panel***)**;** Annexin V or PI flow cytometry histogram (***right panel***). **B.** Flow cytometry SSC/FSC dot plot graph of LPS-stimulated monocyte-HUVECs co-culture (***left panel***)**;** Annexin V or PI flow cytometry histogram (***right panel***).

HUVECs



MO-HUVECs

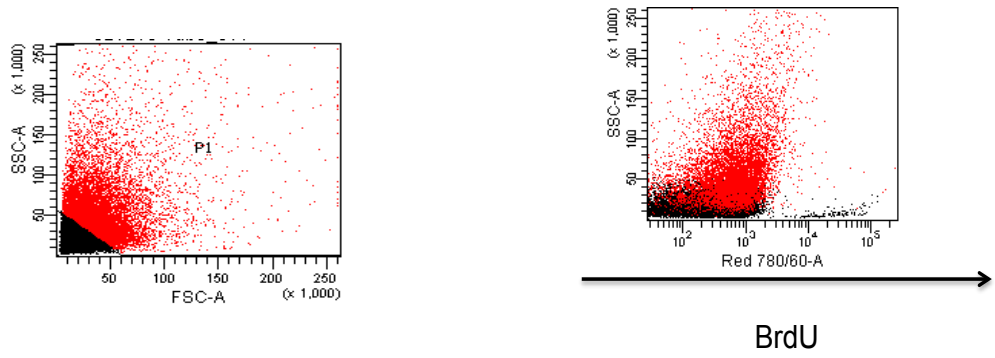


Figure A4.3 Flow cytometry FSC/SSC dot plot graphs representing BrdU labelling of endothelial cells (HUVECS mono- or co-cultures)

Conclusion

It was postulated that NP monocytes would prolong the viability of endothelial cells in monocyte-HUVECs co-cultures. As noticed under microscopic examination of cultures, endothelial cells remained attached to plates and viable for more than 2–5 days when co-cultured with monocytes. The study also aimed to discover any differences between PE and normal pregnant monocytes in viability/apoptosis and proliferation of endothelial cells, and whether PE monocytes induced more apoptosis of endothelial cells in the co-culture experiment.

The apoptosis assay was done with the Annexin V-FITC Apoptosis Detection kit, obtained from e Bioscience and analysed with FACS. Several attempts were carried out to optimize flow cytometry staining for Annexin V, as mentioned above. However, all terminated in negative results. It seems that NP monocytes in the monocyte-HUVECs co-cultures must either enhance endothelial cell viability for a longer time or reduce it to a shorter time. It may be necessary to reduce incubation time of the co-culture from 24 hours to 12, 6 or 4 hours, or to increase it to 48 or 72 hours and study apoptosis for each of these incubation times.

Noble and his colleague demonstrated that primary monocytes induce an expression of endothelial cells bcl-2 homologue A1, a protein that antagonises the apoptosis and promote cell survival, after 6 hours co-cultured monocyte with serum depravation-induced apoptotic ECs and prolonged up to 21 hours (Noble *et al.*, 1999). Also, they confirmed their results by using FACS flow cytometry for Annexin V FITC/PI. The percentages of live cells that they obtained (after 21 hours co-incubation) were as follows: 87.8% from control ECs cultured in complete growth media, 62.7% from serum-starved ECs, and 75.8% from serum-starved ECs co-incubated with primary monocytes (Noble *et al.*, 1999). Another recent study showed that monocytes reduced caspase-3 activity and single-stranded DNA levels (apoptosis markers) from serum-starved ECs or after TNFα exposed ECs; through cell contact-dependent and activation of ECs TIE2/angiopiotein-1 signalling mechanisms (Schubert *et al.*, 2011). Therefore monocytes enhanced ECs survival and protected them against apoptosis through mechanism requested cell-cell contact, which suggests an important role of monocytes in promoting of vascular remodelling.

Perhaps the most serious disadvantage of our apoptosis assay is that we did not include enough positive or negative controls for Annexin V-FITC/PI. Numerous studies have reported that multiple stimuli could induce endothelial cells apoptosis including serum deprivation, induction by TNF-α, oxidative stress, oxidized low-density lipoprotein, LPS and exposure to environmental factors such as irradiation (Geng, 2001, Haimovitz-Friedman *et al.*, 1997, Hogg *et al.*, 1999, Polunovsky *et al.*, 1994, Shin *et al.*, 2004). Therefore, these factors could be used to induce endothelial cells apoptosis and considered as positive controls for our apoptosis assay. On the other hand, vascular growth factors, including fibroblast growth factor-2 (FGF-2), supressed ECs apoptosis (Karsan *et al.*, 1997). Hence, ECs treated with FGF-2 can be used as negative control for Annexin V FITC/PI. However, in our protocol we used only LPS-induced ECs as positive control, but this work was not completed due to time and cost circumstances. Otherwise, for apoptosis assay we can simply incubate ECs, as done by previous work (Schubert *et al.*, 2011), in either complete growth media or serum-starved media as negative or positive controls, respectively. In our protocol HUVECs were incubated with growth medium (DMEM plus 10% heat inactivated FBS) until they reached subconfluence and then cultured for 24 hours with low serum media (DMEM plus 5% heat inactivated FBS) for serum starvation (Chapter 2 Section 2.2.2.1). However, Schubert's protocol HUVECs were cultured up to subconfluence in complete growth media and then exposed the ECs for 2 stages of starvation (Schubert *et al.*, 2011). The first starvation stage HUVECs was incubated with DMEM plus 2% heat inactivated FBS for 24 hours, followed by second starvation stage with incubation of cells in DMEM plus 0.5% heat inactivated FBS for another 24 hours. Hence, it seems that HUVECs from our protocol did not develop enough time for stress with a relatively high amount of serum media. For this reason, ECs may not have picked up Annexin V stain.

In addition, BrdU labelling was carried out to study the role of PE monocytes on endothelial cell proliferation and was analysed with flow cytometry. However, this experiment produced excess debris and lost cells due to multiple washings. Thus, we concluded that it is necessary to label cells with BrdU in tissue culture plates and analyse them with imaging instruments such as confocal microscopy, as has been done previously (Schubert *et al.*, 2008).

Further experiments are required for optimisation of Annexin V and BrdU labelling before carrying out patient recruitment and performing more studies on normal pregnancy and PE women. However, due to time limitations and the cost of flow cytometry/confocal microscopy materials and bookings, which are not feasible for a PhD project, these experiments have been postponed.