Accepted Manuscript

Title: Ligands of toll-like receptors 2/4 differentially alter markers of inflammation, adhesion and angiogenesis by monocytes from women with pre-eclampsia in co-culture with endothelial cells

Authors: Ebtisam A. Al-ofi, Dilly O. Anumba

PII: S0165-0378(16)30668-4
DOI: http://dx.doi.org/doi:10.1016/j.jri.2017.05.002
Reference: JRI 2474

To appear in: *Journal of Reproductive Immunology*

Received date: 16-11-2016
Revised date: 4-5-2017
Accepted date: 16-5-2017

Please cite this article as: {http://dx.doi.org/

Ligands of Toll-Like Receptors 2/4 Differentially Alter Markers of Inflammation, Adhesion and Angiogenesis by Monocytes from Women with Pre-Eclampsia in Co-Culture with Endothelial Cells

Ebtisam A. Al-ofi[a1], and Dilly O. Anumba[a*]

[a] Academic Unit of Reproductive & Developmental Medicine, University of Sheffield, Medical School, Tree Root Walk, Sheffield S10 2SF, United Kingdom

[1] Permanent address: Department of Physiology, King Abdulaziz University, Faculty of Medicine, Branch of Sulaymaniyah, Jeddah 21589, Saudi Arabia

Summary sentence: The effect of fibrinogen, an endogenous ligand of TLR4, on inflammatory cytokine responses, cell adhesion and angiogenesis when human monocytes from women with pre-eclampsia are co-cultured with vascular endothelial cells.

Short title: Fibrinogen alters inflammation and angiogenesis in pre-eclampsia

***Corresponding author information**: Dilly O. Anumba, MBBS FWACS FRCOG MD LL.M, University of Sheffield, Jessop Wing, Tree Root Walk, Sheffield S10 2SF, United Kingdom. Tel +44 114 226 1075. Fax +44 114 226 8538. email: d.o.c.anumba@sheffield.ac.uk

Abstract

Pre-eclampsia (PE) is characterized by an exaggerated systemic inflammatory response and generalized endothelial dysfunction. We have recently demonstrated that fibrinogen, an endogenous ligand of Toll-like receptor (TLR) 4, activates monocytes from women with pre-eclampsia (Al-ofi *et al.*, 2014). Using an experimental co-culture model of primary human monocytes (derived from 9 women with PE (GA=33.18 ± 5.8) and 9 normotensive pregnant women, NP (GA= 33.15 ± 4.0)) and human umbilical venous endothelial cells (HUVECs), we compared the effects of fibrinogen and lipopolysaccharide (LPS, bacterial ligand to TLR4) on the expression levels of inflammatory cytokines (IL-6 and IL-1β), chemokines (IL-8 and MCP-1), and anti-angiogenic factor (soluble fms-like tyrosine kinase-1,sFLT-1), as well as the soluble vascular cell adhesion molecule-1 (sVCAM-1). Cytokines, VEGF and sVCAM-1 were measured in the supernatant media by cytometric array. The levels of sFLT-1 were measured by ELISA. Fibrinogen induced greater expression levels of IL-1β and VCAM-1 from PE HUVEC-monocyte co-culture than from NP HUVEC-monocyte co-culture ($P<0.05$), similar to the effects of LPS. In contrast, unlike LPS, fibrinogen suppressed IL-6, IL-8, MCP-1 and sFLT-1 production by co-cultures that included PE monocytes compared to those with NP monocytes ($P<0.05$). In conclusion, fibrinogen promotes monocyte-endothelial cell adhesion and angiogenesis and suppresses the expression of some inflammatory markers in pre-eclampsia. Although the physiological implications of these intriguing observations are unclear our findings suggest that fibrinogen contributes to the regulation of cell adhesion, angiogenesis and inflammation by mechanisms not wholly dependent on TLR4 stimulation.

Keywords: Pregnancy hypertensive disorder; Endothelial cells; Monocytes; TLR4; Endogenous ligand

1.1 Introduction

Pre-eclampsia (PE) is defined as pregnancy-induced hypertension associated with proteinuria that occurs after mid of pregnancy (Brown *et al.*, 2001). The etiology of PE remains unclear but a number of pathophysiologic mechanisms of the disorder have now been clarified. PE is characterized by increased circulating levels of the anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFLT-1) but suppressed levels of the pro-angiogenic factors free vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) (Widmer *et al.*, 2015). Increased levels of circulating markers of endothelial cell damage such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) have also been described (Veas *et al.*, 2011, Prochazka *et al.*, 2015). Altered activation and function of circulating inflammatory cells such as monocytes (Sibai, 2004, Al-ofi *et al.*, 2012) as well as a changes in their subpopulations, and exaggerated responses to toll-like receptor (TLR) 2 and 4 ligands have been described (Al-ofi *et al.*, 2012). All of these observations suggest that vascular endothelial cell dysfunction and altered immunological responses play major roles in the pathogenesis of PE.

It has been recognized that the interaction between peripheral blood monocytes and vascular endothelial cells alters the expression of various inflammatory mediators: the co-culture of monocytes with endothelial cells has been shown to induce more inflammatory markers (such as IL-6, IL-1β, IL-1α, IL-8, MCP-1, IFN γ-inducible protein 10, and granulocyte-macrophage colony-stimulating factor, GM-CSF) and adhesion molecules than the monoculture of either cell type individually (Lukacs *et al.*, 1995, Takahashi *et al.*, 1996, Kasama *et al.*, 2002, Ward *et al.*, 2009). Monocytes appear to play key roles in the regulation of endothelial cell proliferation and survival, as well as angiogenesis, (Schubert *et al.*, 2011). Furthermore, they are activated in PE, as shown by us (Al-ofi *et al.*, 2012) and others (Gervasi *et al.*, 2001, Luppi and Deloia, 2006). For example, CD11 and reactive oxygen species are upregulated in PE monocytes (Gervasi *et al.*, 2001).

Fibrinogen, an endogenous ligand of TLR4 is also able to bind and activate the signaling of immune cells (i.e. monocytes) via CD11b/CD18 integrin receptors and adhesion molecules (Hodgkinson *et al.*, 2008). During normal pregnancy, plasma fibrinogen is increased and fibrinolysis activity is suppressed, ostensibly to promote coagulability of blood (Thornton and Douglas, 2010). There are conflicting data regarding fibrinogen levels in PE, with some studies, including ours, demonstrating increased (Al-ofi *et al.*, 2014, Kawada, 2015), whilst others demonstrate reduced, circulating levels (Spiezia *et al.*, 2015). 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, Roberts and Post, 2008, Al-ofi *et al.*, 2014)

We recently showed that fibrinogen activates monocytes from women with PE (Al-ofi *et al.*, 2014). We therefore postulated that fibrinogen-induced activation of "inflammatory" monocytes in PE could impair vascular endothelial cell function and alter their chemo-attractant, angiogenic and inflammatory responses, thus playing a key role in the pathogenesis of PE. We tested this hypothesis by comparing the responses to fibrinogen and LPS of HUVECs co-incubated with PE monocytes to the responses of HUVECs co-incubated with monocytes from normotensive women matched for gestation. We chose fibrinogen and HMGB1 as the endogenous ligands of TLR4 having demonstrated in an earlier study that, of the TLR4 ligands studied, fibrinogen was the only factor that appeared to induce inflammatory cytokine release from PE monocyte cultures (Al-ofi *et al.*, 2014). Our co-culture experimental model enabled us to determine the effects of these TLR4 ligands on the expression levels of inflammatory cytokines (IL-6 and IL-1β), chemokines (IL-8 and MCP-1), angiogenic (VEGF) and anti-angiogenic (sFLT-1) factors, as well as the cell adhesion molecule sVCAM-1.

1.2 Material and Method

1.2.1 Ethical approval

Ethical approval for this study was received from the South Yorkshire Research Ethics Committee (09/H1310/12). A detailed information sheet was given to all study participants and written informed consent was obtained from them.

1.2.2 Subjects and samples

Consent for use of umbilical cords was obtained from term normotensive pregnant women in early labour in the delivery suite of the Jessop Wing Hospital (n=12). The harvested umbilical cords were used for the isolation of HUVECs. Monocytes were obtained from blood samples from nine women with PE and nine NP women (**Table1**), and used for HUVEC co-culture experiments. 10ml of fresh blood was collected into a tube containing EDTA to prevent clotting and immediately transported to the research laboratories to isolate monocytes for the experiments.

1.2.3 Stimulation of monocytes and HUVEC cultures

Co-culture experiments of primary human monocytes and endothelial cells were optimized according to previously published studies (Schubert *et al.*, 2008, Ward *et al.*, 2009). Human

vascular endothelial cells were isolated from freshly collected umbilical cords using standard published techniques (Baudin *et al.*, 2007). The HUVECs were then cultured and studied on the second through fifth passages. HUVECs were seeded onto 0.2% gelatin-coated 12-well tissue-culture plates (Greiner bio-one, catalogue number: 665180) at a density of 10^5 cells per well and incubated with one ml of medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-Glutamine and penicillin-streptomycin) at 37°C with 5% CO_2 until they reached 70-90% of confluence, usually within two to three 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.

The monocytes were isolated from the fresh blood of donors (NP or PE), collected in EDTA tubes and separated immediately after the blood was drawn. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on top of Ficoll-Paque (Al-ofi *et al.*, 2012). Monocytes were isolated from PBMCs using the pan monocyte isolation kit (Miltenyi Biotic, Bergisch Gladbach, Germany catalogue number: 130-096-537), which employs indirect magnetic labelling of non-monocyte cells, according to the manufacturer's instructions.

Following 24h of serum starvation, HUVECs were washed with 1ml of pre-warmed PBS and low serum media was replaced (DMEM supplemented with 5% heat inactivated FBS without antibiotics). Monocytes were plated at a density of 20,000 cells/ml on top of HUVECs at the approximate proportion of 1 monocyte to 5 HUVECs. At the same time, monocytes or HUVECs were cultured separately with the equivalent density (mono-cultures). Mono-culture and co-culture assays were performed in DMEM with 5% heat-inactivated FBS without antibiotics media. Thereafter, mono- and co-cultures were immediately stimulated with 100 ng/ml lipopolysaccharides (rough strains) from *Escherichia coli EH 100 (*Sigma-Aldrich), 5 µg/ml peptidoglycan from *Staphylococcus aureus (*Sigma-Aldrich*)*, 2 mg/ml fibrinogen from human plasma *(*Sigma-Aldrich*),* and 0.7 µg/ml recombinant human HMGB1 (R and D Systems), as described in previous studies (Smiley *et al.,* 2001, Yu *et al.,* 2006), by 24h of incubation at 37°C with 5% CO_2. After 24h, the conditioned medium was collected and stored at -80°C.

1.2.4 Flow cytometry

Flow cytometry was used to confirm the presence of monocyte and endothelial cell populations in the co-culture experimental models as depicted in the **Fig 1.**

1.2.5 Cytometric bead array

The cytometric bead array (CBA) technique was used to measure the inflammatory cytokines (IL-1β and IL-6), the anti-inflammatory cytokine (IL-10), and chemokines (IL-8 and MCP-1) within conditioned medium collected from monocytes and the HUVECs cultures. This assay was performed as previously described using kits from BD Biosciences, a BD FACS Array flow cytometry machine and FCAP Array software. The IL-1β and IL-10 cytokines were measured in the undiluted aliquot (30µl). However, IL-6, IL-8 and MCP-1 cytokines were above standard levels for undiluted aliquots. Consequently, serial dilutions (1:10 up to 1:200) were attempted to optimize the desired dilution. Thereafter, according to the optimum concentration within the sample, either 1:50 or 1:100 dilutions were applied to the samples, and each sample was multiplied according to its dilution factor before analysis was initiated. Additionally, The CBA technique was used to measure the VEGF and sVCAM-1 in the supernatant medium collected from monocytes-HUVECs mono- or co-cultures.

1.2.6 Enzyme-linked Immunosorbent Assay

Human sVEGFR-1 */* sFLT-1 Immunoassay (Quantikine ELISA kit catalogue number: DVR100B, R&D Systems, Europe) 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.

1.2.7 Statistical analysis

The results are illustrated as mean ± standard error of the mean (SEM). One-way ANOVA (Kruskal-Wallis test), followed by correction using Dunn's multiple comparisons test, was used to compare differences between mono-cultures of HUVECs and monocytes, as well as between co-cultures of NP/PE monocytes and HUVECs, using GraphPad Prism software version 7.0. The two-way ANOVA test was used to compare differences between the unstimulated cells and the cells stimulated with different agonists (bacterial and endogenous ligands of TLRs). *P* values < 0.05 were considered significantly different.

1.3 Results

1.3.1 Monocytes of women with PE alter inflammatory responses of vascular endothelial cells in co-culture experimental model (Figure 2)

In comparison with NP co-cultures, unstimulated co-cultures of PE monocytes with endothelial cells demonstrated profoundly increased secretion of the inflammatory cytokine IL-6 ($P<0.05$) as well as the chemokines IL-8 and MCP-1(*Fig 2A, 2D and 2E respectively*) ($P<0.01$), along with reduced production of the anti-inflammatory cytokine IL-10 *(Fig 2C*).

Additionally, unstimulated monocyte-HUVEC co-cultures from NP and PE groups released significantly higher IL-6 levels than monocyte monocultures; IL-6 expression from HUVEC-PE monocyte co-cultures was more than 10-fold higher than from PE monocyte monocultures ($P<0.05$) (*Fig 2A*). Furthermore, HUVEC-PE monocyte co-cultures produced much higher basal levels of IL-8 than PE monocyte monocultures ($P<0.05$) (*Fig 2D*). Basal expression levels of IL-1β and IL-10 did not differ between PE mono- and co-cultures (*Fig 2B and 2C respectively*). HUVECs and NP/PE monocytes in monocultures did not produce MCP-1. Conversely, when both cell types interacted during co-cultures experiments, there was a significant increase in MCP-1 production, especially from HUVEC-PE monocyte co-cultures ($P<0.001$) (*Fig 2E*).

1.3.2 Fibrinogen alters inflammatory responses of monocytes from women with PE co-cultured with vascular endothelial cells (Figure 3)

After stimulation with LPS, IL-6 significantly increased in both NP and PE co-culture models ($P<0.05$ and $P<0.001$) (*Fig 3A*). Compared with NP, TLR bacterial ligand-treated HUVEC-PE monocyte co-cultures exhibited markedly increased IL-6 secretion, compared to that seen in ligand-treated HUVEC-NP monocytes ($P<0.01$). In contrast, endogenous ligands of TLR4 evoked no such increase in NP as well as PE monocyte co-cultures.

LPS, PDG, and fibrinogen significantly induced IL-1β secretion by PE monocytes, with or without HUVECs, when compared to the non-stimulated control ($P<0.05$ and $P<0.001$) (*Fig 3B*). Additionally, LPS and PDG significantly induced IL-1β in HUVEC-NP monocytes when compared to non-stimulated control ($P<0.05$). Fibrinogen-treated HUVEC-PE monocytes exhibited a marked increase in IL-1β secretion compared to fibrinogen-treated HUVEC-NP monocytes ($P<0.05$). However, unlike IL-6 and IL- β, IL-10 expression levels did not differ between ligand-treated HUVEC-monocyte co-cultures from PE and NP groups (*Fig 3C*).

Compared to non-stimulated-HUVEC-PE monocyte co-cultures, LPS and PDG induced IL-8 secretion whilst fibrinogen appeared to suppress it and HMGB1 did not alter its basal secretion (*Fig 3D*). In contrast, all ligands significantly induced IL-8 in HUVEC-NP monocyte co-cultures compared to un-stimulated co-cultures ($P<0.05$).

Compared to non-stimulated controls, MCP-1 was released in large amounts from PDG-treated HUVEC-PE monocyte co-cultures ($P<0.001$); was not produced by LPS-treated HUVEC-PE monocyte co-cultures; and was suppressed in fibrinogen-HUVEC-PE monocyte co-cultures (*Fig 3E*).

1.3.3 Fibrinogen attenuates sFLT-1-induced dys-angiogenesis (Figure 4)

Human vascular endothelial cell monocultures produced high basal levels of sFLT-1. However, this appeared down-regulated by co-culture with monocytes from both groups of pregnant women (P<0.001), NP monocytes more so than PE monocytes (P<0.05) (*Fig 4A1*). Although the treatment of HUVEC monocultures with LPS or PDG appeared to reduce sFLT-1 production, this observation did not attain statistical significance (*Fig 4A2*). On the other hand, stimulation of co-cultures with bacterial ligands induced sFLT-1 production in both NP and women with PE (*Fig 4A2*). Fibrinogen treatment of HUVEC-PE monocyte co-cultures significantly reduced sFLT-1 production (*Fig 4A2*) (P<0.001) but did not alter sFLT-1 production by HUVEC-NP monocytes co-culture (*Fig 4A2*).

The basal expression levels of VEGF differed significantly between the various culture models as depicted in *Fig 4B1*. HUVEC monocultures expressed relatively higher levels of VEGF than HUVEC with NP or PE monocyte co-cultures (*Fig 4B1*). Stimulation of mono or co-cultures with TLR ligands did not result in altered VEGF production, except for HMGB1 which appeared to induce production in by PE monocytes in culture (P<0.05) (*Fig 4B2*).

1.3.4 Fibrinogen enhanced vascular adhesion in PE (Figure 4C)

We also determined that soluble vascular cell adhesion molecule is induced by the interaction between monocytes from women with PE and vascular endothelial cells. As depicted in (*Fig 4C1*), HUVECs and NP/PE monocytes in monocultures produced very low levels of VCAM-1. However, cell-cell interactions during co-cultures experiments resulted in significant increases in VCAM-1 production, especially from HUVEC-PE monocytes co-cultures (P<0.001) (*Fig 4C1*). Unstimulated HUVECs produced extremely low levels of VCAM-1. However, LPS, but not other TLR ligands, induced higher expression levels of VCAM-1 (P<0.05) (*Fig 4C2*). Compared to the HUVEC-NP monocyte co-culture, the HUVEC-PE monocyte co-culture was associated with higher basal expression levels of VCAM-1 (P<0.05) (*Fig 4C1*), and an exaggerated VCAM-1 response on exposure to TLR bacterial ligands was also associated with (P<0.05) (*Fig 4C2*). Furthermore, the HUVEC-PE monocyte co-culture exhibited amplified VCAM-1 secretion in response to fibrinogen compared to the HUVEC-NP monocyte co-culture (P<0.001) (*Fig 4C2*).

1.4 Discussion

This study develops on our previous observation that fibrinogen, an endogenous ligand of TLR4, appears to induce PE monocytes to release inflammatory cytokines (Al-ofi *et al.*, 2014), consistent with several studies that suggest a role for this receptor in the pathogenesis of PE (Al-ofi *et al.*, 2012, Chen *et al.*, 2015, Kulikova *et al.*, 2016). We now show that fibrinogen

also appears to suppress the expression of inflammatory cytokine markers, induce the expression of cell adhesion molecules, and promote angiogenesis (by decreasing sFLT-1 production) when PE monocytes and endothelial cells are established in co-culture.

To the best of our knowledge, this is the first study to report the influence of activated human peripheral blood monocytes from patients with PE on endothelial cell function. We successfully established a co-culture model of human primary peripheral blood monocytes and HUVECs, and showed that PE monocytes in co-culture with HUVECs elaborated more IL-6 (pro-inflammatory cytokine), IL-18 and MCP-1 (chemokines) compared to NP monocyte-HUVECS co-cultures, whereas the anti-inflammatory cytokine IL-10 expression levels was attenuated.

Several studies have shown that IL-6 levels are elevated in the plasma of women with pre-eclampsia and is involved in the pathogenesis of the condition (Freeman *et al.*, 2004, Jonsson *et al.*, 2006, Xiao *et al.*, 2012). Being a multifunctional cytokine, IL-6 could play a key role in the modulation of angiogenesis in normotensive or hypertensive pregnancy (Desai *et al.*, 2002). We have observed the elevation of IL-6 levels when HUVECs are established in co-culture with monocytes from women with PE, suggesting that IL-6 may promote vascular endothelial dysfunction in PE. Similarly, bacterial TLR2 and TLR4 ligands induced very high levels of IL-6 in co-cultures of PE monocytes with HUVECs, suggesting that vascular endothelial damage in PE may involve TLR-mediated mechanisms. It is intriguing that fibrinogen appeared to induce IL-6 production from monocultures of PE, but not NP monocytes (consistent with our previous report (Al-ofi *et al.*, 2014)), but reduced its release from PE monocyte-HUVECs co-cultures. The potential 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 endothelial cells in the microvasculature. It is plausible that there is potential gene-gene association between IL-6 and fibrinogen, which could influence fibrinogen levels without influencing the development of cardiovascular disease (Carty *et al.*, 2010). It may also be that fibrinogen at this interface regulates monocyte activation and the release of cytokines in order to protect endothelial cells from inflammatory damage.

It has been shown in porcine and murine models of vascular disease, and through *in vitro* experiments employing co-cultures of primary human monocytes and endothelial cell, that IL-1β is a critical mediator of vascular inflammation (Morton *et al.*, 2005, Chamberlain *et al.*, 2006, Ward *et al.*, 2009). Data from the current study demonstrate that fibrinogen significantly induced IL-1β production by PE monocytes in mono- as well as co-cultures with

HUVECs, compared to NP monocytes. In another study, IL-1β, but not IL-1α, binds to fibrinogen and augments endothelial cell activity with increased NF-kβ, MCP-1, and nitric oxide production (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, as well as a potent angiogenic marker (Koch *et al.*, 1992), whilst MCP-1 is a chemo-attractant for mononuclear leukocytes (Leonard and Yoshimura, 1990). Kauma *et al*., demonstrated that plasma of women with severe PE expressed higher levels of IL-8 and MCP-1, and induced HUVEC to release IL-8 and MCP-1 (Kauma *et al.*, 2002). We have observed increased chemokine production from NP monocyte-HUVEC co-cultures, indicating their role in normal angiogenesis. Their amplified production from PE monocyte-HUVEC co-cultures is consistent with dysregulated angiogenesis in this model. The apparent modulatory effect of fibrinogen on the production of IL-8 from PE monocyte-HUVEC co-cultures suggests some role for fibrinogen during chemotaxis. MCP-1 production appeared amplified in PDG-treated HUVEC-PE monocyte co-cultures, but reduced in fibrinogen-treated PE monocyte-HUVEC co-cultures. Taken together, our data suggest a complex role for TLR-2 and -4 receptors, and their ligands, in vascular endothelial homeostasis in PE, which merits further investigation.

Circulating sFLT-1 is an anti-angiogenic protein whose levels are increased in PE, a condition in which it appears to suppress circulating PIGF and VEGF (Maynard *et al.*, 2003, Chappell *et al.*, 2013, Widmer *et al.*, 2015). We have demonstrated that endothelial cells produced higher basal levels of sFLT-1, consistent with previous studies (Kendall *et al.*, 1996, Barleon *et al.*, 2001). For the first time however, we show that sFLT-1 production by HUVECs can be inhibited by fibrinogen. At variance with a previous study that reported 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 monocyte mono-cultures, even after TLR2 or TLR4 stimulation. One explanation for the differences between these studies could be the differing duration of culturing the monocytes – we incubated monocytes for 24 hours whilst Barleon et al (Barleon *et al.*, 2001) did so for 72 hours. We also observed that monocytes from both pregnant groups attenuated sFLT-1 production by endothelial cells, but that 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 sFLT-1 production, an intriguing finding is that fibrinogen suppressed sFLT-1 production in the HUVEC-PE monocyte but not in the HUVEC-NP monocyte co-culture. The plausible explanation for this observation is unclear but a role for fibrinogen in counteracting dysregulated angiogenesis in PE seems likely. We observed reduced soluble VEGFR-1 (sFLT-1) from HUVEC or HUVEC-PE monocyte co-cultures, in

agreement with one study that shows that endothelial cells cultured on fibrin matrices had markedly reduced growth factor receptors; particularly FGFR-1, FGFR-2 and VEGFR- 1 (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.

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 (Lam et al., 2005). One unanticipated finding was that HMGB-1 treatment of the PE monocytes monoculture induced VEGF production whilst other TLR ligands did not. This may be because HMGB-1 appears to stimulate VEGF production from PE monocytes through a different signalling pathway, such as the advanced glycation end product (RAGE) (Huang et al., 2010), as has been shown in oesophageal squamous cell carcinoma (Chen et al., 2012).

Soluble adhesion molecules (VCAM-1, ICAM-1 and E-selectin) are elevated in the serum of patients with PE (Sibai, 2004, Al-ofi *et al.*, 2012). Serum from women with PE also activates endothelial cells to express ICAM-1 but only in the presence of monocytes (Faas *et al.*, 2010). Consistent with these previous studies (Sibai, 2004, Faas *et al.*, 2010, Al-ofi *et al.*, 2012), we observed increased expression levels of VCAM-1 by HUVEC-PE monocyte co-culture, and further enhanced induction by the bacterial TLR2/4 ligands (LPS and PDG) as well as by fibrinogen. Languino *et al*. inferred from their studies that fibrinogen acts as a molecular bridge between endothelial cells and leukocytes, enhancing the adhesion of leukocytes to the vascular endothelium and facilitating leukocyte trans-endothelial migration (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). Moreover, fibrinogen stimulates TLR4 signalling in macrophages to release MMP-1 and MMP-9 (Hodgkinson *et al.*, 2008). 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 (Lominadze *et al.*, 2010).

1.4.1 Conclusion

We have shown that fibrinogen and HMGB1 variably induce pro-inflammatory cytokine production in co-cultures of monocytes and HUVECs similar to, but less strongly than, the TLR2 and TL4 ligands peptidoglycan and lipopolysaccharide. Our observations suggest that

fibrinogen appears to promote monocyte-endothelial cell adhesion thus may contribute to endothelial repair in PE by promoting angiogenesis through unknown mechanisms that may include strong suppression of sFLT-1 production. How fibrinogen modulates cell adhesion, angiogenesis and inflammation in normotensive and hypertensive pregnancy, and whether these functions are mediated by the TLR4 receptor or other fibrinogen receptors remain to be determined.

1.5 Acknowledgments

The authors thank Dr. Andrea King (University of Sheffield, Department of Cardiovascular Science of the Medical School) for her assistance in the techniques used for human umbilical vascular endothelial cells (HUVECs) isolation and cultures. E.A. was funded by the King Abdul Aziz University, the Kingdom of Saudi Arabia. No additional external funding received for this study.

1.6 References

Al-ofi, E., Coffelt, S. B. & Anumba, D. O. 2012. Monocyte subpopulations from pre-eclamptic patients are abnormally skewed and exhibit exaggerated responses to Toll-like receptor ligands. *PloS one,* 7, e42217.

Al-ofi, E., Coffelt, S. B. & Anumba, D. O. 2014. Fibrinogen, an endogenous ligand of Toll-like receptor 4, activates monocytes in pre-eclamptic patients. *J Reprod Immunol,* 103, 23-8.

Arias, F. & Mancilla-Jimenez, R. 1976. Hepatic fibrinogen deposits in pre-eclampsia. Immunofluorescent evidence. *The New England journal of medicine,* 295, 578-82.

Barleon, B., Reusch, P., Totzke, F.*, et al.* 2001. Soluble VEGFR-1 secreted by endothelial cells and monocytes is present in human serum and plasma from healthy donors. *Angiogenesis,* 4, 143-54.

Baudin, B., Bruneel, A., Bosselut, N.*, et al.* 2007. A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat Protoc,* 2, 481-5.

Brown, M. A., Lindheimer, M. D., de Swiet, M.*, et al.* 2001. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertension in pregnancy : official journal of the International Society for the Study of Hypertension in Pregnancy,* 20, IX-XIV.

Carty, C. L., Heagerty, P., Heckbert, S. R.*, et al.* 2010. Interaction between fibrinogen and IL-6 genetic variants and associations with cardiovascular disease risk in the Cardiovascular Health Study. *Annals of human genetics,* 74, 1-10.

Chamberlain, J., Evans, D., King, A.*, et al.* 2006. Interleukin-1beta and signaling of interleukin-1 in vascular wall and circulating cells modulates the extent of neointima formation in mice. *Am J Pathol,* 168, 1396-403.

Chappell, L. C., Duckworth, S., Seed, P. T.*, et al.* 2013. Diagnostic accuracy of placental growth factor in women with suspected preeclampsia: a prospective multicenter study. *Circulation,* 128, 2121-31.

Chen, W., Qian, L., Wu, F.*, et al.* 2015. Significance of Toll-like Receptor 4 Signaling in Peripheral Blood Monocytes of Pre-eclamptic Patients. *Hypertens Pregnancy,* 34, 486-494.

Chen, C. G., Tang, P. & Yu, Z. T. 2012. Effect of HMGB1 on the VEGF-C expression and proliferation of esophageal squamous cancer cells. Zhonghua Zhong Liu Za Zhi, 34, 566-70.

Desai, T. R., Leeper, N. J., Hynes, K. L.*, et al.* 2002. Interleukin-6 causes endothelial barrier dysfunction via the protein kinase C pathway. *J Surg Res,* 104, 118-23.

Faas, M. M., van Pampus, M. G., Anninga, Z. A.*, et al.* 2010. Plasma from preeclamptic women activates endothelial cells via monocyte activation in vitro. *J Reprod Immunol,* 87, 28-38.

Freeman, D. J., McManus, F., Brown, E. A.*, et al.* 2004. Short- and long-term changes in plasma inflammatory markers associated with preeclampsia. *Hypertension,* 44, 708-14.

Gervasi, M. T., Chaiworapongsa, T., Pacora, P.*, et al.* 2001. Phenotypic and metabolic characteristics of monocytes and granulocytes in preeclampsia. *Am J Obstet Gynecol,* 185, 792-7.

Hodgkinson, C. P., Patel, K. & Ye, S. 2008. Functional Toll-like receptor 4 mutations modulate the response to fibrinogen. *Thrombosis and haemostasis,* 100, 301-7.

Huang, W., Tang, Y. & Li, L. 2010. HMGB1, a potent proinflammatory cytokine in sepsis. Cytokine, 51, 119-26.

Jonsson, Y., Ruber, M., Matthiesen, L.*, et al.* 2006. Cytokine mapping of sera from women with preeclampsia and normal pregnancies. *J Reprod Immunol,* 70, 83-91.

Kasama, T., Muramatsu, M., Kobayashi, K.*, et al.* 2002. Interaction of monocytes with vascular endothelial cells synergistically induces interferon gamma-inducible protein 10 expression through activation of specific cell surface molecules and cytokines. *Cellular immunology,* 219, 131-9.

Kauma, S., Takacs, P., Scordalakes, C.*, et al.* 2002. Increased endothelial monocyte chemoattractant protein-1 and interleukin-8 in preeclampsia. *Obstet Gynecol,* 100, 706-14.

Kawada, T. 2015. Effect of serum C-reactive protein and plasma fibrinogen levels on recurrent preeclampsia. *J Hypertens,* 33, 421.

Kendall, R. L., Wang, G. & Thomas, K. A. 1996. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochemical and biophysical research communications,* 226, 324-8.

Koch, A. E., Polverini, P. J., Kunkel, S. L.*, et al.* 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science,* 258, 1798-801.

Kulikova, G. V., Nizyaeva, N. V., Nagovitsina, M. N.*, et al.* 2016. Specific Features of TLR4 Expression in Structural Elements of Placenta in Patients with Preeclampsia. *Bull Exp Biol Med,* 160, 718-21.

Lam, C., Lim, K. H. & Karumanchi, S. A. 2005. Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia. Hypertension, 46, 1077-85.

Languino, L. R., Duperray, A., Joganic, K. J.*, et al.* 1995. Regulation of leukocyte-endothelium interaction and leukocyte transendothelial migration by intercellular adhesion molecule 1-fibrinogen recognition. *Proceedings of the National Academy of Sciences of the United States of America,* 92, 1505-9.

Leonard, E. J. & Yoshimura, T. 1990. Human monocyte chemoattractant protein-1 (MCP-1). *Immunol Today,* 11, 97-101.

Lominadze, D., Dean, W. L., Tyagi, S. C.*, et al.* 2010. Mechanisms of fibrinogen-induced microvascular dysfunction during cardiovascular disease. *Acta physiologica,* 198, 1-13.

Lukacs, N. W., Strieter, R. M., Elner, V.*, et al.* 1995. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. *Blood,* 86, 2767-73.

Luppi, P. & Deloia, J. A. 2006. Monocytes of preeclamptic women spontaneously synthesize pro-inflammatory cytokines. *Clin Immunol,* 118, 268-75.

Maynard, S. E., Min, J. Y., Merchan, J.*, et al.* 2003. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest,* 111, 649-58.

Morton, A. C., Arnold, N. D., Gunn, J.*, et al.* 2005. Interleukin-1 receptor antagonist alters the response to vessel wall injury in a porcine coronary artery model. *Cardiovasc Res,* 68, 493-501.

Prochazka, M., Prochazkova, J., Lubusky, M.*, et al.* 2015. Markers of endothelial activation in preeclampsia. *Clin Lab,* 61, 39-46.

Roberts, D. J. & Post, M. D. 2008. The placenta in pre-eclampsia and intrauterine growth restriction. *Journal of clinical pathology,* 61, 1254-60.

Sahni, A., Guo, M., Sahni, S. K., *et al.* 2004. Interleukin-1beta but not IL-1alpha binds to fibrinogen and fibrin and has enhanced activity in the bound form. *Blood,* 104, 409-14.

Schubert, S. Y., Benarroch, A., Monter-Solans, J., *et al.* 2011. Primary monocytes regulate endothelial cell survival through secretion of angiopoietin-1 and activation of endothelial Tie2. *Arteriosclerosis, thrombosis, and vascular biology,* 31, 870-5.

Schubert, S. Y., Benarroch, A., Ostvang, J., *et al.* 2008. Regulation of endothelial cell proliferation by primary monocytes. *Arterioscler Thromb Vasc Biol,* 28, 97-104.

Sibai, B. M. 2004. Preeclampsia: an inflammatory syndrome? *Am J Obstet Gynecol,* 191, 1061-2.

Smiley, S. T., King, J. A. & Hancock, W. W. 2001. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. J Immunol, 167, 2887-94.

Spiezia, L., Bogana, G., Campello, E., *et al.* 2015. Whole blood thromboelastometry profiles in women with preeclampsia. *Clin Chem Lab Med*.

Takahashi, M., Ikeda, U., Masuyama, J., *et al.* 1996. Monocyte-endothelial cell interaction induces expression of adhesion molecules on human umbilical cord endothelial cells. *Cardiovascular research,* 32, 422-9.

Thornton, P. & Douglas, J. 2010. Coagulation in pregnancy. *Best practice & research. Clinical obstetrics & gynaecology,* 24, 339-52.

Tsou, R. & Isik, F. F. 2001. Integrin activation is required for VEGF and FGF receptor protein presence on human microvascular endothelial cells. *Molecular and cellular biochemistry,* 224, 81-9.

Veas, C. J., Aguilera, V. C., Munoz, I. J., *et al.* 2011. Fetal endothelium dysfunction is associated with circulating maternal levels of sE-selectin, sVCAM1, and sFlt-1 during pre-eclampsia. *J Matern Fetal Neonatal Med,* 24, 1371-77.

Ward, J. R., Francis, S. E., Marsden, L., *et al.* 2009. A central role for monocytes in Toll-like receptor-mediated activation of the vasculature. *Immunology,* 128, 58-68.

Widmer, M., Cuesta, C. B., Khan, K., *et al.* 2015. [93-OR]: Accuracy of angiogenic biomarkers for predicting preeclampsia: An observational study. *Pregnancy Hypertens,* 5, 50.

Xiao, J. P., Yin, Y. X., Gao, Y. F., *et al.* 2012. The increased maternal serum levels of IL-6 are associated with the severity and onset of preeclampsia. *Cytokine,* 60, 856-60.

Yu, M., Wang, H., Ding, A., *et al*. 2006. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock, 26, 174-9

1.8 Figure legends

Fig 1. Flow cytometry graphs representing monocyte and HUVECs co-culture

Flow cytometry pseudo-popular graph of monocyte-HUVECs co-culture (***left panel***-FSC/SSC plot and ***right panel***-CD14/CD146 plot); G1 is gated monocytes and G2 is gated endothelial cells.

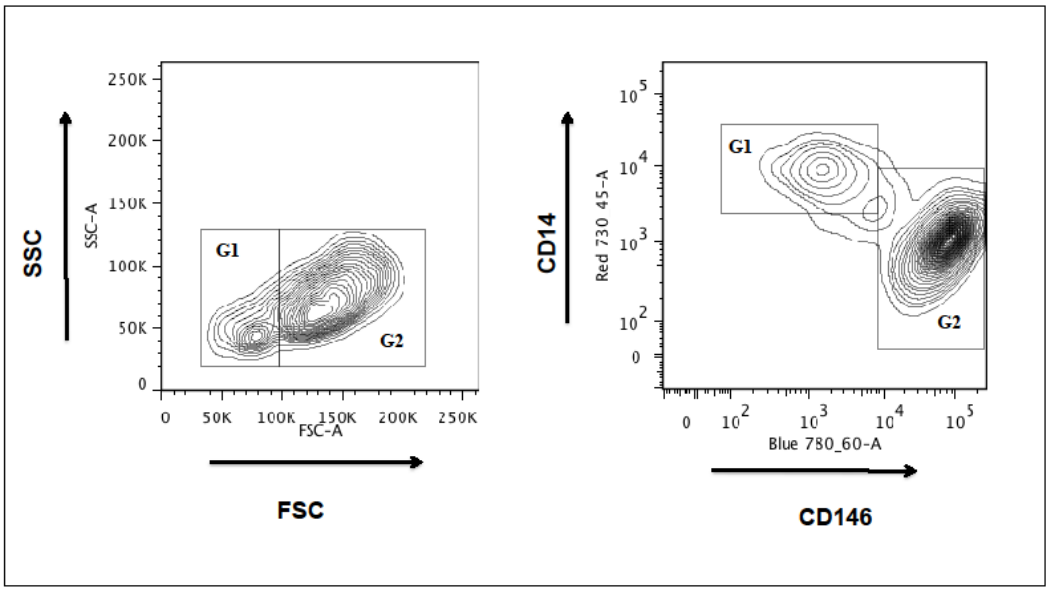


Fig 2. Monocytes (MO) from patients with PE co-cultured with HUVECs exhibit an amplified inflammatory response of cytokines

Basal levels of **A**. IL-6, **B.** IL-1β, **C.**IL-10, **D.** IL-8 and **E.** MCP-1productions by HUVEC monoculture, NP/PE MO monocultures, HUVEC-NP MO co-culture or HUVEC-PE MO co-culture. Comparisons were made between NP and PE co-cultures versus HUVECs monocultures $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, and $^{\wedge\wedge\wedge}p < 0.001$; or versus NP/PE monocytes monocultures $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$. Values were illustrated as mean ± SEM. Statistical significance was determined by one-way ANOVA (Kruskal-Wallis test) (n=7-9).

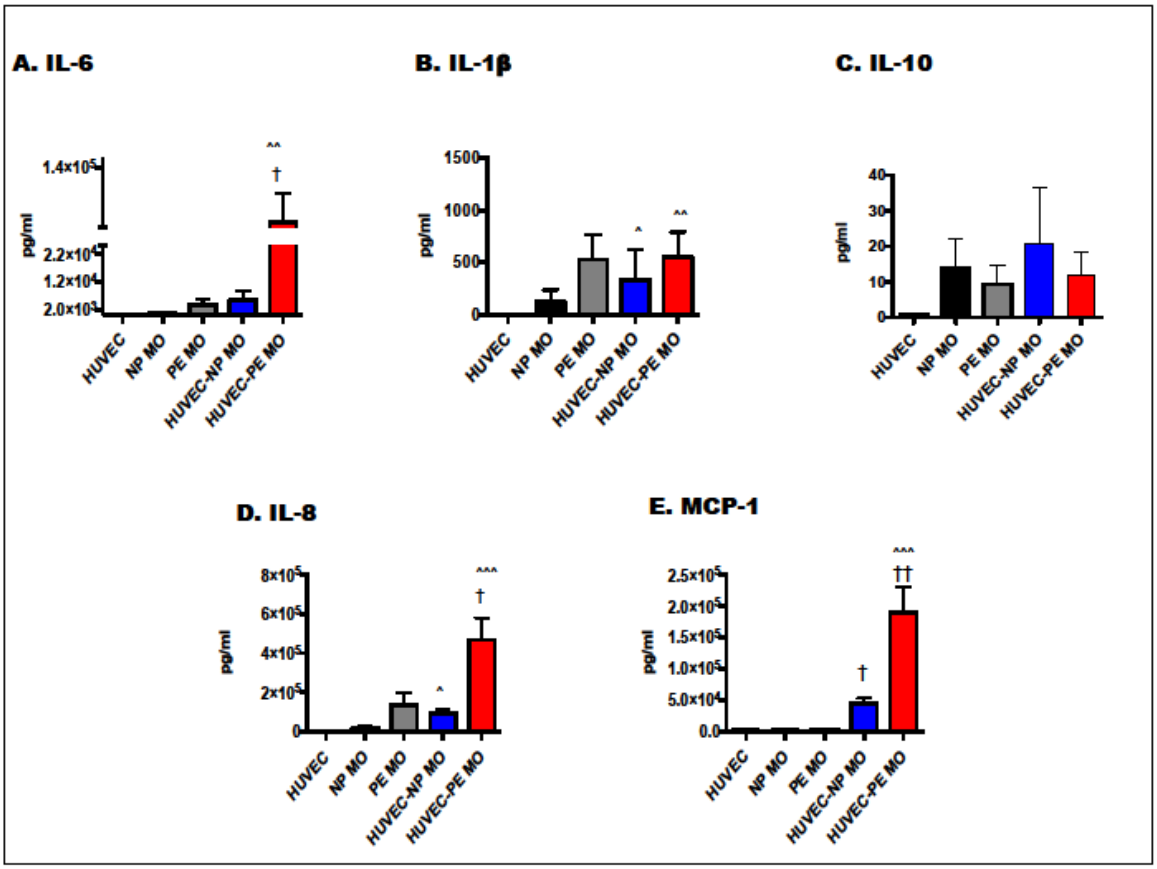


Fig 3. Fibrinogen altered inflammatory cytokines levels from monocytes (MO) of patients with PE co-cultured with HUVECs

Graphic representation of **A**. IL-6, **B.** IL-1β, **C.**IL-10, **D.** IL-8 and **E.** MCP-1 levels by mono- or co-cultures in response to LPS, PDG, fibrinogen and HMGB1. Comparisons were made between unstimulated control versus stimulated cultures; and NP versus PE mono- or co-cultures $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Values were illustrated as mean ± SEM. Statistical significance was tow-way ANOVA (n=7-9).

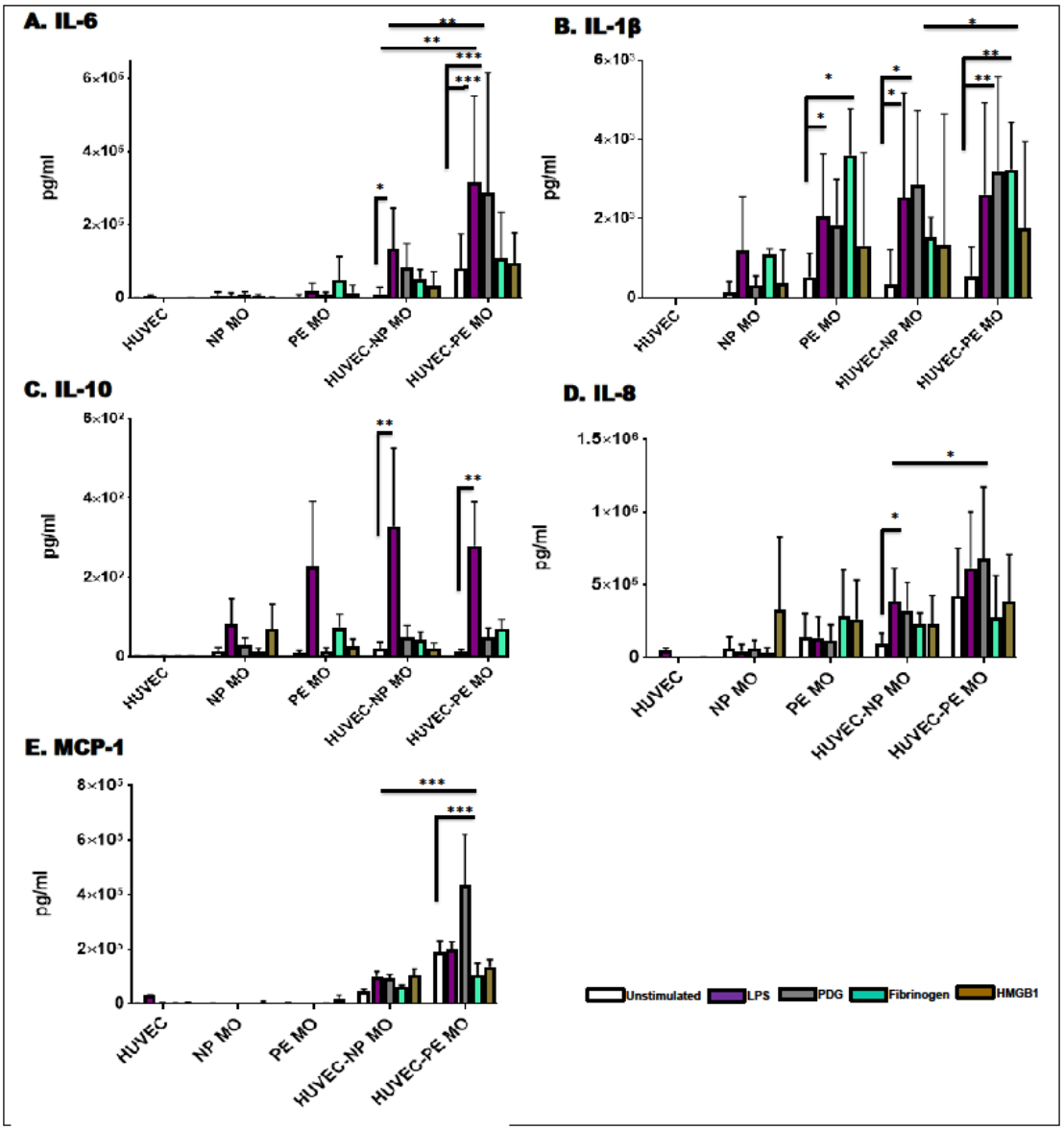
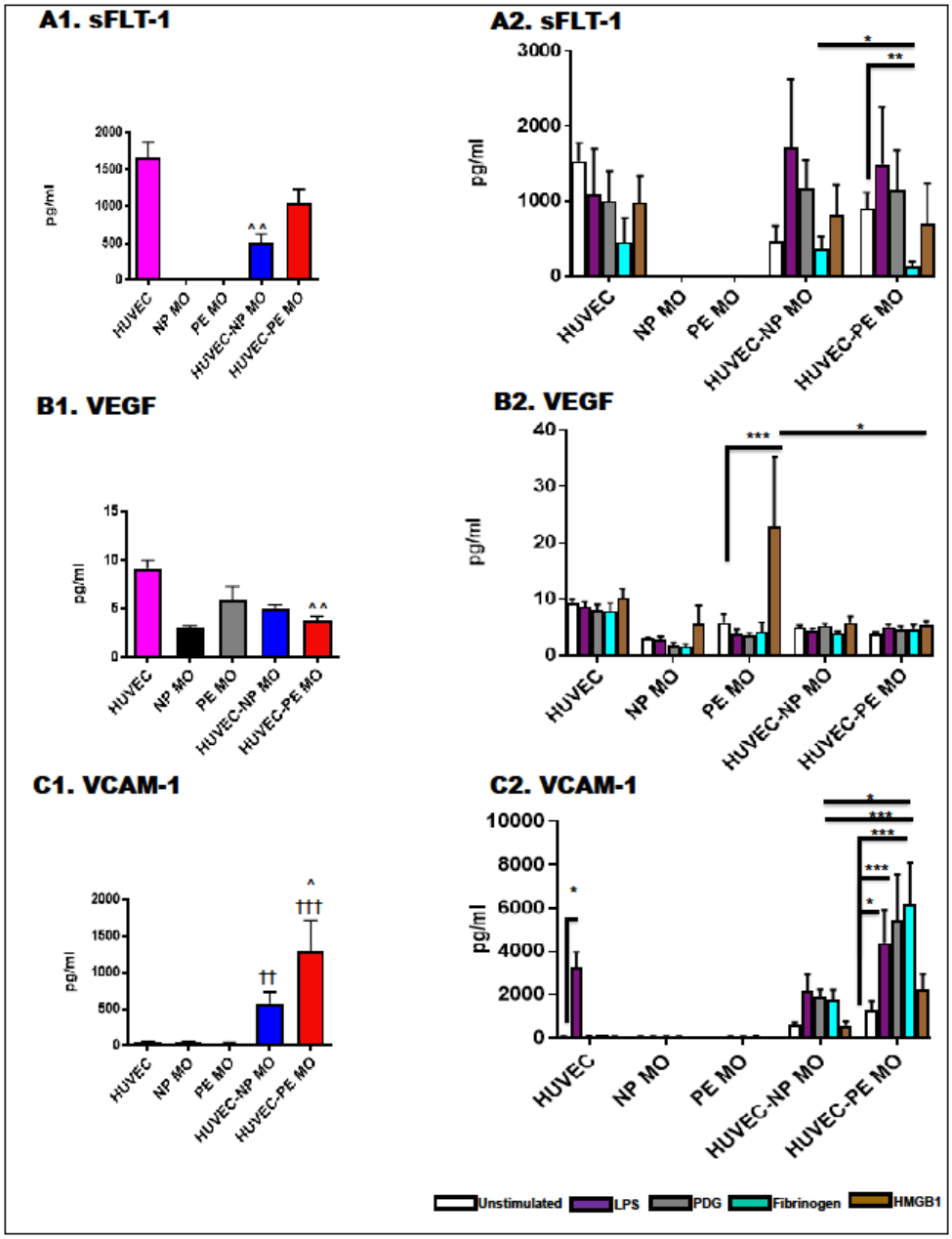


Fig 4. Fibrinogen attenuates sFLT-1-expression and enhanced vascular adhesion molecule expression in PE

Basal levels of **A1**. sFLT-1, **B1.** VEGF and **C1.** VCAM-1 productions by HUVEC monoculture, NP/PE monocytes monocultures, HUVEC-NP monocytes co-cultures or HUVEC-PE monocytes co-cultures. Graphic representation of **A2**. sFLT-1, **B2.** VEGF and **C2.** VCAM-1 levels by mono- or co-cultures in response to LPS, PDG, fibrinogen and HMGB1. Comparisons were made between NP and PE co-cultures versus HUVECs monocultures $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, and $^{\wedge\wedge\wedge}p < 0.001$; or versus monocytes monocultures $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$; unstimulated versus stimulated cultures; and NP versus PE mono- or co-cultures $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Values were illustrated as mean ± SEM. Statistical significance was determined by one-way ANOVA (Kruskal-Wallis test); or tow-way ANOVA (n=7-9).



1.7 Table 1 Subject 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+)	