

**Preeclampsia and increased permeability over the blood brain barrier - a role of vascular endothelial growth receptor 2.**

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## Abstract

### BACKGROUND

Cerebral complications in preeclampsia are leading causes of maternal mortality worldwide but the underlying pathophysiology is largely unknown and a challenge to study. Using an *in vitro* model of the human blood brain barrier (BBB), we explored the role of vascular endothelial growth factor receptor 2 (VEGFR2) in preeclampsia.

### METHODS

The human brain endothelial cell line (hCMEC/D3) cultured on Tranwells insert were exposed (12 h) to plasma from women with preeclampsia (n=28), normal pregnancy (n=28) and non-pregnant (n=16) controls. Transendothelial electrical resistance (TEER) and permeability to 70 kDa FITC-dextran were measured for assessment of BBB integrity. We explored possible underlying mechanisms, with focus on expression of tight junction proteins and phosphorylation of two tyrosine residues of VEGFR2, associated with vascular permeability and migration (pY951) and cell proliferation (pY1175). Plasma concentrations of soluble FMS like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) were measured in order to establish correlations with *in vitro* results.

### RESULTS

hCMEC/D3 exposed to plasma from women with preeclampsia exhibited reduced TEER and increased permeability to 70 kDa FITC-dextran. Further, these cells up-regulated the mRNA levels of VEGFR2, as well as pY951-VEGFR2; but reduced pY1175-VEGFR2 ( $p < 0.05$  in all cases). No difference in mRNA expression of tight junction protein was observed between groups. There was no correlation between angiogenic biomarkers and BBB permeability.

## CONCLUSION

We present a promising *in vitro* model of the BBB in preeclampsia. Selective tyrosine phosphorylation of VEGFR2 may participate in the increased BBB permeability in preeclampsia irrespective of plasma concentrations of angiogenic biomarkers.

**Key words:** Blood brain barrier; eclampsia; *in vitro* studies; preeclampsia; PlGF; sFlt-1; VEGFR2

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## Introduction

Preeclampsia is disorder that affects 2-8% of pregnancies and is characterized by hypertension and multi-organ impairment after 20 weeks of gestation <sup>1</sup>. Cerebral complications represent 40 % of preeclampsia- and eclampsia related deaths <sup>2, 3</sup> and include eclampsia, cerebral edema, increased intracranial pressure and stroke <sup>4</sup>. Regarding eclampsia, 90% of cases occur after 28 weeks of gestation <sup>5</sup> and a majority after 32 weeks of gestation <sup>6</sup>.

The pathophysiology of preeclampsia involves different pathways. Impaired placental invasion of the maternal vascular bed with potential release of harmful molecules <sup>7, 8</sup>, preexisting maternal endothelial dysfunction <sup>9</sup> and increased demand from the pregnancy itself such as multi-fetal pregnancies <sup>10</sup> have all been explored as underlying contributors to the endothelial injury and subsequent organ impairment observed in preeclampsia. The pathophysiology of eclampsia remains unknown, but evidence from animal studies suggests involvement of brain endothelial dysfunction that generate intracerebral edema, increased intracerebral inflammation and intracerebral hypoxia <sup>11, 12</sup>, which may be caused by, or lead to increased permeability of the blood brain barrier (BBB). There are conflicting evidence about the pathophysiology of BBB dysfunction in preeclampsia, and this issue might be related to the relevance of the animal models employed <sup>13-15</sup> and lack of data collected from studies in human. In addition, studies of the BBB in pregnant women are ethically questionable and technically difficult to set up; or are limited in the characterization of events occurring at a cellular level. To overcome this issue, cell-based models are an useful alternative to study events at a cellular level, but the availability of fresh human tissue for isolation of brain endothelial cells is scarce. Immortalized cell lines such as the human brain microvascular endothelial cell line hCMEC/D3 derived from healthy brain tissue of an adult epileptic woman <sup>16, 17</sup> may constitute a reliable alternative to explore underlying mechanisms, including the involvement of tight junction proteins and vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) <sup>18</sup>.

VEGF binds and activates the tyrosine kinase receptors VEGF receptor type 1 (VEGFR1), type 2 (VEGFR2) and type 3 (VEGFR3). In addition, VEGF binds to the VEGFR1 decoy receptor (or sFlt-1), a soluble splice variant of VEGFR1 lacking the intracellular tyrosine kinase domain. Women with preeclampsia demonstrate increased maternal plasma concentrations of sFlt-1<sup>19</sup> which, by sequestering VEGF, could act as a physiological inhibitor of VEGFRs and potentially decrease BBB permeability. Conversely, plasma from women with preeclampsia added to rodent brain blood vessels *ex vivo* activated VEGFR2<sup>20</sup> but no further characterization of VEGFR2 activation was done.

VEGFR2 is predominately expressed in vascular endothelial cells, including brain endothelial cells, where activation of VEGFR2, but not VEGFR1, leads to increased permeability of the BBB<sup>21</sup>. At least eleven major autophosphorylation sites on VEGFR2 have been described, which can activate different intracellular signaling pathways<sup>22</sup>. Among these sites, phosphorylation of the tyrosine 951 residue (pY951-VEGFR2) has been reported to regulate endothelium permeability and cell migration<sup>23, 24</sup>; while phosphorylation of Y1175 (pY1175-VEGFR2) is associated to regulation of cell proliferation<sup>22</sup>.

Therefore, using hCMEC/D3 as promising *in vitro* BBB model, we investigated the potential underlying causes of impaired preeclampsia-induced permeability of BBB analyzing VEGFR2 phosphorylation.

## Methods

### *Study population*

Detailed information about the participants can be found in earlier published work<sup>25</sup>. Briefly, women with preeclampsia (n=28) diagnosed according to the recommendations of the International Society for Studies on Hypertension in Pregnancy (ISSHP)<sup>26</sup> were recruited at Uppsala University Hospital, Sweden, during 2013-2016<sup>25</sup>. Within the preeclampsia group, 15 had late-onset

preeclampsia and 13 had early-onset preeclampsia (diagnosed  $\geq 34$  and  $< 34$  weeks of gestation respectively). Preeclampsia with severe features was defined according to the definition of ISSHP <sup>26</sup>.

Two control groups were recruited; normal pregnant women (n=28), matched for gestational length at recruitment; and non-pregnant women (n=16).

Uppsala Ethical Review Board approved the study and written informed consent was obtained from all participants.

#### *Laboratory analyses*

All analyses were carried out in duplicates by researchers blinded to groups.

#### Plasma concentration of sFlt-1 and PIGF

Li-Heparin plasma samples were analyzed for sFlt-1 and placental growth factor (PIGF) using commercially available enzyme-linked immunosorbent assay (ELISA) kits (DY321B and DY264, R&D Systems, Minneapolis, MN, USA). Quantifications were performed according to the manufacturer's instructions. The inter-assay coefficient of variation was 6%. The lower limit of detection for PIGF was 50 ng/L and for sFlt-1 100 ng/L.

#### Culturing of hCMEC / D3 cell line

For the *in vitro* experiments, monolayers of the female human brain endothelial cell line hCMEC/D3 (Merck Millipore, Darmstadt, Germany) were used. Cells were seeded on plates coated with rat-tail type I collagen (Discovery Labware, Bedford, MA, USA) and incubated at 37°C, 5% CO<sub>2</sub>, using Medium EndoGro™ MV Supplement Kit (Merck Millipore) as culturing medium. Prior to experiments

(6 hours), culture medium was replaced with medium without growth supplements. Cultured hCMEC/D3 cells were treated with plasma (1:10 v/v; 12 h) from the three groups.

#### Measurement of the transendothelial electrical resistance (TEER) and permeability to FITC-dextran 70 kDa

Cells were cultured in semipermeable polycarbonate (8  $\mu\text{m}$  pore size, 0.33  $\text{cm}^2$  area) 24-well Transwell inserts (Sigma-Aldrich, St Louis, MO, USA) previously coated with rat tail type I collagen, at a density of 20000 cells/well. TEER measurements were performed with an Epithelial Volt/Ohm meter (EVOM2, World Precision Instruments, Sarasota, FL, USA). Transwells with cells demonstrating basal electrical resistance below 20  $\Omega\text{cm}^2$  were excluded prior to any experimental treatment.

In order to estimate the effect of plasma on BBB permeability, we calculated the arithmetic difference of TEER values prior and after plasma exposure, resulting in a  $\Delta\text{TEER}$  value, expressed as decrease from baseline.

For permeability experiments, cells were incubated (1 hour) with 1  $\mu\text{M}$  FITC-dextran 70 kDa solution, which was added into the apical chambers. The medium in the basolateral compartment was collected in ambert Eppendorf tubes and stored at  $-20^\circ\text{C}$ . The fluorescence in the collected media was measured using a Synergy 2 multiplate reader setup with filters at 485 nm and 540 nm (BioTek Instruments, Winnoski, VT, USA). For calculations, the background values (obtained from transwells without any cells or collagen coating) were subtracted from every measurement.

### Quantitative PCR for measuring mRNA levels of VEGF, VEGFR2 and tight junction proteins

After exposure to plasma as described above, the mRNA levels of VEGF, VEGFR2, and the tight junction proteins occludin and zonula occludens 1 (ZO-1) were quantified in hCMEC/D3 by quantitative PCR (QPCR) as previously described<sup>27</sup> and according to the manufacturer's instructions.

Specific primers for VEGF and VEGFR2<sup>28</sup>, as well as occludin, ZO-1,  $\beta$ -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are described in Supplemental Table 1. Product specificity was confirmed by agarose gel electrophoresis (2% v/v) and melting curve analysis. Quantification of gene expression was performed following the delta-delta CT method<sup>29</sup>.

### Phosphorylation of VEGFR2

Protein phosphorylation was analyzed using dot-blot and Western blot. For dot-blot analysis, hCMEC/D3 cells were treated with randomly selected plasmas (n=6 per subgroup) (1:10 v/v; 30 min) after that, cells were used for protein extraction using lysis buffer (Tris HCL, pH 8, 20 mM; NaCl 137 mM; EDTA 2 mM; glycerol 10%, Nonidet P-40 1%) and protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA) and centrifuged at 14,000  $\times$  g at 4°C for 10 minutes. Proteins (20  $\mu$ g) were directly transferred into nitrocellulose membranes (BioRad Laboratories, Hertfordshire, UK) and probed with primary anti-VEGFR2 (Cell Signaling Technology, Danvers, MA, USA; #2479, dilution 1:1000 v/v), and anti-pY951-VEGFR2 (Cell Signaling Technology; #4991, dilution 1:1000 v/v). Membranes were rinsed in Tris buffer saline Tween (TBS/T), and incubated (1 h) in TBS-T/0.2% BSA containing respective horseradish peroxidase-conjugated antibody. Proteins were detected by enhanced chemiluminescence and quantified by densitometry.

Subsequently, in the next set of experiments, hCMEC/D3 cells were treated with plasma from late-onset preeclampsia, late-normal pregnancy and non-pregnant women (1:10 v/v) in a time course analysis (0 - 24 h). A minimal of four randomly selected plasmas per group were used. After protein extraction described above, proteins (70 µg) were separated by SDS-PAGE (10%), transferred to nitrocellulose membranes, and probed with the same primary antibodies used for dot-blot analysis (i.e., anti-VEGFR2 and pY951-VEGFR2), as well as for anti-pY1175-VEGFR2 (Cell Signaling Technology; #2478, dilution 1:1000 v/v) in order to perform Western blot assays as previously validated in our laboratory <sup>28, 30</sup>. Bands on gels were scanned and images quantified by ImageJ 1.48 software (National Institute of Health, Bethesda, MD, USA). Red-Ponceau staining was used as loading control.

#### *Statistical analyses*

Background characteristics are presented as mean ± standard deviation (SD) and percentage as appropriate and compared between groups by student's t-test or Chi square test.

For the BBB permeability analyses, values are presented as medians with interquartile range (IQR) and differences between groups were compared by non-parametric analysis and in case of statistical significance, Bonferroni post hoc test was used. Associations between TEER values and expression of the angiogenic biomarkers sFlt-1 and PlGF were analyzed using a generalized additive model (GAM) <sup>31</sup> allowing for different, possibly non-linear, associations in the three groups. The model was further adjusted for baseline TEER, BMI, parity and maternal age. Data and statistical analyses were performed using SPSS version 25, GraphPad Prism 6.00 (GraphPad Software, CA, USA) and R version 3.6.1 using the add-on package mgcv.

## Results

### *Population characteristics*

Clinical characteristics of the population are described in Table 1. Women with preeclampsia had a higher body mass index (BMI) and were more often nulliparous compared to women with normal pregnancy and non-pregnant women. In the group of women with preeclampsia, most women had antihypertensive treatment (79%) and at recruitment, 10 women had preeclampsia with severe features (Table 2) but none of these experienced neurological complications and none had cerebral edema on magnetic resonance imaging (previously published data)<sup>25</sup>. Women with preeclampsia experienced increased plasma concentrations of sFlt-1 and decreased concentrations of PlGF compared to both control groups (Table 2).

### *Effects of plasma from preeclamptic women on BBB permeability*

The effects of plasma from women with preeclampsia on the BBB were measured by TEER and permeability to FITC-dextran (Figure 1).

Basal TEER prior to plasma treatment was not statistically different among cell monolayers that were exposed to plasma from non-pregnant (median 31.1, IQR 27.9 to 34.8  $\Omega\cdot\text{cm}^2$ ), pregnant (median 32.0, IQR 28.7 to 36.9  $\Omega\cdot\text{cm}^2$ ), or from women with preeclampsia (median 33.5, IQR 31.3 to 36.9  $\Omega\cdot\text{cm}^2$ ). A larger reduction in TEER (i.e., a larger  $\Delta\text{TEER}$ ) was seen in cell monolayers exposed to plasma from women with preeclampsia compared to those exposed to plasma from control groups (Figure 1A). In addition, a larger  $\Delta\text{TEER}$  was found in cells exposed to plasma from late-onset preeclampsia compared to both late normal pregnancy and early-onset preeclampsia (Figure 1B).

Cells treated with plasma from women with preeclampsia demonstrated increased permeability to 70 kDa FITC-dextran compared to those treated with plasma from women with normal pregnancies

(Figure 1C). In cell exposed to plasma from late-onset preeclampsia, permeability to FITC-dextran was greater than in those exposed to plasma from late normal pregnancy or early-onset preeclampsia (Figure 1D).

*Underlying mechanisms to BBB injury: VEGF, VEGFR2 and tight junction proteins.*

Cells exposed to plasma from women with preeclampsia expressed higher mRNA levels of VEGFR2 compared to cells exposed to plasma from normal pregnancy (Figure 2A). In addition, cells exposed to plasma from women with late-onset preeclampsia expressed higher mRNA levels of VEGFR2 than those exposed to plasma from late normal pregnancy or to plasma from early-onset preeclampsia (Figure 2B).

In dot-blot analysis, there were no significant changes after short exposure (30 min) to plasma from any of the groups or subgroups of plasmas (Figure 3A-C). In the time course analysis carried out by Western blot, pY951-VEGFR2 levels were significantly higher in cells exposed to plasma from preeclampsia compared to non-pregnant or normal pregnancy at 6 hours and 24 hours after exposure (Figure 3D-F). In addition, higher pY951-VEGFR2 levels were found in preeclampsia compared to non-pregnant at 30 min.

In contrast, pY1175-VEGFR2 expression was significantly lower in cells exposed to plasma from normal pregnancy and preeclampsia compared to those exposed to plasma from non-pregnant women at 24 hours exposure.

There were no differences in the mRNA levels of VEGF (Supplemental Figure S1) or tight junction (Supplemental Figure S2) in cells exposed to plasma among the studied groups.

### *Correlation of plasma biomarkers with in vitro results*

There were no correlations between sFlt-1 or PlGF and  $\Delta$ TEER, permeability to FITC-dextran, VEGFR2 or tight junction protein expression for any of the three groups.  $\Delta$ TEER differed 1.52 (95% CI: -0.90 ; 3.94) for preeclampsia, 0.03 (-0.02 ; 0.08) for non-pregnancy and 0.18 (-0.11 ; 0.47) for normal pregnancy when comparing the group specific outer quartiles (i.e. the 75<sup>th</sup> and 25<sup>th</sup> percentiles of the distribution within each group) of sFlt-1 while holding the rest of the adjustment variables at their median or most frequent categories. Similar estimates for PlGF were -0.03 (-0.10 ; 0.04) for preeclampsia, -0.01 (-0.03 ; 0.014) for non-pregnant and -0.22 (-0.70; 0.25) for normal pregnancy (Figure 4).

## **Discussion**

### *Main findings*

Using a human brain endothelial cell line (hCMEC/D3), we confirm earlier findings from animal studies that plasma from preeclampsia impairs the BBB, an effect associated with increased activation of VEGFR2. We further extend these results suggesting that selective activation of VEGFR2 in the tyrosine 951, a specific residue linked with enhanced endothelial permeability<sup>23, 24</sup> could be involved in the pathophysiological pathway without alterations in mRNA levels of key proteins such as tight junctions and VEGF.

### *Interpretation*

Previously, it has been shown that serum from women with preeclampsia increases the permeability of human umbilical vein endothelial cells (HUVEC)<sup>32, 33</sup> and cerebral rat veins<sup>20</sup>. But, to our knowledge, this is the first time where plasma from women with preeclampsia has been

demonstrated to increase permeability of an *in vitro* BBB model employing human brain endothelial cells.

We showed that in late-onset preeclampsia, the disruption of the *in vitro* BBB was more pronounced. This finding is in line with eclampsia being more commonly reported in late pregnancy<sup>34</sup>. However, our findings contrast with those presented by Schreurs *et al.*, in which plasma from early-onset preeclampsia had a more pronounced effect on BBB permeability in rat cerebral veins<sup>35</sup>. This could imply that the cerebral response in rodents is not similar to the response in humans.

The increased BBB permeability is more likely due to activation of the VEGFR2 rather than VEGFR1<sup>21</sup>. A previous study showed that plasma from women with preeclampsia increased the permeability of rat cerebral veins *ex vivo* and that this effect was abrogated after inhibition of VEGFR2<sup>20</sup>. In our study, we confirmed these findings in a human model. Firstly, we found a significant increase in the mRNA expression of VEGFR2. We further showed that in response to plasma from women with preeclampsia, phosphorylation at pY951-VEGFR2 was increased in brain endothelial cells, which corresponds to increased endothelial permeability<sup>24</sup>.

We also analyzed the phosphorylation of the Y1175 residue of VEGFR2 that is related to VEGF-stimulated proliferation of endothelial cells<sup>36</sup>. This particular phosphorylation site was reduced in cells exposed to plasma from preeclampsia and normal pregnancy compared to non-pregnant women, in line with previous findings from peripheral endothelial cells<sup>37</sup>. Thus, a reduction in pY1175 of VEGFR2 may indicate impaired cell proliferation, which could also compromise BBB integrity.

In preeclampsia, increased plasma concentrations of sFlt-1 (the decoy receptor of VEGF) and reduced concentrations of PlGF (a member of the VEGF family) are a well established hallmark of this condition<sup>38</sup>. In the brain circulation, sFlt-1 would reduce BBB permeability, while PlGF exert the opposite effect by inhibition or activation of VEGFRs, respectively<sup>38</sup>. However, we did not find any

direct correlation between plasma concentrations of these angiogenic biomarkers and BBB permeability, then we encourage further molecular analysis aiming to understand the complexity of VEGF-VEGFRs signalling on BBB integrity in the context of preeclampsia.

Tight junctions contribute to the unique properties of the tightly sealed BBB, but animal studies show conflicting results regarding the involvement of these proteins on the augmented BBB permeability seen in those animal models of preeclampsia <sup>13, 14, 39</sup>. In our study, mRNA levels of the tight junction proteins were not affected, but changes in their localization morphology or function cannot be discarded. This issue warrants further research.

#### *Strengths and limitations*

Previous animal studies have reported impaired BBB in preeclampsia but this is the first study investigating the effect of preeclampsia on human brain endothelial cells. In addition, mechanistic questions were explored regarding participation of key proteins including VEGF and tight junctions; as well as specific analyses of activation sites of VEGFR2. Also, we correlated *in vitro* experiments with maternal circulating angiogenic biomarkers that have an established role in progress and severity of preeclampsia.

The brain endothelial cell line used in this study does not originate from a woman with preeclampsia, but despite that limitation, brain endothelial dysfunction occurred upon exposure of these cells to plasma from women with preeclampsia. This outcome points towards a direct effect exerted by potentially harmful endogenous molecules present in plasma in preeclampsia <sup>40</sup>.

Primary cell cultures might display a superior phenotype compared to an endothelial cell line, but use of primary cell cultures from human brain tissue have several ethical concerns and limitations, thus a cell line provides a feasible alternative.

## *Perspectives*

The BBB model employed for this work is simple, reproducible, mimics the human BBB <sup>41, 42</sup> and could be used for further characterization of the mechanisms by which preeclampsia alter the functionality of brain endothelial cells. Additional questions need to be answered regarding underlying mechanisms of VEGFR2 activation in preeclampsia. These include the role of selective intracellular pathways driven by this receptor. The high plasma concentrations of sFlt-1 in preeclampsia, associated with endothelial dysfunction <sup>19</sup> might in fact be protective for the BBB. This hypothesis is supported by our data showing increased activation of VEGFR2 in preeclampsia and by a previous study showing that inhibition of VEGFR2 (which is the effect of sFlt-1) revert the enhanced brain vessels permeability <sup>20</sup>. Despite that, we encourage future studies in this regards. Further, PlGF is a key modulator of brain vascular formation <sup>43</sup> and function <sup>44</sup> and needs to be investigated regarding its role in BBB permeability in preeclampsia. These questions are crucial since current therapeutics for cerebral complications in preeclampsia are limited and therefore there is a need to localize new drug targets for restoring BBB integrity in preeclampsia.

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## **Author contributions**

CE and LB conceptualized the study and conducted the statistical analyses. CE, LB and AKW wrote the manuscript. JA, JL, AL, EL, CA, and PT-V performed the experiments. TF, MN, JW, AKW, and PT-V edited the manuscript. All co-authors approved the final version of this manuscript.

## **Disclosure**

The authors do not have any conflict of interest to declare

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## Table legends

### Table 1. Clinical characteristics of study population

BMI. body mass index; n.s. non-significant. <sup>a</sup>Early pregnancy BMI for the pregnant study groups. <sup>b</sup>Blood pressure measurement missing for one woman in the normal pregnant group. Values are presented as mean  $\pm$  SD and medians with IQR (sFlt-1 and PlGF).

### Table 2. Characteristics of the preeclampsia group

BMI. body mass index; n.s. non-significant. <sup>a</sup>Early pregnancy BMI (body mass index) for the pregnant study group. <sup>b</sup>According to the guidelines from the International Society for the study of Hypertension in Pregnancy (ISSHP)

## Figure legends

**Figure 1. Reduction in (A-B) transendothelial electrical resistance ( $\Delta$ TEER) and (C-D) enhanced permeability to dextran (fluorescence units).** Human brain endothelial cells (hCMEC/D3) were exposed (1/10 v/v; 24 h) to plasma from women with preeclampsia (PE), normal pregnancy (NP) and non-pregnant women (Non-P). **A)** Non-P women (4.0; 1.4-5.3); NP (5.2; 2.6-8.2) and PE (8.2; 5.8-10.2). **B)** Non-P (4.0; 1.4-5.3), Early Normal Pregnancy (ENP) (7.6; 4.3-8.6), Late Normal Pregnancy (LNP) (4.2; 1.1-6.5), Early-Onset Preeclampsia (EOPE) (6.7; 5.5-8.7) and Late-Onset Preeclampsia (LOPE) (10.1; 7.8-10.6). **C)** Non-P (2.7; 1.2-4.2), NP (1.2; 0.6-1.9) and PE (3.4; 1.5-10.0). **D)** Non-P (2.7; 1.2-4.2), ENP (1.8; 0.5-6.9), LNP (0.9; 0.6-1.4), EOPE (2.9; 1.2-3.2) and LOPE (10.0; 5.4-16.3). Values are represented by medians with interquartile range (IQR). Comparisons between groups by Mann Whitney u-test and Bonferroni correction. \*P<0.0001; \*\*P<0.001; †P<0.01; ‡P<0.05 versus respective comparison.

**Figure 2. mRNA levels of VEGFR2 in hCMEC/D3.** Cells were exposed (1/10 v/v; 24 h) to plasma from women with preeclampsia (PE), normal pregnancy (NP) and non-pregnant women (Non-P) and used for quantitative PCR for VEGFR2 (see Methods). **A)** Non-P (1.0; 0.5-1.9), NP (0.5; 0.1-1.0) and PE (1.0; 0.5-1.6). **B)** Non-P (1.0; 0.5-1.9), Early Normal Pregnancy (ENP) (0.3; 0.0-1.0), Late Normal Pregnancy (LNP) (0.7; 0.5-1.0), Early-Onset Preeclampsia (EOPE) (0.8; 0.4-1.0) and Late-Onset Preeclampsia (LOPE) (1.6; 0.7-2.8). Comparisons between groups by Mann Whitney u-test and Bonferroni correction. \*P<0.05 versus respective comparison.

**Figure 3. Phosphorylation of VEGFR2 in hCMEC/D3.** **A)** Representative images of dot-blot for total (unphosphorylated) and tyrosine 951 (pY951-VEGFR2) phosphorylated VEGFR2 in cell exposed (30 min, 1/10 v/v) of random plasmas from non-pregnant, normal pregnancy or preeclamptic women (n=6 per subgroup). Protein extraction of human placentas from normal pregnancy was used as a positive control (C+). Numbers below images represent cell extractions exposed to plasma from late-onset (1) and early-onset preeclampsia (2); as well as late normal pregnancy (3), early pregnancy (4) and non-pregnant women (5). **B)** Densitometry of pY951-VEGFR2/VEGFR2 in each experimental group, or **C)** subgroup analysis. Ponceau staining is used as a loading control. **D)** Representative image of Western blot analysis of phosphorylation of tyrosine 951 (pY951-VEGFR2) and tyrosine 1175 (pY1175-VEGFR2) in cell exposed (0-24 h, 1/10 v/v) of random plasmas from non-pregnant, normal pregnancy or preeclamptic women (n=4 per group). **E)** Densitometry of pY951-VEGFR2 and **E)** pY951-VEGFR2 in cell treated as in C. Ponceau staining is used as a loading control. Values are represented by medians with interquartile range (IQR). Comparisons between groups by Mann Whitney u-test and Bonferroni correction. In E \*P<0.05 vs non-pregnant. †P<0.05 vs normal pregnancy.

**Figure 4. Correlation of TEER with PlGF and sFlt-1.** Associations between TEER values and expression of the angiogenic biomarkers PlGF and sFlt-1 analyzed by a generalized additive model stratified by group and adjusted for baseline TEER, BMI, parity and maternal age.

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**Table 1. Clinical characteristics of the study population**

Characteristics of the women	Preeclampsia (n=28)	Normal pregnant (n=28)	Non-pregnant (n=16)	ANOVA p-value
Age (years)	29.2 ± 5.6	32.1 ± 4.6	29.2 ± 6.4	n.s.
BMI <sup>a</sup> (kg/m <sup>2</sup> )	27.3 ± 5.3	24.4 ± 2.8	22.2 ± 2.6	<0.0001
Prior births, number (%)	5 (17.9 %)	18 (64.5 %)	6 (37.5 %)	<0.0001
<i>At examination</i>				
Gestational week	33.5 ± 4.8	33.1 ± 5.1		n.s.
Blood pressure (mm Hg)				
Systolic	148 ± 11	112 ± 8 <sup>b</sup>	112 ± 6	<0.0001
Diastolic	94 ± 12	68 ± 8 <sup>b</sup>	68 ± 7	<0.0001
Plasma- PIGF (pg/ml)	340 (273-477)	1428 (979-2386)	122 (63-174)	<0.0001
Plasma- sFlt-1 (pg/ml)	82044 (50024-118489)	12288 (7866-16116)	2160 (100-1533)	<0.0001
sFlt-1/PIGF ratio	218 (115.6-387.4)	7.1 (3.1-15.6)	9.2 (0.4-14.4)	<0.0001

Numbers presenting mean ± SD. BMI, body mass index; n.s. non-significant; PIGF, Placental growth factor; sFlt-1 soluble FMS like tyrosine kinase. <sup>a</sup>Early pregnancy BMI for the pregnant study group. <sup>b</sup>Blood pressure measurement missing for one woman in the normal pregnant group

**Table 2. Characteristics of the preeclampsia group**

Characteristics of the women	Early-onset	Late-onset	p-value
	Preeclampsia (n= 13)	Preeclampsia (n= 15)	
Onset of preeclampsia (weeks)	28.4 ± 3.4	37.0 ± 1.8	<0.0001
Age (years)	28.2 ± 5.2	30.1 ± 5.9	n.s.
BMI <sup>a</sup> (kg/m <sup>2</sup> )	28.5 ± 6.6	26.1 ± 3.7	n.s.
Blood pressure (mm Hg)			
Systolic	148 ± 11	147 ± 11	n.s.
Diastolic	91 ± 14	96 ± 9	n.s.
<i>At examination</i>			
Gestational weeks	29.1 ± 3.2	37.2 ± 1.7	<0.0001
Severe preeclampsia <sup>b</sup> , n (%)	7 (53.8 %)	3 (20 %)	n.s.
Antihypertensive medication, n (%)	11 (84.6 %)	11 (73.3 %)	n.s.
Plasma- PIGF (pg/ml)	308 (199-439)	568 (298-568)	
Plasma- sFlt-1 (pg/ml)	94379 (66141-156544)	51646 (44824- 115737)	
<i>At delivery</i>			
Gestational weeks	30.0 ± 3.3	37.9 ± 1.5	<0.0001
Severe preeclampsia <sup>b</sup> , n (%)	9 (69.2 %)	7 (46.7 %)	n.s.

Numbers presenting mean ± SD BMI, body mass index; n.s. non-significant. <sup>a</sup>Early pregnancy BMI (body mass index) for the pregnant study group. <sup>b</sup>According to the guidelines from the International Society for the study of Hypertension in Pregnancy (ISSHP).

Figure 1

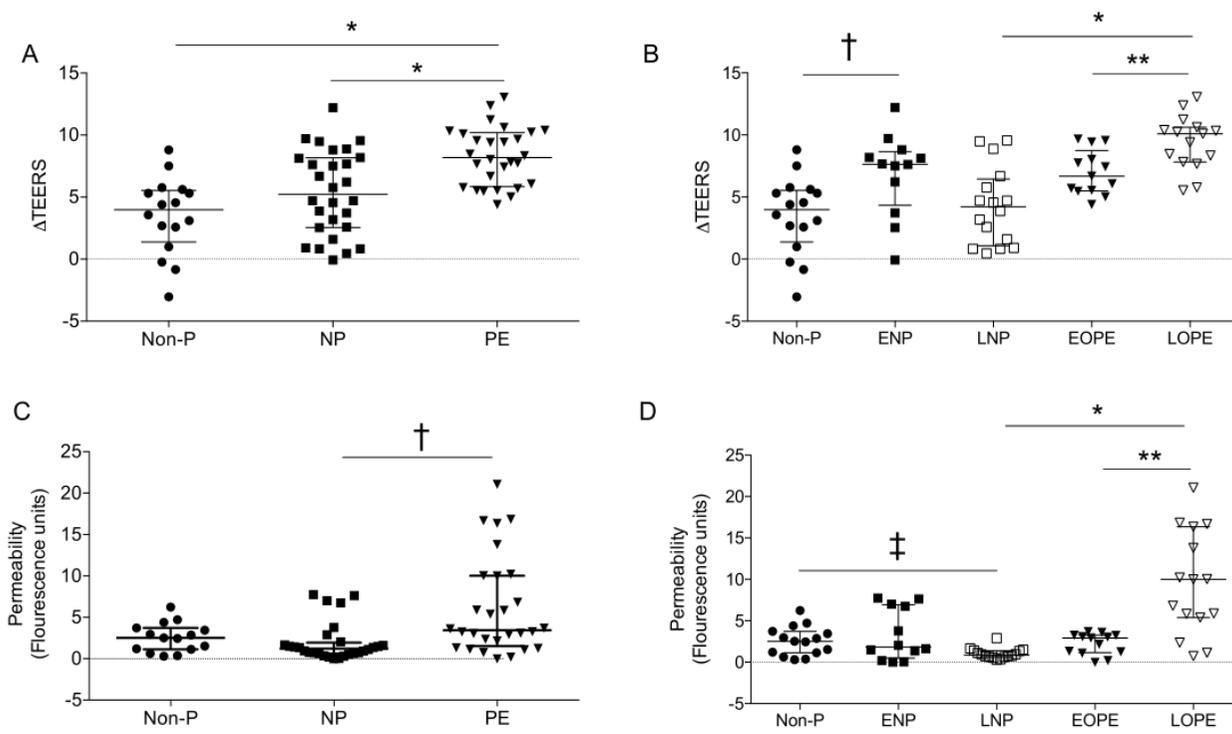


Figure 2

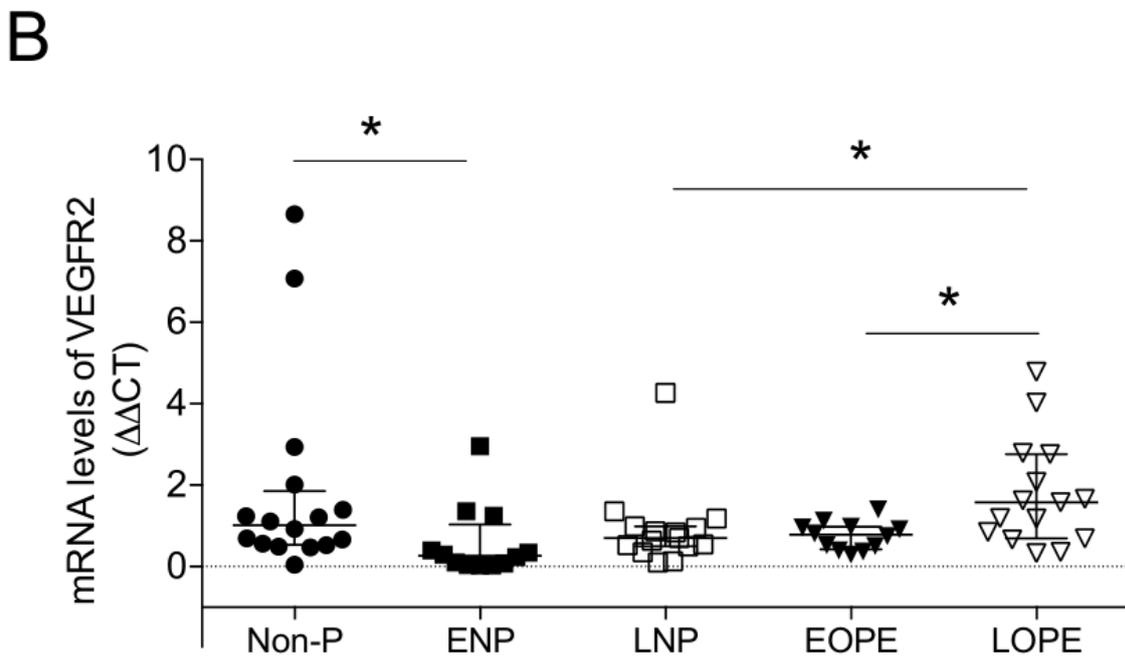
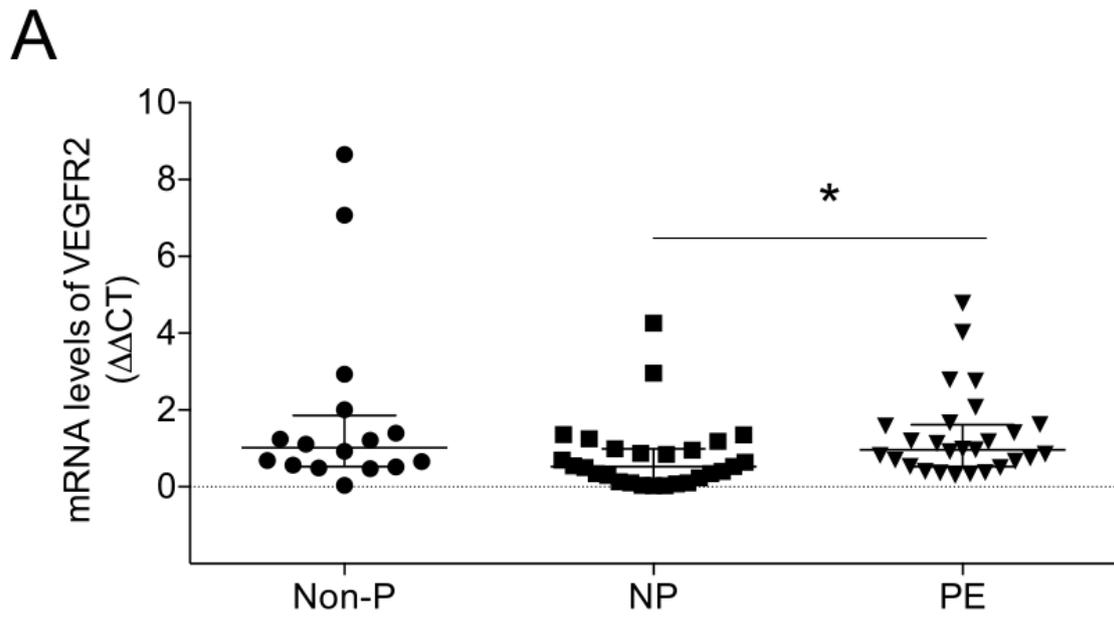


Figure 3

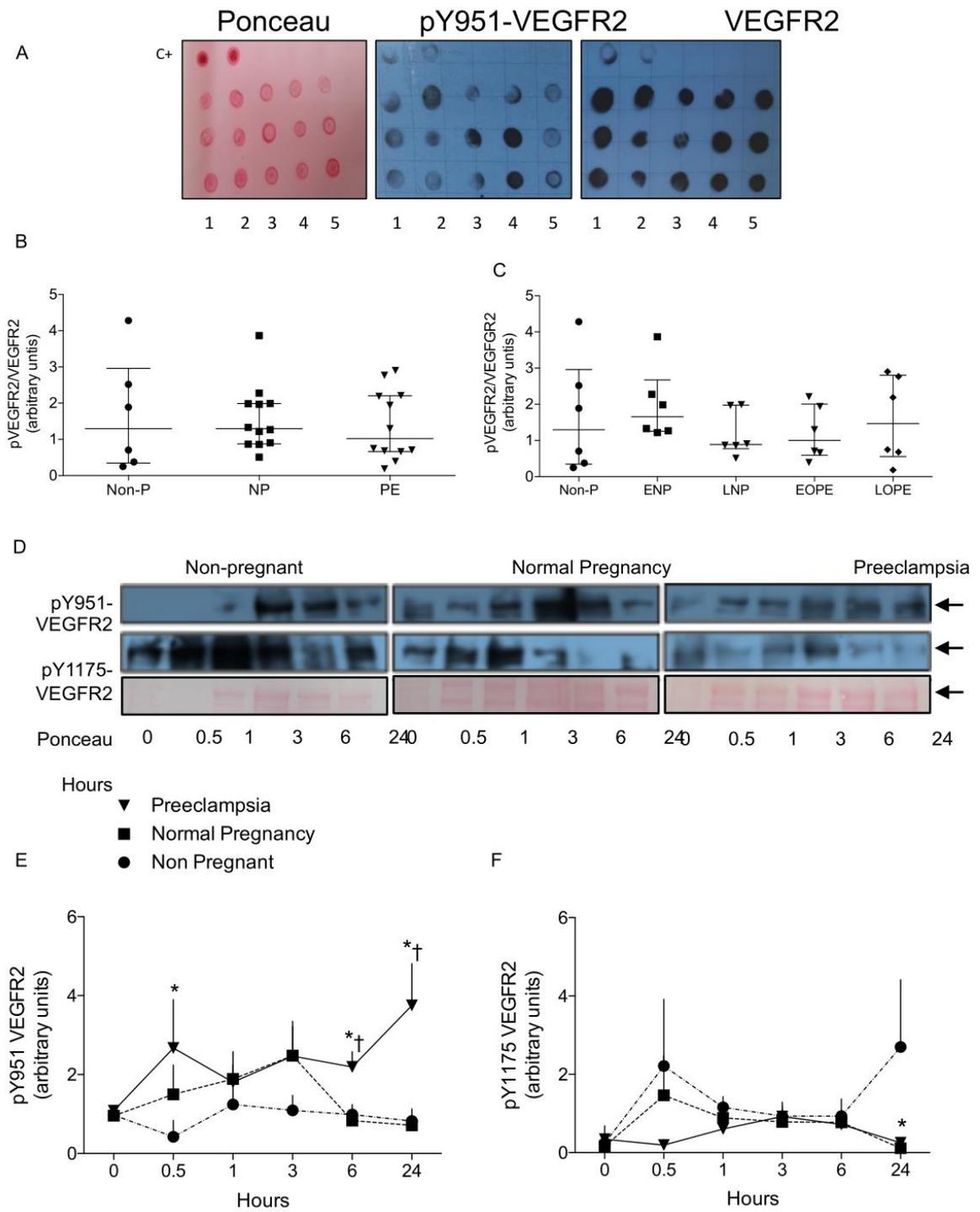
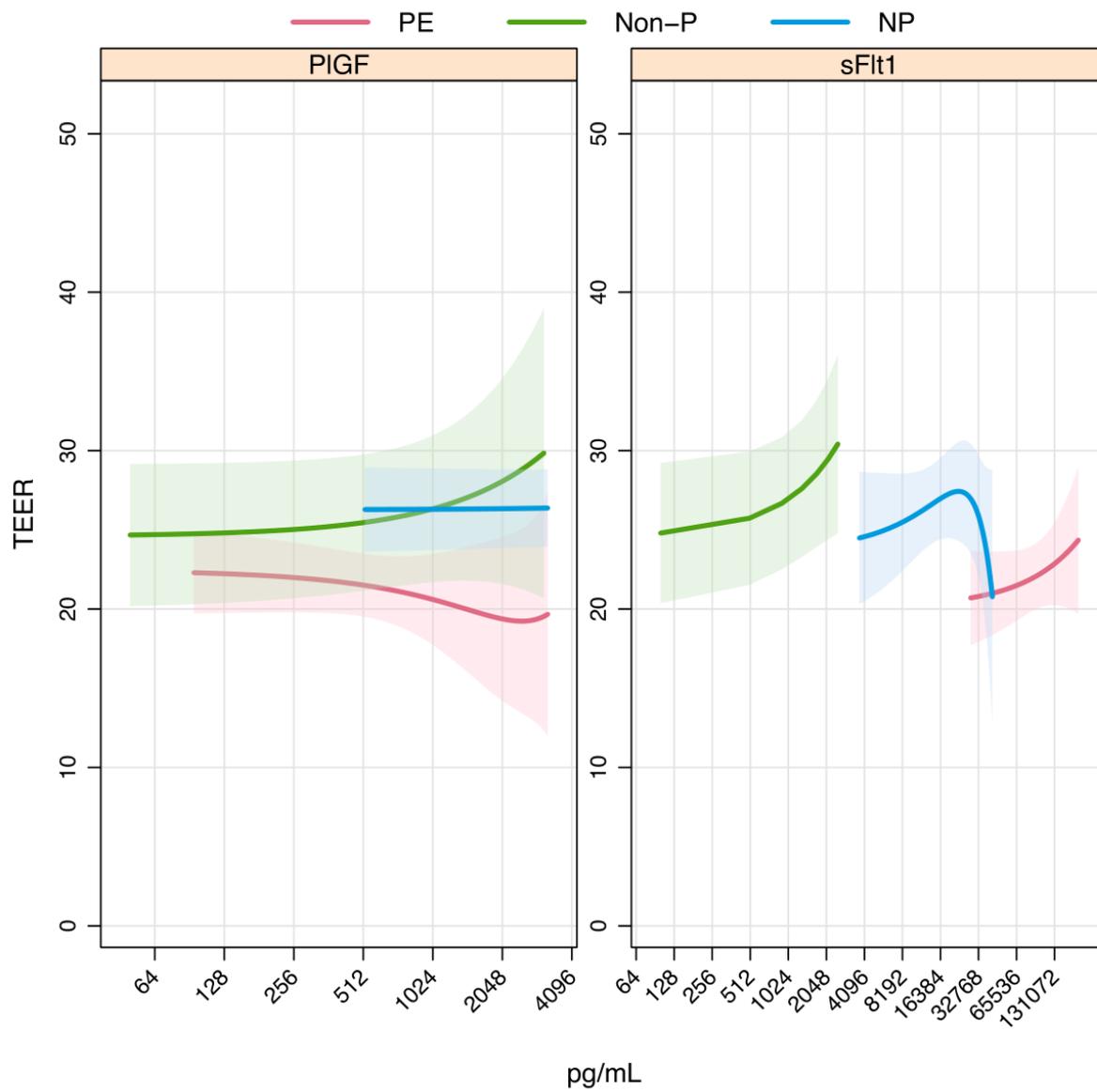


Figure 4



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