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# Increased placental expression of Placental Protein 5 (PP5) / Tissue Factor Pathway Inhibitor-2 (TFPI-2) in women with preeclampsia and HELLP syndrome: Relevance to impaired trophoblast invasion?

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ABSTRACT

*Introduction:* Placental Protein 5 (PP5)/Tissue Factor Pathway Inhibitor-2 (TFPI-2) is an extracellular matrix-associated protein mainly expressed by the syncytiotrophoblast that may regulate trophoblast invasion. Our aim was to study placental PP5/TFPI-2 expression and its relation to placental pathology in various forms of pre-eclampsia and HELLP syndrome.

*Methods:* Placental and maternal blood specimens were collected at the time of delivery from the same women in the following groups: 1) early controls; 2) early preeclampsia; 3) early preeclampsia with HELLP syndrome; 4) late controls; and 5) late preeclampsia. After histopathological examination, placental specimens were immunostained with polyclonal anti-PP5/TFPI-2 antibody on Western blot and tissue microarray immunohistochemistry. Placental PP5/TFPI-2 immunoscores were assessed manually and with a semi-automated method. Maternal sera were immunoassayed for PP5/TFPI-2.

*Results:* PP5/TFPI-2 was localized to the cytoplasm of syncytiotrophoblast. Manual and semi-automated PP5/TFPI-2 immunoscores were higher in early preeclampsia with or without HELLP syndrome but not in late preeclampsia than in respective controls. In patients with preeclampsia, the correlation of placental PP5/TFPI-2 expression with maternal vascular malperfusion score of the placenta was positive while it was negative with birthweight and placental weight. Maternal serum PP5/TFPI-2 concentration was higher in early preeclampsia and it tended to be higher in early preeclampsia with HELLP syndrome than in early controls.

*Discussion:* Our findings suggest that an increased placental PP5/TFPI-2 expression may be associated with abnormal placentation in early preeclampsia, with or without HELLP syndrome.

## 1. Introduction

Preeclampsia (PE) is one of the most severe obstetrical syndromes, leading cause of maternal and perinatal morbidity and mortality [1]. According to gestational age at the time of its clinical presentation, preeclampsia was subdivided into early and late subforms [2]. Early preeclampsia is more severe than late preeclampsia as it affects the placenta and the fetus, and is more often associated with HELLP (*He*molysis, *E*levated *L*iver enzymes, and *L*ow *P*latelet count) syndrome, intrauterine growth restriction (IUGR) or the delivery of a small-for-gestational age (SGA) neonate [1–4].

There are several mechanisms that contribute to the development of

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preeclampsia. In early preeclampsia, abnormal placentation and defective spiral artery remodeling by invasive trophoblasts is associated with placental lesions consistent with maternal vascular malperfusion (MVMP) and oxidative stress, ultimately leading to the release of syncytiotrophoblast debris, pro-inflammatory and anti-angiogenic molecules into the maternal circulation [5,6]. These will result in endothelial dysfunction and an exaggerated maternal systemic inflammatory response, which also includes leukocyte activation as well as complement- and thrombin generation [1,7–13]. Uteroplacental vascular insufficiency, placental lesions consistent with MVMP and increased shedding of syncytiotrophoblast microparticles are less characteristic of late preeclampsia [14,15]. Indeed, this late subform may be induced by stress factors distinct from placental ischemic stress [16]. For example, pro-inflammatory and metabolic conditions in various maternal diseases such as diabetes, kidney disease, autoimmune diseases may lead to the development of late preeclampsia [3,7,17]. Based on these observations, it has been proposed that early preeclampsia is principally a placental disease, while late preeclampsia is predominantly a maternal disease [9,18]. Although the complex pathophysiology and triggering conditions are still not fully understood, and the two forms may share elements of pathogenesis resulting in the underlying maternal vascular disease, it is evident that the placenta plays an important role in preeclampsia development. This is also substantiated by the fact that currently the only effective therapy of preeclampsia is the delivery of the placenta [1,3]. Therefore, there is an increased interest in the identification of placental factors involved in the pathogenesis of preeclampsia in order to develop new diagnostic and therapeutic tools.

Placental Protein 5 (PP5) was originally described as a placenta-specific protein when isolated from the human placenta and characterized by Hans Bohn and his colleagues [19]. Later it was recognized to be placenta specific based on BioGPS data [20]. PP5 was localized to the syncytiotrophoblast [21] as well as detected in maternal and umbilical blood, urine and amniotic fluid [22,23]. Subsequently, PP5 was found to be identical by its amino acid sequence to Tissue Factor Pathway Inhibitor-2 (TFPI-2) [24], a glycoprotein with serine proteinase inhibitor activity [25] abundantly produced by the syncytiotrophoblast [26]. TFPI-2 belongs to the Kunitz-type serine proteinase inhibitor (serpin) family, having three Kunitz-type inhibitor domains [27,28]. TFPI-2 is an extracellular matrix-associated protein also produced by various tumors, in which it regulates invasion [29,30].

Several studies examined tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in pregnancy complications [31,32]. Of importance, PP5/TFPI-2 has been investigated by a few studies focusing on the pathologic events in preeclampsia [21,31,33–37]. Although, there were some contradictory observations, the results of most of these studies may suggest that this serpin is related to the pathophysiology of preeclampsia. The early pathogenesis of preeclampsia includes impaired trophoblast invasion, which may be the result of altered balance of proteinases and their inhibitors at the maternal-fetal interface [38–40], also substantiated by a recent proteomic study [41]. However, their contribution to these pathologic events has not been fully elucidated in various subforms of preeclampsia.

Here, we aimed at revealing the changes in PP5/TFPI-2 quantities parallel in placental and maternal compartments in various subtypes of preeclampsia. Moreover, as no data have been published on the placental behavior of PP5/TFPI-2 in patients with HELLP syndrome, we aimed to investigate the placental expression and localization as well as maternal serum concentrations of PP5/TFPI-2 also in patients with this severe syndrome. Our results indeed indicate that the altered expression of PP5/TFPI-2 may be related to the placental pathogenesis of the early forms of preeclampsia and HELLP syndrome.

## 2. Materials and methods

### 2.1. Clinical samples and definitions

The study was approved by the Health Science Board of Hungary (TUKEB: 22–164/2007-1018EKU; 4834-0/2011-1018EKU). Samples were collected after written informed consent had been obtained. Specimens and data were stored anonymously.

A couple of first trimester placentas were collected prospectively at the Maternity Private Department, Semmelweis University (Budapest, Hungary). Pregnancies were dated according to ultrasound scans between 5 and 13 weeks of gestation (GW). Patients with twin gestation were excluded. Third trimester placental samples were obtained following Caesarean sections and maternal blood specimens were collected from the same women at delivery at the First Department of Obstetrics and Gynecology, Semmelweis University (Budapest, Hungary). According to ultrasound scans, pregnancies were dated within 8–12 GW. After exclusion of cases with multiple births, fetal congenital or chromosomal abnormalities, women were enrolled into

**Table 1**
Demographic and clinical characteristics of the placental study groups.

| Groups | Early control | Early preeclampsia | | Late control | Late preeclampsia |
|---|---|---|---|---|---|
| | | without HELLP sy. | with HELLP sy. | | |
| Number of cases[a] | 5 | 7 | 8 | 9 | 8 |
| Maternal age (years)[b] | 31.6 (31.1–34.3) | 34.0 (27.6–35) | 29.4 (27.1–30.1) | 30.8 (30.1–34.2) | 31.3 (26–34.2) |
| Maternal BMI (kg/m2)[b] | 23.4 (20.1–24.6) | 24.4 (23.4–25.2) | 24.7 (21.3–26.8) | 26.7 (23.1–28) | 21.9 (19.6–23.1) |
| Gestational age at delivery (weeks)[b] | 31.7 (31–34) | 32.6 (31.2–34.4) | 29.4 (28.4–32.3) | 38.9 (38.7–39.7) | 37.4 (36.8–38)$^{\#}$ |
| Systolic blood pressure (mmHg)[b] | 120 (120–133) | 160 (156–160)* | 165 (148–170)* | 130 (125–135) | 157 (151–168)$^{\#\#}$ |
| Diastolic blood pressure (mmHg)[b] | 80 (70–80) | 100 (100-100)* | 100 (98–110)* | 80 (78–85) | 95 (90–100)$^{\#\#}$ |
| Severe preeclampsia[c] | – | 57** | 75** | – | 37.5$^{\#}$ |
| Proteinuria[c] | 0 | 100** | 100** | 0 | 100$^{\#\#}$ |
| Birthweight (g)[b] | 1,990 (910-2,210) | 1,100 (1,010–1,280) | 965 (885-1,513) | 3,470 (3,400–4,030) | 2,955 (2,588–3,163)$^{\#\#}$ |
| Placental weight (g)[b] | 294 (290–301) | 217 (211–227)* | 185 (141–279)* | 518 (481–650) | 470 (431–486)$^{\#}$ |

All women were Caucasian.
$^{**}p < 0.01$ compared to gestational-age-matched early controls.
$^{*}p < 0.05$ compared to gestational-age-matched early controls.
$^{\#\#}p < 0.01$ compared to gestational-age-matched late controls.
$^{\#}p < 0.05$ compared to gestational-age-matched late controls.
[a] Values are presented as number.
[b] Values are presented as median (interquartile (IQR) range).
[c] Values are presented as percentage.

the following groups: 1) early controls (n = 5); 2) early preeclampsia (n = 7); 3) early preeclampsia with HELLP syndrome (n = 8); 4) late preeclampsia (n = 8) and 5) late controls (n = 9) (Table 1). Late controls delivered an appropriate-for-gestation-age (AGA) neonate without medical or obstetrical complications. Early controls had episode of preterm labor leading to preterm birth without clinical or histological signs of chorioamnionitis and delivered an AGA newborn [42]. Preeclampsia was defined by new onset hypertension (systolic/diastolic blood pressure ≥140/90 mmHg, respectively, measured at two timepoints, ≥4 h apart) and proteinuria (24-h urine protein of ≥300 mg, or one dipsctick measurement of ≥2) that developed after 20 GW [1,43]. Severe preeclampsia was defined according to Sibai et al. [1]. HELLP syndrome was characterized by hemolysis (serum LDH > 600IU/l; bilirubin > 1.2 mg/dl; presence of schistocytes in peripheral blood), elevated liver enzymes (serum ALT and/or AST > 70IU/l) and thrombocytopenia (platelet count < 100,000/mm$^3$) [4,44]. Severe clinical symptoms or previous Caesarean sections necessitated Caesarean section in all cases or controls, respectively.

### 2.2. Sample collections and histopathological examinations

Blood specimens were collected at the time of delivery. Sera were separated by centrifuging blood (1,300 × *g*, 10min, 4 °C), and then were stored at −70 °C. Placentas were examined immediately after delivery; 6–8 samples were taken including 4–5 full thickness tissue blocks from central and peripheral cotyledons, and an extra block from the maternal side of placenta. These blocks were either fixed in formalin and embedded in paraffin (FFPE) or deep frozen and stored at −80 °C. For microscopic examinations, 4 μm sections were cut from FFPE tissue blocks and mounted on SuperFrost/Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany). After deparaffinization and rehydration, slides were stained with hematoxylin&eosin (H&E). Placentas were histopathologically examined by a perinatal pathologist blinded to the clinical history, except for the gestational age, using standard perinatal pathological protocol and previously published diagnostic criteria [45–49]. Histopathological signs of MVMP were summarized and a composite MVMP score was generated for each placenta as described earlier [20].

### 2.3. Placental tissue preparations for protein determination

Total protein was extracted from snap frozen placental tissues. Villous tissue samples were quickly pulverized into a fine powder using liquid nitrogen and mortar, and were further homogenized on ice in 200 μl lysis buffer (10 mM Tris-HCl; pH = 7.5; 1% SDS; 2 mM sodium orthovanadate; 10 mM sodium fluoride; 0.5% protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO, USA). After brief sonication and incubation on ice (30min), homogenates were centrifuged (13,000 *g*, 20min, 4 °C). Supernatants containing soluble or solubilized cytoplasmic, membrane and nuclear proteins were collected, then equal amounts of protein samples were precipitated with absolute ethanol and then reconstituted with 0.1 N sodium hydroxide solution to measure protein concentrations with Bradford reagent using Ultrospec-2000 UV/VIS Spectrophotometer (Hoefer Pharmacia Biotech, Inc., San Francisco, CA, USA) [50]. Subsequently, samples were equalized for protein content before immunoblotting.

### 2.4. SDS-PAGE and Western blot

To verify the specificity of our anti-PP5/TFPI-2 antibody, PP5/TFPI-2 was determined from placental protein extracts on Western blot. Three samples from each study group were investigated on Western blot using equal protein amount from each sample. Total pooled protein (10 μg) from placental protein extracts was incubated with loading buffer containing β-mercaptoethanol (95 °C, 5min). Denatured samples were run on 10% polyacrylamide gels on Mini Protean electrophoresis equipment (Bio-Rad, Hercules, CA, USA). Proteins were electroblotted (16 h, 75 mA, 4 °C) onto PVDF membranes (Millipore, Billerica, MA, USA), and the efficiency of protein transfer was determined with Ponceau staining. The endogenous biotin and avidin were blocked (15-15min, room temperature - RT) with the avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA USA). Non-specific binding was blocked (1 h, RT) with TBS containing 0.05% Tween 20, 5% non-fat dry milk (Bio-Rad), 1% BSA (A2153, Sigma-Aldrich) and goat serum (S-1000, Vector Laboratories, 1:1,500) followed by the incubation with rabbit polyclonal anti-PP5/TFPI-2 (1:1,000; kind gift from Dr. Hans Bohn, Behringwerke AG, Marburg-Lahn, Germany [19]) or mouse monoclonal anti-beta-actin (A2228, Sigma-Aldrich; 1:4,000, 4 °C, overnight). Beta-actin served as loading control. Membranes were washed five times with TBS containing 0.05% Tween 20, and then incubated (RT, 1 h) with polyclonal goat anti-mouse HRP-conjugated secondary antibody (P0447, DakoCytomation, Glostrup, Denmark; 1:2,000) or biotinylated polyclonal goat anti-rabbit immunoglobulin (E0447, DakoCytomation; 1:1,000). Sensitivity of PP5/TFPI-2 immunostaining was enhanced with Vectastain Elite ABC Kit (Standard; PK-6100; Vector Lab.). Signals were visualized by SuperSignal West Pico ECL reagent (Pierce/Thermo Fisher Scientific, Waltham, MA) and documented using iBright Imaging Systems (Thermo Fisher Scientific). Signal quantification was done with ImageJ software (Bethesda, MD, USA).

### 2.5. Tissue microarrays and PP5/TFPI-2 immunostaining

Placental FFPE tissue sections were stained with H&E at the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University. As previously described [46,48,51,52], representative areas were selected for the construction of tissue microarrays (TMAs), which contained 2 mm cores in diameter from five tissue blocks of each case (n = 37).

Five μm sections of TMAs were mounted on SuperFrost/Plus slides, deparaffinized and rehydrated. After inhibition of endogenous peroxidases with 10% $H_2O_2$ in methanol (20min, RT), antigen retrieval was carried out by incubating slides at 100 °C in TRS (10 mM Tris; 1 mM EDTA; 0.05% Tween 20; pH = 9; 3min). PP5/TFPI-2 immunostaining was performed using the Novolink Polymer Detection System (Peroxidase/DAB+, Rabbit, Novocastra Laboratories, Newcastle, UK). Unspecific binding was blocked (10min, RT) using Novocastra™ Protein Block. Slides were then incubated (overnight, 4 °C) with rabbit polyclonal anti-PP5/TFPI-2 antibody (kind gift from Dr. Hans Bohn; 1:10,000 dilution in all stainings except for extravillous trophoblasts (EVTs) in third trimester for which 1:6,000 dilution was used). Subsequently, the post-primary block and the polymer-peroxidase conjugate of the Novolink kit were used (30-30min, RT). PLAP immunostaining was carried out using a Leica BOND-MAX™ autostainer (Leica GmbH, Nussloch, Germany), according to the manufacturer's protocol. Slides were dewaxed in Bond™ Dewax Solution (Leica Microsystems) and rehydrated in Bond Wash Solution (Leica Microsystems). Antigen retrieval was performed at pH = 9 using Bond Epitope Retrieval 2 Solution (Leica Microsystems) for 20 min at 100 °C. Slides were incubated for 20 min at room temperature with a mouse monoclonal anti-PLAP antibody (clone M7191; DakoCytomation; 1:60). After post-primary amplification (8min) biotin-free Bond Polymer Refine Detection (15min) was used. The reactions were visualized using 3,3'-diaminobenzidine (DAB)-hydrogen peroxide chromogen-substrate kit (10min), and counterstained with hematoxylin.

### 2.6. Evaluation of immunostainings

Placental TMAs immunostained for PP5/TFPI-2 were digitally scanned by a high-resolution bright field slide scanner (Pannoramic Scan, 3DHistech Ltd., Budapest, Hungary). PP5/TFPI-2 cytoplasmic staining in the syncytiotrophoblast was evaluated by three examiners

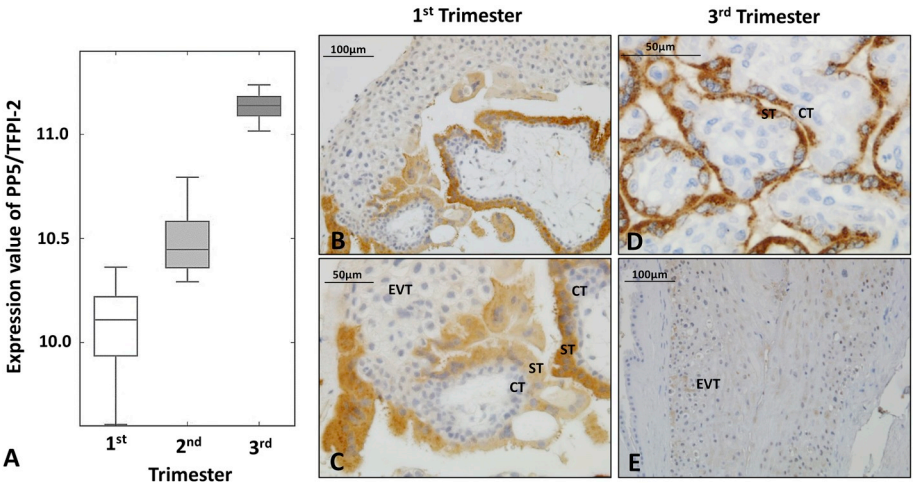


**Fig. 1. Placental TFPI2 (PP5) expression increases with advancing gestation.** Reanalysis of microarray data of normal placentas (GEO Accession No: GDS4037) showed that *TFPI2* (PP5) mRNA expression increases from first trimester towards term (first vs second trimester: $p = 0.07$; first trimester vs term: $p = 0.005$; second trimester vs term: $p = 0.006$)(**A**). Villous trophoblast expressed strongly while EVTs faintly PP5/TFPI-2 in both first and third trimesters (**B-E**). Magnified representative images of first (**C**) and third (**D**) trimester placentas showed the specific staining of the syncytiotrophoblast but not the cytotrophoblast, suggesting that PP5/TFPI-2 expression is dependent on trophoblast differentiation. ST: syncytiotrophoblast, CT: cytotrophoblast, EVT: extravillous trophoblast, 400× magnifications with scale bar 50 µm, 200× magnifications with scale bar 100 µm.

blinded to the clinical information (KK, SzSz, BKD) on virtual slides using Pannoramic Viewer 1.15.4 (3DHistech Ltd.). At least 25 terminal or intermediate villi (diameter of 20–150 µm) were scored semi-quantitatively in each core with a scoring system modified from that previously published [47]. The intensity of PP5/TFPI-2 immunoscores were graded as 0 to +3. The average intensity was determined for each core, then the average intensity was calculated for each placenta, and then the overall mean intensity score was assigned to each patient group. PP5/TFPI-2 immunostaining in EVTs was evaluated by the semiquantitaive scoring of 100 cells in each core graded similarly as to describe above.

PP5/TFPI-2 immunostaining on digitized, "virtual" TMA slides were also quantified by the DensitoQuant software of Pannoramic Viewer v1.15 (3DHistech Ltd.), an unbiased semi-automated analyser of bright field digital slides. Two-five areas (at least 25 terminal or intermediate villi with diameter of 20–150 µm) were marked on each core and analyzed after calibration of parameters. Pixels in these areas were classified into intensity ranges (+1, +2, +3) (Supplementary Fig. 1) and averaged for each core, for each placenta and then for each patient group. These scores were then used as the representative data for the patient groups.

### 2.7. Immunoassays

Serum PP5/TFPI-2 concentrations were measured parallel to placental PP5/TFPI-2 immunostainings in samples of the same patients, 1) early controls (n = 3); 2) early preeclampsia (n = 6); 3) early preeclampsia with HELLP syndrome (n = 8); 4) late preeclampsia (n = 5) and 5) late controls (n = 9). PP5/TFPI-2 was measured in duplicate with a human TFPI-2 sandwich ELISA Kit (KA4494, Abnova Corporation, Taipei, Taiwan) according to the manufacturer's instructions. Optical density was measured at 450 nm and translated into quantitative amounts using a calibration curve consisting of TFPI-2 standards (156-10,000 pg/ml). The sensitivity of the assay was < 10 pg/ml, and the coefficients of intra-assay variation and inter-assay variations were < 5.6% and < 6.1%, respectively.

### 2.8. Data and statistical analysis

Demographic data were analyzed using Microsoft Excel v.2016 (Microsoft Corp., Redmond, WA, USA) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Comparisons among groups were performed using Chi-square and Fisher's exact tests for proportions, Kruskal–Wallis and Mann–Whitney tests for non-normally distributed continuous variables, and the Student's t-test for normally distributed continuous variables. Statistical significance was considered at $p < 0.05$.

Normalized placental *TFPI2* microarray data [53] were obtained from GEO (Accession No: GDS4037) and plotted with "R" (www.r-project.org) as boxplot.

The correlations between PP5/TFPI-2 immunoscores and MVMP score of the placenta, placental weight or birthweight were also investigated.

## 3. Results

### 3.1. Demographic and clinical data

Demographic and clinical characteristics are displayed in Table 1. Systolic and diastolic blood pressures were higher in the disease groups than in controls. Proteinuria was detected in all cases but not in controls. Although controls were matched to cases within two weeks of gestational age, the median gestational age of late controls was slightly higher than that of cases with late preeclampsia. Placental weights were lower in all disease groups compared to respective controls. Birthweights were lower in late preeclampsia cases than in controls, and they tended to be lower in early preeclampsia with or without HELLP syndrome than in respective controls.

### 3.2. Placental PP5/TFPI2 expression in placentas from normal and complicated pregnancies

According to GEO database microarray data, *TFPI2* (PP5) mRNA is increasingly expressed in the placenta with advancing gestation (Fig. 1A). Our initial immunostainings of first and third trimester placentas supported this finding at the protein level (Fig. 1B–E). Remarkably, villous trophoblast expressed strongly while EVTs faintly PP5/TFPI-2 in both trimesters. Moreover, magnified images of first and third trimester placentas showed the specific staining of the syncytiotrophoblast but not the cytotrophoblast, suggesting that PP5/TFPI-2 expression is dependent on trophoblast differentiation.

Next, we were interested whether PP5/TFPI-2 placental expression is changed in preeclampsia, syndrome affecting trophoblast differentiation invasion. PP5/TFPI-2 was recognized as 36–37 kDa and 30–31 kDa bands on immunoblots (Fig. 2), which well correspond with the molecular weight of PP5/TFPI-2 in the original publication on PP5 by Bohn et al. [19] and by later studies [25,54]. Western blot also showed that the same molecular size PP5/TFPI-2 was detectable both in control and preeclamptic placentas, and PP5/TFPI-2 expression tended to be higher in preeclampsia compared to controls. This suggested that placental PP5/TFPI-2 expression is altered in preeclampsia affected by abnormal placentation. Therefore, we decided to investigate placental PP5/TFPI-2 expression on TMAs immunostained for this protein and study how PP5/TFPI-2 expression is related to histopathological

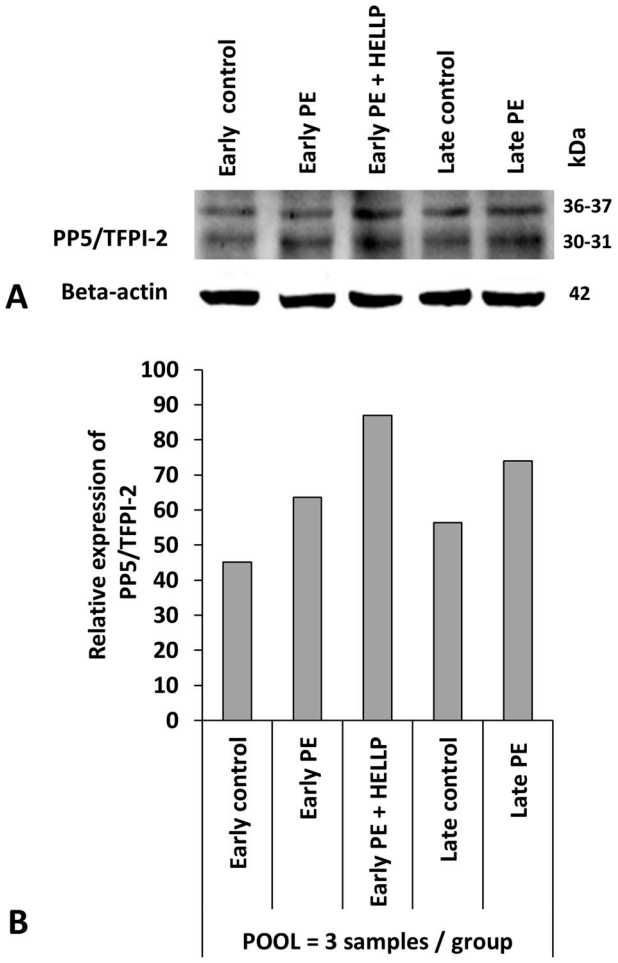


**Fig. 2. PP5/TFPI-2 immunoreactivity in the placenta**. PP5/TFPI-2 immunoreactivity in placentas from all study groups (3 samples pooled in each group) was determined by anti-PP5/TFPI-2 antibody on Western blot (**A**). Densitometrical analysis of band intensities determined PP5/TFPI-2 values normalized to beta-actin loading control (**B**).

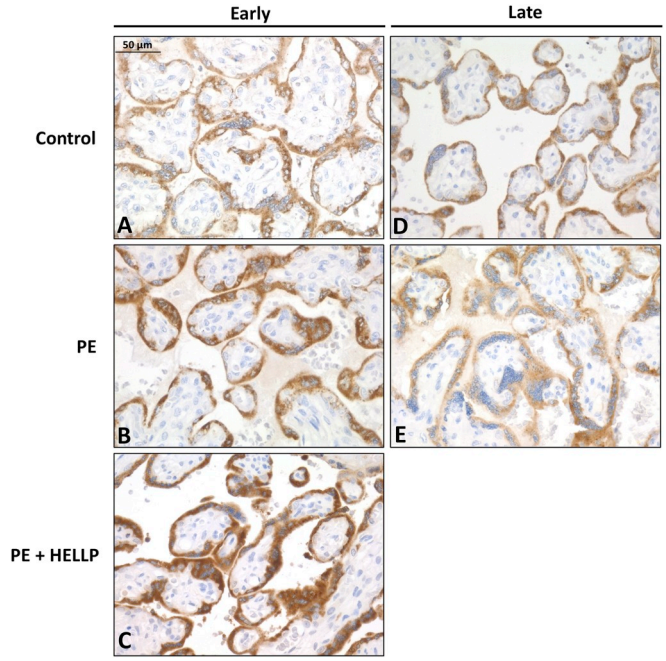


**Fig. 3. Representative images of PP5/TFPI-2 immunostaining on placental TMAs.** PP5/TFPI-2 immunostaining was detected in the syncytiotrophoblast but not in other cell types of the chorionic villi. Syncytiotrophoblastic PP5/TFPI-2 immunostaining was similar in early controls (**A**: GW 34) and late controls (**D**: GW 40). PP5/TFPI-2 immunostaining was stronger in early preeclampsia with HELLP syndrome (**C**: GW 32) or without HELLP syndrome (**B**: GW 32) than in early controls. Similar syncytiotrophoblastic PP5/TFPI-2 immunostaining was observed in late preeclampsia cases (**E**: GW 40) as in late controls. Representative images, hematoxylin counterstain, 400× magnifications, scale bar 50 µm.

changes of the placenta.

PP5/TFPI-2 immunostaining of TMAs allowed the simultaneous examination of PP5/TFPI-2 expression in 37 placentas (Fig. 4A). PP5/TFPI-2 immunostaining was clearly detected in the cytoplasm of the syncytiotrophoblast, while other cell types of the chorionic villi did not show PP5/TFPI-2 staining (Fig. 1B–D, Fig. 3A–E).

PP5/TFPI-2 immunoscores of the syncytiotrophoblast did not change with gestational age (early controls: mean ± SE: 2.02 ± 0.08, late controls: 1.99 ± 0.07, $p = 0.75$) (Fig. 3A,D, Fig. 4B and C). However, there was an increased syncytiotrophoblastic immunostaining in early preeclampsia compared to controls, irrespective of the presence of HELLP syndrome (PE: 2.37 ± 0.07, $p = 0.001$; PE with HELLP syndrome: 2.35 ± 0.07, $p = 0.003$; early controls: 2.02 ± 0.08) (Fig. 3A–C, Fig. 4B and C). There was no difference in syncytiotrophoblast cytoplasmic immunostaining between late preeclampsia (1.86 ± 0.1, $p = 0.26$) and late controls (1.99 ± 0.07) (Fig. 3D and E, Fig. 4B and C).

These results were further confirmed by computerized image analysis (Supplementary Figs. 1 and 2). Mean PP5/TFPI-2 immunoscores were higher in placentas of women with early preeclampsia with (2.21 ± 0.04, $p < 0.017$) and without HELLP syndrome (2.21 ± 0.04, $p < 0.015$) than in early controls (2.08 ± 0.04). However, there was no difference between late preeclampsia (1.97 ± 0.04, $p = 0.86$) and late controls (1.98 ± 0.03) (Supplementary Figs. 2A and B).

Our tissue collection also allowed us to investigate PP5/TFPI-2 immunoscores in EVTs in a few samples in each group (Supplementary Fig. 4). Overall, EVT staining was very faint compared to syncytiotrophoblast. There was a larger proportion of EVTs with 1 + immunoscore in early preeclampsia than in early controls, however, a large proportion of cells with 2 + immunoscore was observable in late preeclampsia (Supplementary Fig. 4A). Cytoplasmic PP5/TFPI-2 immunoscores of EVTs tended to be higher in early preeclampsia (mean ± SE: 0.33 ± 0.14) and in early preeclampsia with HELLP syndrome (0.46 ± 0.45) than in early controls (0.05 ± 0.00). PP5/TFPI-2 immunoscores tended to be higher in late preeclampsia (0.55 ± 0.39) than in late controls (0.29 ± 0.17) (Supplementary Fig. 4B).

To investigate how specific the observed phenomenon was for PP5/TFPI-2, we studied other placental proteins' expression in preeclampsia. In previous studies we showed the increased expression of hCG, PAPPA2, Siglec-6, sFLT1, syndecan-1 and decreased expression of galectin-13 and galectin-14 in the syncytiotrophoblast in early preeclampsia [20,46,47,51,52,55,56]. Here, we selected a gene with unchanged expression in early preeclampsia (*ALPP*) in our microarray study (Gene Expression Omnibus accession number GSE65866) [20], and showed its unaltered expression in the syncytiotrophoblast in any of the preeclampsia groups (Supplementary Fig. 5).

### 3.3. Histopathological examination of placentas

There was difference in mean MVMP scores between early preeclampsia and early controls, irrespective of the presence of HELLP syndrome (PE: mean ± SE: 6.57 ± 1.36, $p = 0.029$; PE with HELLP syndrome: 6.88 ± 0.74, $p = 0.002$; early controls: 1.8 ± 1.11). There was no difference in mean MVMP scores between late preeclampsia (2.88 ± 0.79, $p = 0.39$) and late controls (2 ± 0.62) (Fig. 5A).

A

| | Early control | Early PE | Early PE + HELLP | Late control | Late PE | Total |
|---|---|---|---|---|---|---|
| Case | 5 | 7 | 8 | 9 | 8 | 37 |
| Core | 24 | 31 | 31 | 45 | 35 | 166 |
| Villi | 1,795 | 2,326 | 2,325 | 3,375 | 2,615 | 12,436 |

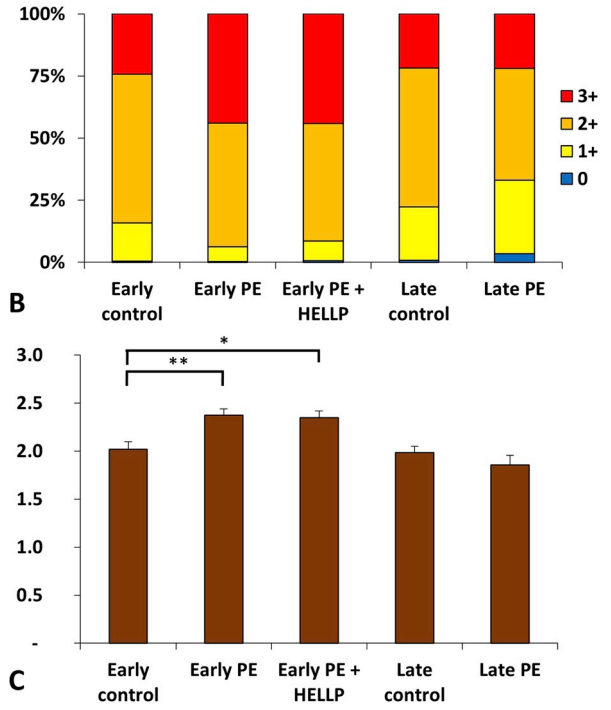


**Fig. 4. Villous trophoblastic PP5/TFPI-2 immunoscores.** The number of placentas on TMAs, analyzed TMA cores and villi immunoscored by the three examiners in each study group are summarized on the first panel (**A**). There was a larger proportion of villi with 3 + immunoscore in cases of early preeclampsia than in early controls, however, this change was not observable in late preeclampsia cases (**B**). Syncytiotrophoblastic PP5/TFPI-2 immunoscores of TMA cores were higher in early preeclampsia (mean ± SE: 2.37 ± 0.07, $p = 0.001$) and in early preeclampsia with HELLP syndrome (2.35 ± 0.07, $p = 0.003$) than in early controls (2.02 ± 0.08). PP5/TFPI-2 immunoscores were not different between late preeclampsia (1.86 ± 0.1, $p = 0.26$) and late controls (1.99 ± 0.07) (**C**). PE: preeclampsia; HELLP: HELLP syndrome; PE + HELLP: preeclampsia with HELLP syndrome, $*p < 0.05$, $**p < 0.01$, SE: standard error.

### 3.4. Correlation of placental PP5/TFPI-2 expression with maternal vascular malperfusion, birthweight and placental weight

PP5/TFPI-2 immunoscore positively correlated with placental MVMP score of the placenta of patients with preeclampsia (R = 0.43, $p = 0.04$), but not with those of controls (R = −0.18, $p = 0.54$) (Fig. 5B and Supplementary Fig. 3A). PP5/TFPI-2 immunoscore negatively correlated with birthweight among patients with preeclampsia (R = −0.68, $p = 0.0003$), however, this was not significant among controls (R = −0.27, $p = 0.36$)(Fig. 5C and Supplementary Fig. 3B). Moreover, PP5/TFPI-2 immunoscore negatively correlated with placental weight among patients with preeclampsia (R = −0.74, $p = 0.0001$), however, this was not significant among controls (R = −0.52, $p = 0.08$)(Fig. 5D and Supplementary Fig. 3C).

### 3.5. Maternal serum PP5/TFPI-2 concentrations are increased in preeclampsia

In controls, maternal serum PP5/TFPI-2 concentration increased with gestational age (early controls: mean ± SE: 8.88 ng/ml ± 4.18; late controls: 33.4 ng/ml ± 5.28, $p = 0.036$) (Fig. 6). There was higher maternal serum PP5/TFPI-2 concentration in early preeclampsia (29.75 ng/ml ± 6.68, $p = 0.048$) than in early controls (8.88 ng/ml ± 4.18), and it tended to be higher in cases with early preeclampsia with HELLP syndrome (21.55 ng/ml ± 5.35, $p = 0.19$). However, there was no difference between late preeclampsia (38.15 ng/ml ± 14.37, $p = 0.60$) and late controls (33.4 ng/ml ± 5.28).

## 4. Discussion

### 4.1. Principal findings of this study

(1) PP5/TFPI-2 was localized to the cytoplasm of the syncytiotrophoblast and this PP5/TFPI-2 immunostaining did not change with gestational age in control placentas; (2) There was a stronger PP5/TFPI-2 immunostaining of the syncytiotrophoblast in cases of early preeclampsia with or without HELLP syndrome than in early controls, however, no such difference was observed in late preeclampsia; (3) Maternal serum PP5/TFPI-2 concentration increased with gestational age in controls; (4) Maternal serum PP5/TFPI-2 concentration was higher in early preeclampsia and tended to be higher in preeclampsia with HELLP syndrome than in early controls; however, there was no such difference in late preeclampsia; and (5) In cases of preeclampsia, PP5/TFPI-2 immunoscores positively correlated with the MVMP score of the placenta, while negatively correlated with placental weights and birthweight.

Abnormal placentation is associated with preeclampsia and HELLP syndrome [1,9,10,12,13], and placental pathologic changes are more dominant in early than in late preeclampsia [11]. Early preeclampsia is more often complicated with HELLP syndrome than late preeclampsia, with which it shares similar placental morphological changes [47,57] and genome-wide gene expression patterns [46]. In accord, our histopathological analysis showed consistent results; the composite placental MVMP score was higher in early preeclampsia, with or without HELLP syndrome, than in early controls, while late preeclampsia cases did not differ from late controls. These data reflect that there is an extensive abnormality of trophoblast invasion and spiral artery remodeling in early preeclampsia, which leads to malperfusion and hypoxic-ischemic stress of the placenta, while this pathological condition is less frequent and extensive in late preeclampsia.

Based on these, we hypothesized that PP5/TFPI-2, which inhibits tumor cell invasion [30,58], may have expressional changes in preeclampsia, possibly connected with abnormal trophoblast invasion, mainly in early preeclampsia and HELLP syndrome. To investigate this hypothesis, we immunostained TMAs constructed using 37 placentas from early and late preeclampsia and gestational age-matched controls. In addition, we firstly examined placental PP5/TFPI-2 expression in early preeclampsia with HELLP syndrome, as well. Similar to previous studies, PP5/TFPI-2 localized to the syncytiotrophoblast cytoplasm [26,34,59,60], but there was no immunostaining in other cells of the chorionic villi in spite of reports on cytotrophoblastic PP5/TFPI-2 staining [61–63].

In controls, no changes were found in syncytiotrophoblast PP5/TFPI-2 immunostaining with increasing gestational age. In contrast, PP5/TFPI-2 immunoscores were increased in early preeclampsia with or without HELLP syndrome by both manual and semi-automated image analysis, which latter has become a considerable method for immunostaining assessment [64]. These findings were similar to the results of Xiong et al. and Xiao et al., who used a semi-quantitative immunoscoring system and showed that PP5/TFPI-2 immunostaining was higher in preeclampsia than in normal pregnancy although these authors did not divide preeclampsia into early and late subtypes [34,35]. Xiao et al. showed that the overexpression of PP5/TFPI-2 in preeclampsia was probably due to the hypomethylation of the *TFPI2* gene promoter [35]. Interestingly, Ogawa et al. [21] reported decreased placental PP5/TFPI-2 levels in severe preeclampsia. The discrepancy between these studies may lie in critical differences in the applied

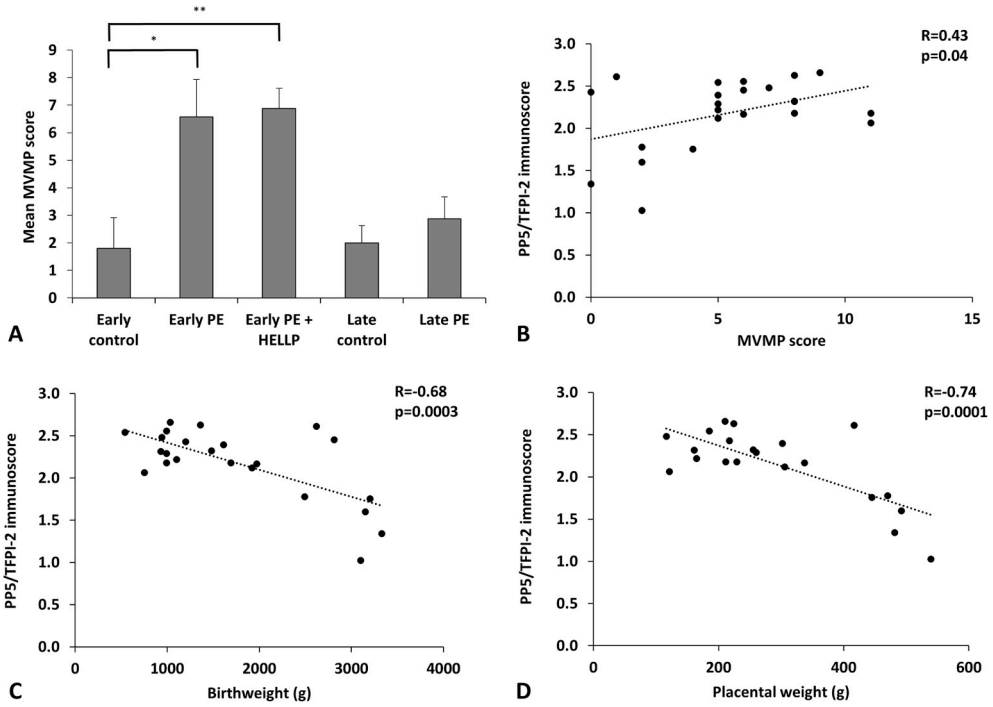


**Fig. 5. Mean MVMP score of placentas and correlation of PP5/TFPI-2 immunoscores with clinical parameters in preeclampsia.** There was a significant difference in early preeclampsia compared to gestational age-matched controls, irrespective of the presence of HELLP syndrome (PE without HELLP syndrome: mean ± SE: 6.57 ± 1.36, $p = 0.029$; PE with HELLP syndrome: 6.88 ± 0.74, $p = 0.002$; early controls: 1.8 ± 1.11). There was no difference between late preeclampsia (2.88 ± 0.79, $p = 0.39$) and late control immunoscores (2 ± 0.62) (**A**). PP5/TFPI-2 immunoscore positively correlated with maternal vascular malperfusion (MVMP) score of the placenta (R = 0.43, $p = 0.04$) (**B**), negatively correlated with birthweight (R = −0.68, $p = 0.0003$)(**C**) and with placental weight (R = −0.74, $p = 0.0001$)(**D**) among patients with preeclampsia.

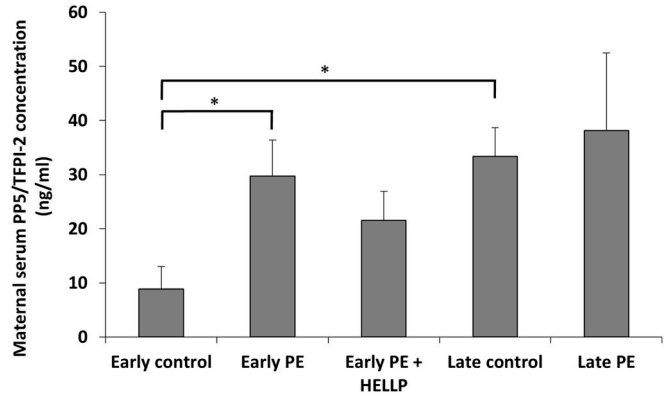


**Fig. 6. Maternal serum PP5/TFPI-2 concentrations.** Maternal serum PP5/TFPI-2 concentration significantly changed with gestational age in controls (early controls: mean ± SE: 8.88 ng/ml ± 4.18 versus late controls: 33.4 ng/ml ± 5.28, $p = 0.036$). PP5/TFPI-2 concentration was higher in all disease groups than in respective controls. This difference was significant in women with early preeclampsia without HELLP syndrome (29.75 ng/ml ± 6.68, $p = 0.048$) compared to early controls (8.88 ng/ml ± 4.18). PE: preeclampsia; HELLP: HELLP syndrome; PE + HELLP: preeclampsia with HELLP syndrome, $*p < 0.05$, SE: standard error.

methodologies, used anti-PP5/TFPI-2 antibodies and the composition of the enrolled clinical study groups.

To examine whether placental PP5/TFPI-2 expression is related to trophoblast invasion, we investigated the clinical proxies of this cell biological phenomenon. First, we correlated histopathological signs of placental MVMP with PP5/TFPI-2 immunoscores, and found a positive correlation in all cases of preeclampsia; however, we did not find this in controls, suggesting a link between PP5/TFPI-2 overexpression and problems with trophoblast invasion and placental perfusion. Moreover, PP5/TFPI-2 immunoscore negatively correlated with placental weight and birthweight among patients with preeclampsia; however, this was not the case in controls, suggesting that PP5/TFPI-2 expression is also linked to restricted placental and fetal development due to decreased placental perfusion.

We detected that maternal blood PP5/TFPI-2 concentrations reflect placental PP5/TFPI-2 expression. In control women, our findings corresponded with some studies showing increasing maternal serum PP5/TFPI-2 levels with gestational age [23,34,36,65,66]. Although the syncytiotrophoblast is the main source of the pregnancy-related rise in PP5/TFPI-2 serum levels, other cell types may also contribute to circulating levels of PP5/TFPI-2 including perivascular and/or endovascular EVTs and platelets as recently demonstrated Vadivel et al. [67]. Indeed, a recent publication confirms that EVT-derived factors appear in the serum of pregnant women [68].

We found that maternal serum PP5/TFPI-2 concentration was increased in women with preeclampsia compared to gestational-age-matched controls. Due to the limited number of cases, this difference was only significant in cases of early preeclampsia and tended to be higher in those with preeclampsia with HELLP syndrome than in early controls. The observations of other studies on PP5/TFPI-2 levels in maternal serum [21,36,37,69,70] are in accordance with our results. Two studies experienced decreased PP5/TFPI-2 level in plasma in preeclampsia compared to control group [34,61], which may be the result of the difference in the behavior of PP5/TFPI-2 in plasma vs. serum due to the coagulation processes.

As discussed above, the overexpression of PP5/TFPI-2 is linked to placental MVMP and the consequent malnutrition and growth restriction of the placenta and fetus, the histopathological and clinico-pathological proxies of abnormal trophoblast invasion. Hence, PP5/TFPI-2 may be a factor regulating trophoblast invasion in the placenta. Indeed, PP5/TFPI-2 is less expressed in the placenta in the first trimester when trophoblasts are invasive than in the third trimester when trophoblast invasion is not detectable (Fig. 1A). Remarkably, PP5/TFPI-2 is also produced by various tumors, in which it regulates invasion [29,30], and is assumed to be a putative tumor suppressor gene that is frequently inactive in tumors [58,71–73]. Since the invasion of the trophoblast is somewhat similar to the invasion of tumors in spite of the remarkable differences in its tight regulation [74,75], PP5/TFPI-2 may act in a common trophoblast-tumor invasion pathway.

PP5/TFPI-2 is a potent protease inhibitor of various MMPs (i.e. MMP-1, -2, -3, -9,-13 [76–80], MMPs and their inhibitor proteases are among the machinery that regulates physiological trophoblast invasion [38–40,81–91], and their dysregulation can be detected in preeclampsia [92,93]. Therefore, the overproduction of PP5/TFPI-2 may

lead to abnormal trophoblast invasion via an altered MMP balance at the maternal-fetal interface. However, no data can currently support that indeed there is an overproduction of PP5/TFPI-2 in the first trimester in early preeclampsia cases, which would subsequently and consequently lead to abnormal trophoblast invasion via this MMP pathway.

Alternatively, PP5/TFPI-2 overproduction can be the result of hypoxic/ischemic placental stress response in early preeclampsia, in a later stage of the pathogenesis. Since PP5/TFPI-2 inhibits tissue factor and factor VII and reduce thrombin generation and coagulation, this would act against a hypercoagulation state, which is frequently an underlying disease in placental hypoxia and ischemia [94–98]. However, this hypothesis has also to be carefully investigated by further studies also employing functional experiments that were not feasible in our study.

### 4.2. Strengths and limitations of the study

The strengths of the study are: (1) strict clinical definitions and homogenous patient groups; (2) sample collection from a standardized location in placentas taken from C-sections; (3) histopathological examination based on international criteria; (4) protein expression profiling with tissue microarray and immunostaining followed by both manual and automatic immunoscorings; (5) correlation of PP5/TFPI-2 expression with clinico-pathological parameters of the placenta and the fetus; and (6) parallel investigation of serum and placental PP5/TFPI-2.

A limitation of the study is the relatively modest number of cases in each group due to the strict clinical and histopathological inclusion criteria we used for patient enrollment. On the other hand, this was one of the most important strengths of our study. Another limitation is that we mainly investigated PP5/TFPI-2 expression in the syncytiotrophoblast while trophoblast invasion is related to EVTs. However, PP5/TFPI-2 probably acts and inhibits EVT invasion as a paracrine substance. This needs to be investigated by *in vitro* assays. Finally, we investigated PP5/TFPI-2 expression in the second half of pregnancy, while trophoblast invasion problem is related to the first trimester.

## 5. Conclusions

Our findings suggest that an increased placental PP5/TFPI-2 expression may be associated with abnormal placentation in early preeclampsia, with or without HELLP syndrome.

## Author contributions

KK and NGT conceptualized study and designed research; KK, KJ, and BH performed research; TK, PH, ZP, IK, and NGT contributed new reagents/analytic tools/clinical specimens; KK, SzSz, KJ, PK, BKD, BH, OE, ZP, IK, and NGT analyzed and interpreted data; all authors contributed to the writing of the paper.

## Conflicts of interest

The authors report no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.01.011.

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