Pathogenetic markers in preeclampsia

Ph.D. Thesis

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Introduction

Preeclampsia (PE) is a severe and common complication of human pregnancy with a worldwide incidence of 2%–10%. As PE is responsible for the majority of maternal and fetal morbidity and mortality, it is one of the main challenges of obstetrics of our times, even in the developed countries. This systemic disorder is characterized by the development of a maternal syndrome that includes hypertension and proteinuria. In the most cases, these symptoms develop in the third trimester of pregnancy and usually disappear within a few weeks after delivery. The syndrome of haemolysis, elevated liver enzymes and low platelet count (HELLP) is a less frequent, but rapidly progressing, life-threatening complication, which develops mostly on the basis of severe PE.

Despite intensive research efforts, the etiology and pathogenesis of PE are not completely understood. The development of preeclampsia is influenced by both genetic, immunologic and environmental risk factors, suggesting a multifactorial origin. Preeclampsia appears to progress in two stages: preclinical and clinical. It arises from poor development of the early placenta and its maternal blood supply, called poor placentation. In the second stage, an increasingly hypoxic placenta causes the maternal signs of the condition. In severe, particularly early onset disease (before 34 weeks gestation), the fetus may suffer increasing nutritional and respiratory insufficiency, asphyxia, or death. Increasing evidence suggests that an excessive maternal systemic inflammatory response to pregnancy with systemic oxidative stress and an imbalance between pro-angiogenic and anti-angiogenic factors play an important role in the pathogenetic processes. The levels of substances with anti-angiogenic effects, such as soluble fms-like tyrosine kinase-1 (sFlt1), also known as soluble vascular endothelial growth factor receptor-1 (VEGFR-1), are increased in the peripheral blood of mothers with PE, while amount of factors with pro-angiogenic properties, such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF), are decreased. VEGF and PlGF have crucial role in the maintenance of the stability and physiologic functions of vascular endothelial cells. sFlt1 binds circulating PlGF and VEGF, thus preventing their action on the endothelial receptors, which leads to endothelial dysfunction and injury. This anti-angiogenic shift is strongly related to clinical features and to severity of the disease. Systemic inflammation and the impairment of maternal

immune tolerance characteristic for healthy pregnancy are also considered to be dominant components of the pathogenesis of this pregnancy specific disorder. An important feature of systemic inflammation in PE is the absence of Th2 skewness, and thus the predominance of Th1-type immunity and pro-inflammatory cytokines. A further characteristic pathogenetic process is the activation of the haemostatic system, which can lead to intravascular coagulation and consumptive coagulopathy, resulting in the decrease of circulating platelet count in HELLP syndrome and in severe cases of PE. Osteopontin (OPN) is a multifunctional protein expressed by endothelial, vascular smooth muscle and immune cells. It was demonstrated to be present in the trophoblast of the human placenta, and it can enhance the invasiveness of trophoblast cells. It is involved in the regulation of the immune responses and acts as a pro-inflammatory Th1 type cytokine. OPN is considered in several physiological and pathological states, such as embryo implantation, placentation, chronic inflammation and autoimmune diseases. Several clinical surveys reported an association between altered OPN concentrations and cardiovascular diseases. Local overexpression and elevated plasma concentrations of OPN were observed in aortic, coronary and carotid atherosclerosis. Atherosclerosis shares many risk factors (obesity, dyslipidemia, insulin resistance) and pathogenetic features (inflammation, oxidative stress and endothelial injury) with preeclampsia. In addition, women who develop preeclampsia are at an increased risk of atherosclerotic cardiovascular disorders later in life.

Due to the complexity of PE, several other factors have been investigated that might play a role in the pathogenesis. Among these are alterations of iron homeostasis. It has been demonstrated that plasma iron concentrations, ferritin, and saturation of transferrin are increased, whereas total iron binding capacity (TIBC), unsaturated iron binding capacity and apotransferrin are decreased in PE when compared to healthy pregnant women. Since iron may induce the generation of reactive oxygen species (ROS), higher than normal iron concentrations can exacerbate lipid peroxidation and endothelial cell injury in PE. However, increased plasma iron concentrations are contrary to the ongoing inflammation in PE. Several findings and general clinical experience support the notion that chronic inflammation decreases iron availability, which might even result in inflammation-induced anaemia. Based on these observations, one would expect a decrease in plasma iron concentrations in PE, instead of increased concentrations that

have been reported. The link between iron homeostasis and inflammation is hepcidin, a recently described acute phase protein. It acts by down regulating intestinal iron absorption and iron release from enterocytes and macrophages through internalization and degradation of ferroportin. Hepcidin expression is regulated by several factors. Its primary triggers include inflammatory signals, such as interleukin-6 (IL-6) and high concentrations of iron.

Soluble urokinase plasminogen activator receptor (suPAR) is a biomarker increasingly used for the monitoring of systemic inflammation. suPAR is derived from the cleavage and release of the membrane-bound uPAR, and is present in plasma, blood, cerebrospinal fluid and urine. Increased activation of the immune system and increased inflammatory response – which is also characteristic for PE - lead to elevated plasma suPAR levels. This is confirmed by findings of increased suPAR levels in cases of viral, bacterial or parasitical infections, as well as in autoimmune disorders and cancer. Interestingly, in all of these conditions suPAR concentrations are directly proportional to a worse prognosis of the disease.

Thrombospondin-1 (TSP-1) is produced and secreted into the circulation mainly by activated platelets and endothelial cells. Its highest concentrations can be seen in the α-granules of platelets, and its expression is regulated among others by hypoxia. In conjunction with von Willebrand factor and fibrinogen, TSP-1 contributes to clot formation. Furthermore, TSP-1 is a well-known inhibitor of angiogenesis and regulator of apoptosis. Through binding to its receptor, CD47, TSP-1 interrupts NO-induced generation of pro-angiogenic and vasodilatory cGMP, therefore it may participate in the regulation of blood pressure, as well. According to recent clinical studies, TSP-1 has pro-atherosclerotic properties, however, there is a limited number of studies examining the circulating TSP-1 levels in cardiovascular diseases.

Thrombospondin 2 (TSP-2) is a multifunctional molecule primarily described as a non-structural regulator component of the extracellular matrix, where it modulates activity and bioavailability of proteases and growth factors. It is expressed in the vessel walls, and presumably it is mainly secreted into the circulation by endothelial cells. In contrast to other types of thrombospondins, platelets do not contain detectable levels of TSP-2. Its expression is modulated by hypoxia. Thrombospondin 2 affects several cellular functions, including proliferation, motility, adhesion, apoptosis and platelet aggregation,

and takes part in the regulation of angiogenesis, tumor growth, wound healing and hemostasis. Several authors have reported on the anti-angiogenic properties of TSP-2. Based on these data we have to raise the question: does TSPs participate in the pathogenesis of PE?

The mainspring of my work was that there is a strong need for better understanding of the pathogenetic background of PE and for finding sensitive markers to improve the clinical issues of this severe disease.

Aims

My aim was to find new circulating factors, which could participate in the pathogenetic processes of praeeclampsia, and which probably could serve as biomarkers of the disease, in the future. Furthermore, I analyzed their relation to other known biomarkers and to the clinical features of PE, to study their exact role in the pathogenesis.

1. I studied, if there is any difference in plasma osteopontin (OPN) concentrations in healthy pregnant women and in patients with preeclampsia. I determined several markers of processes involved in the pathogenesis of PE, such as systemic inflammation (C-reactive protein (CRP)), endothelial activation (von Willebrand factor antigen (VWF:Ag)), endothelial injury (fibronectin), systemic oxidative stress (malondialdehyde (MDA)) and elevation of the amount of circulating trophoblast debris (cell-free fetal DNA). I examined whether these laboratory markers, as well as the clinical features of the study subjects, were related to OPN.

2. I aimed to describe hepcidin concentrations in PE when compared to healthy pregnant women. I studied its associations with iron homeostasis (plasma iron, ferritin, transferrin, total iron binding capacity (TIBC), complete blood cell count), and with a marker of systemic inflammation (IL-6), which enhances its expression. My goal was to find the explanation of the contradiction of elevated iron levels, despite the ongoing systemic inflammation in PE.

3. I assessed the circulating levels of soluble urokinase plasminogen activator receptor (suPAR) in comparison with other markers of systemic inflammation (IL-6, C-reactive protein) in PE and healthy pregnancy, in order to evaluate suPAR as a possible marker for the characterization of the inflammatory status during pregnancy. I examined its diagnostic accuracy for distinguishing preeclamptic women and healthy controls, based on the degree of the systemic inflammation.

4. My aim was to determine serum TSP-1 levels in non-pregnant, healthy and preeclamptic pregnant women, as well as in patients with HELLP syndrome. Furthermore, I examined whether there are any associations between TSP-1 levels and the clinical features and routine laboratory results of the study subjects.

5. I studied, if there are differences in the serum levels of the anti-angiogenic TSP-2 between preeclamptic patients and healthy normotensive pregnant women. I also analysed, whether TSP-2 concentrations are related to the clinical features of the disease and whether there are any associations between TSP-2 and the anti-angiogenic sFlt-1 or the pro-angiogenic PlGF levels.

Patients and methods

My studies were designed using a case-control approach. The study participants were enrolled in the First Department of Obstetrics and Gynecology and in the Department of Obstetrics and Gynecology of Kútvölgyi Clinical Center, at the Semmelweis University, Budapest, Hungary, between 2007 and 2011. Preeclampsia was diagnosed according to the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (NHBPEP). Early onset PE was diagnosed if hypertension and proteinuria started before the 34th completed gestational week. The HELLP syndrome patients were classified into three groups, according to the Mississippi Classification. Exclusion criteria were multifetal gestation, chronic hypertension, renal disorder, maternal or fetal infection and fetal congenital anomaly. Fetal growth restriction was diagnosed if the fetal birth weight was below the 10th percentile for gestational age and gender, based on Hungarian birth weight percentiles.

Peripheral blood samples were obtained into blank or anticoagulated tubes, and then immediately centrifuged. The aliquots were stored at –80°C until the analyses.
Statistical analyses were carried out using the following software: STATISTICA (version 8.0; StatSoft, Inc., Tulsa, OK, USA) and Statistical Package for the Social Sciences (version 15.0 for Windows; SPSS, Inc., Chicago, IL, USA). Following assessment of the normal distribution of continuous variables, adequate parametric or non-parametric statistical methods were used. Data are reported as median (interquartile range) for continuous variables and as number (percent) for categorical variables. For all statistical analyses, $p<0.05$ was considered statistically significant. We applied multiple logistic regression, or analysis of covariance (ANCOVA) for adjusting for confounding variables.

1. In my first study forty-four preeclamptic patients (19 with severe disease) and 44 normotensive, healthy pregnant women matched for age and gestational age were involved. Quantitative detection of plasma OPN was performed using the Human Osteopontin ELISA assay. Serum CRP concentrations were determined using an automated analyzer. Plasma VWF:Ag concentrations were quantified using ELISA, while plasma fibronectin concentrations were measured by nephelometry. Plasma MDA concentrations were estimated by the thiobarbituric acid-based colorimetric assay. The amount of cell-free fetal DNA was determined in patients with male newborns by quantitative real-time PCR analysis of the sex determining region Y (SRY) gene.

2. To determine plasma hepcidin, iron, ferritin, transferrin, total iron binding capacity (TIBC) and complete blood cell count, I obtained blood samples from thirty preeclamptic and 37 healthy pregnant women. All participants received regular oral iron supplementation in the form of iron (II) sulphate (30 mg/day). Hepcidin concentrations were measured using a HPLC system, which was coupled to a mass spectrometer. IL-6 was measured by ELISA. Plasma iron, transferrin and ferritin concentrations and TIBC were measured using an automated laboratory analyzer. CRP concentrations were determined by an immuno-turbidimetric assay.

3. I enrolled sixty-two healthy pregnant women and 41 preeclamptic patients in my third study. Gestational age at the blood draw and maternal age were comparable. Plasma suPAR concentrations were measured using an ELISA assay. IL-6 levels were determined by the Roche Elecsys IL-6 kit. CRP levels were measured using immuno-turbidimetric method.

4. To study serum TSP-1 levels, I enrolled 45 pregnant women with early-onset (EOPE) and 43 with late-onset preeclampsia (LOPE), 21 patients with HELLP-syndrome, 45 women with uncomplicated pregnancies (HP), as well as 20 non-pregnant controls (NP) in my fourth study. 14 patients with HELLP syndrome were classified into Class II, while 7 patients into Class III, according to the Mississippi Classification. In non-pregnant subjects the blood sampling occurred between the 5^{th} and 7^{th} days of the menstrual cycles. Serum TSP-1 levels were measured by enzyme-linked immunosorbent assay on an automated ELISA analyzer.

5. To determine circulating TSP-2, sFlt1 and PlGF concentrations, I enrolled 35 pre-eclamptic patients (23 with severe and 18 with early onset disease) and 35 healthy pregnant women. Serum TSP-2 levels were measured by enzyme-linked immunosorbent assay on an automated ELISA analyzer. Serum total sFlt1 and biologically active PlGF concentrations were determined by electrochemiluminescence immunoassay on a Cobas e 411 analyzer.

Results

1. In patients with preeclampsia, I demonstrated significantly elevated circulating concentrations of CRP (6.11 (1.92–12.12) vs. 3.59 (1.68–7.40) mg/L, $p<0,05$), VWF:Ag (183.0 (128.7–242.8) vs. 148.4 (106.6–199.0) %, $p<0.05$), fibronectin (0.58 (0.39–0.82) vs. 0.36 (0.32–0.47) g/L, $p<0.001$), MDA (18.17 (14.98–20.31) vs. 13.13 (8.38–18.61) nmol/mL, $p<0.05$) and cell-free fetal DNA (0.065 (0.034–0.267) vs. 0.005 (0.0–0.178) pg/μL, $p<0.05$), compared to normotensive, healthy pregnant women. However, there was no significant difference in plasma OPN concentrations between the preeclamptic and the control group (7.77 (6.60–9.67) vs. 7.40 (6.51–8.80) ng/mL, NS).

In preeclamptic patients, plasma OPN showed a positive linear association with plasma fibronectin concentrations (Spearman R=0.38, p<0.05). However, no other relationship was found between clinical features and the measured laboratory parameters and plasma OPN concentrations. Preeclamptic patients with plasma fibronectin in the upper quartile (≥0.82 g/L) had significantly higher plasma OPN concentrations than those below the 75th percentile (<0.82 g/L), as well as healthy pregnant women (9.38 (8.10–11.99) vs. 7.54 (6.31–9.40) and 7.40 (6.51–8.80) ng/mL, respectively, p<0.05 for both). This subgroup of patients with preeclampsia was also characterized by more frequent occurrence of the severe form of the disease (9 of 13 (69.2%) vs. 10 of 31 (32.3%), p<0.05), as compared to preeclamptic women with fibronectin concentrations in the lower three quartiles.

2. Plasma hepcidin concentrations were increased in women with PE compared to healthy pregnant individuals (5.68 (0.72–9.25) vs. 3.74 (0.73–8.14) ng/mL, p=0.003). Median IL-6 concentrations were elevated in PE (p<0.001), however, plasma IL-6 concentrations were below the limit of detection in 28 of the healthy and eight of the women with PE. Plasma iron and ferritin concentrations were also higher (19.1 (7.1–51.6) vs. 15.0 (6.8–29.5) μmol/L, p=0.02 and 34 (5–78) vs. 15 (5–69) μg/L, p=0.003), while plasma transferrin concentrations and TIBC values were lower in women with PE compared to healthy pregnant women (4.1 (2.8–5.7) vs. 4.4 (3.6–6.2) μmol/L, p=0.02 and 81.2 (55.4–112.9) vs. 87.1 (71.3–122.8) μmol/L, p=0.02). No difference was detected in RBC, WBC, PLT, MCV, MCH, blood hemoglobin, hematocrit and plasma CRP values. Hepcidin levels were not related to the markers of the iron-homeostasis, neither to IL-6 concentrations.

3. Plasma suPAR levels were elevated in PE compared with healthy controls (3.18 (2.30–4.71) vs. 2.02 (1.81–2.40) ng/mL, p<0.001). The difference remained significant after adjustment for gestational age at blood sampling and maternal age. IL-6 levels were also higher in PE than in controls (5.99 (2.97–18.12) vs. 1.41 (1.00–2.70) pg/mL, p<0.001). Plasma IL-6 levels were below the level of detection in one PE and 22 control subjects. CRP levels were also higher in PE than in healthy pregnant women (6.60 (3.55–15.40) vs. 3.90 (2.10–7.25) mg/L, p=0.006).

I found a positive linear association between plasma suPAR and IL-6 levels (R=0.58, p<0.001,), and between suPAR and CRP levels (R=0.29, p=0.003). No association was observed between IL-6 and CRP levels. The above associations were present when the values were analyzed among PE patients only (R=0.62, p<0.001 and R=0.27, p=0.005, respectively). ROC curve analysis indicated a ROC AUC value of 0.83 (95% CI: 0.74–0.91, p<0.001) for suPAR, 0.83 (95% CI: 0.76–0.92, p<0.001) for IL-6 and 0.66 (95% CI: 0.55–0.76, p<0.008) for CRP.

4. TSP-1 levels were lower in HELLP syndrome compared to all of the other study groups (93.5 (62.0-103.4) ng/mL vs. EOPE: 141.2 (102.5-177.3) ng/mL, p=0.006; LOPE: 138.4 (111.8-158.2) ng/mL, p=0.008; HP: 133.4 (106.2-149.8) ng/mL, p=0.02; NP: 171.7 (135.1-201.3) ng/mL, p<0.001)). Among the other groups, there was no statistically significant difference in TSP-1 levels. There was a significant positive correlation between TSP-1 levels and platelet count in the PE and HELLP syndrome groups (r=0.33 and 0.53, p=0.002 and 0.01, respectively). In patients with more severe HELLP syndrome (Mississippi Class II), TSP-1 levels were significantly lower compared to women suffering from a milder form of HELLP syndrome (Mississippi Class III), ((74.6 (57.0-97.6) vs. 103.4 (87.4-164.2) ng/mL, p=0.04). PE patients with thrombocytopenia had lower TSP-1 levels compared to those without thrombocytopenia (96.2 (62.5-131.1) vs. 145.8 (112.0-171.0) ng/mL, p=0.004). There was no difference in TSP-1 concentrations in the subgroups of PE (mild or severe; early- or late-onset, or in presence or absence of IUGR). I could not demonstrate any association of TSP-1 levels with the clinical features and routine laboratory results of the patients. Adjustment for gestational age at the blood sampling in analysis of covariance did not change my results.

5. Thrombospondin 2 serum levels were significantly elevated in PE patients compared with normotensive pregnant women [13.2 (9.4–18.1) vs. 7.9 (7.2–11.2) ng/ml, p<0.001]. I found significantly increased sFlt1 concentrations in the preeclamptic group [9 263 (5 460–16 110) vs. 2 414 (1 448–2 868) pg/ml, p<0.001], while PlGF concentrations were lower in PE patients [50.9 (27.9–87.4) vs. 220.1 (137.3–339.8) pg/ml, p<0.001] than in control women. The differences in serum TSP-2, sFlt1 and

PlGF levels between the two groups remained significant even after adjustment for age, smoking status, primiparity and pre-pregnancy body mass index in analyses of covariance. I could not demonstrate significant differences in TSP-2 levels comparing early onset with late onset, or mild with severely preeclamptic patients. Likewise, there was no difference in TSP-2 concentrations between PE groups with or without small-for-gestational age newborns. I did not find any association between TSP-2 and sFlt1 or PlGF levels. Furthermore, TSP-2 concentrations were not related to the clinical features (maternal age, gestational age at blood collection, body mass index, smoking and parity) or to the routine laboratory parameters (white blood cell count, platelet count and serum lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase and creatinine) in the study groups.

Conclusion

1. Preeclamptic patients with extensive endothelial injury had significantly higher plasma OPN concentrations compared to those without extensive injury as well as healthy pregnant women. In addition, plasma OPN showed a significant positive linear association with plasma fibronectin concentrations in preeclampsia. I suppose that OPN may be released from the vessel wall into the peripheral circulation together with fibronectin in cases of extensive endothelial injury. However, there were no significant differences in OPN levels, compared PE patients to healthy controls. Furthermore, I could not demonstrate any associations between OPN levels and known markers of pathogenetic processes, such as systemic inflammation, endothelial activation, oxidative stress or elevated amount of placental debris. OPN can be a marker of endothelial injury in PE.

2. I demonstrated that hepcidin concentrations are increased in PE when compared to healthy pregnant women. However, plasma iron concentrations did not decrease, but instead showed an increase in PE. This finding might indicate resistance to the iron decreasing action of hepcidin in PE. In accordance with previous reports, I demonstrated higher plasma concentrations of IL-6 in PE. As IL-6 is one of the main triggers of hepcidin expression, this finding supports the notion that increased IL-6 may

be responsible for higher hepcidin concentrations. However, no association between hepcidin and other parameters of iron status were noted. The lack of correlation between hepcidin and iron homeostasis may indicate that factors specific for pregnancy may interact with the iron decreasing action of hepcidin.

3. My results indicate that plasma suPAR levels are elevated in PE compared with healthy pregnancy. This could be the consequence of the systemic inflammatory response, observed in PE. Circulating concentrations of classic inflammatory markers, such as IL-6 and CRP, were linked to suPAR levels. In previous studies, suPAR levels showed lower variability and higher stability compared to IL-6 and CRP. Taking this and my results into consideration, on the basis of the degree of the inflammatory processes, suPAR levels could differentiate healthy and preeclamptic pregnant women more efficiently than IL-6 and CRP. suPAR is a good indicator of inflammatory responses and thus it is a suitable tool for the detection of systemic inflammation and pathological immune activation in the pregnant population, and in PE. suPAR might be a useful marker for the characterization of the inflammatory status during pregnancy.

4. Circulating TSP-1 levels are decreased in HELLP syndrome, while they are unaltered in preeclampsia. In light of the association between TSP-1 levels and platelet count, concentrations of circulating TSP-1 seem to reflect disease severity in HELLP syndrome, lower levels representing more severe disease. Nevertheless, despite its potent anti-angiogenic, pro-thrombotic and immunomodulatory effects, my results suggest that circulating TSP-1 does not play a significant role in the pathogenesis of preeclampsia. Furthermore, its levels do not differ in healthy pregnancy and in non-pregnant state. This new marker may provide useful additional information in the future, to assess the disease severity in HELLP syndrome.

5. I have shown that serum levels of the anti-angiogenic thrombospondin 2 are significantly elevated in PE. I interpret my findings as an overexpression of TSP-2 by vascular endothelial cells in association with systemic endothelial activation/dysfunction prevailing in PE. Furthermore, in agreement with previous findings, I demonstrated significantly elevated sFlt-1 and decreased PlGF levels in PE.

Despite the potent anti-angiogenic properties of TSP-2, I could not demonstrate any association with sFlt-1 and PlGF. Therefore, I hypothesize that in pre-eclampsia, TSP-2 exerts its anti-angiogenic effect in a distinct way from sFlt-1, for instance by direct inhibition of proliferation, or by inducing apoptosis of endothelial cells. Thrombospondin 2 could be a novel marker of PE, which might participate in the pathogenesis of the disease via its anti-angiogenic, pro-apoptotic and immunomodulatory effects.

In conclusion, in my five studies I managed to identify five new factors, which presumably participate in the pathogenesis of PE and HELLP syndrome, and which probably might be used in the future, as pathogenetic biomarkers of these severe complications of human pregnancy.

List of publications

Publications in connection with the thesis

1. Toldi G, Biro E, Szalay B, **Stenczer B**, Molvarec A, Rigo J, Vásárhelyi B, Bekő G. (2011) Soluble urokinase plazminogen activator receptor (suPAR) levels in healthy pregnancy and preeclampsia. Clin Chem Lab Med. doi: 10.1515/CCLM.2011.656. (IF: 2,069)
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