

Plasma osteopontin concentrations in preeclampsia – is there an association with endothelial injury?

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Abstract

Background: It has been previously reported that plasma osteopontin (OPN) concentrations are increased in cardiovascular disorders. The goal of the present study was to determine plasma OPN concentrations in healthy pregnant women and preeclamptic patients, and to investigate their relationship to the clinical characteristics of the study subjects and to markers of inflammation [C-reactive protein (CRP)], endothelial activation [von Willebrand factor antigen (VWF:Ag)] or endothelial injury (fibronectin), oxidative stress [malondialdehyde (MDA)] and trophoblast debris (cell-free fetal DNA).

Methods: Forty-four patients with preeclampsia and 44 healthy pregnant women matched for age and gestational age were involved in this case-control study. Plasma OPN concentrations were measured with ELISA. Serum CRP concentrations were determined with an autoanalyzer using the manufacturer's reagents. Plasma VWF:Ag was quantified by 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 in maternal plasma was determined by quantitative real-time PCR analysis of the sex-determining region Y (*SRY*) gene. For statistical analyses, non-parametric methods were applied.

Results: Serum levels of CRP, as well as plasma concentrations of VWF:Ag, fibronectin, MDA and cell-free fetal DNA were significantly higher in preeclamptic patients than in healthy pregnant women. There was no significant difference in plasma OPN concentrations between controls and the preeclamptic group. However, preeclamptic patients with plasma fibronectin concentrations in the upper quartile had significantly higher plasma OPN concentrations than those below the 75th percentile, as well as healthy pregnant women [median (interquartile range): 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]. Furthermore, in preeclamptic patients, plasma OPN concentrations showed a significant positive linear association with plasma fibronectin (Spearman $R = 0.38$, standardized regression coefficient (β) = 0.41, $p < 0.05$ for both).

Conclusions: Plasma OPN concentrations are increased in preeclamptic patients with extensive endothelial injury. However, further studies are warranted to explore the relationship between OPN and endothelial damage.

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Keywords: endothelial injury; fibronectin; osteopontin; preeclampsia; pregnancy.

Introduction

Preeclampsia is a severe complication of human pregnancy with a worldwide incidence of 2%–10% (1). It is one of the leading causes of maternal, as well as perinatal morbidity and mortality, even in developed countries. Despite intensive research efforts, the etiology and pathogenesis of preeclampsia are not completely understood. Increasing evidence suggests that an excessive maternal systemic inflammatory response to pregnancy with systemic oxidative stress and resultant endothelial damage plays a crucial role in the pathogenesis of the disease (2, 3). The development of preeclampsia is influenced by both genetic and environmental risk factors, suggesting a multifactorial inheritance (4–9).

Osteopontin (OPN) is a 70-kDa glycoprotein which was demonstrated to be present in the endometrium (10), where it is indicative of a decidualization-like process during pregnancy. It is involved in adhesion and signal transduction at the uterine-placental interface (11). OPN has also been found in the trophoblast of the human placenta, and it can enhance the invasiveness of trophoblast cells in vitro (12). Furthermore, OPN is expressed by endothelial, vascular smooth muscle cells (VSMCs) and immune cells (13, 14), and it acts as a proinflammatory Th1 type cytokine (15). The role of

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OPN is considered in several physiological and pathological states, such as embryo implantation, placentation, chronic inflammation and autoimmune diseases (11, 16–20).

Local overexpression and elevated plasma concentrations of OPN were observed in aortic, coronary and carotid atherosclerosis (21–24). It was shown that OPN is a component of atherosclerotic plaques and mediates arterial neointima formation as well as dystrophic calcification in the vessel walls (25). These indicate the significance of OPN in the maintenance of chronic inflammation and in plaque formation in atherosclerosis. Interestingly, 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 (26).

In the present study, we measured plasma OPN concentrations in healthy pregnant women and patients with preeclampsia. Given that plasma OPN has recently been observed to be increased in atherosclerotic cardiovascular disorders and that atherosclerosis shares many common pathophysiological mechanisms with preeclampsia, including inflammation and oxidative stress in which OPN has been implicated, we hypothesized that plasma OPN concentrations are increased in preeclampsia. To our knowledge, this is the first report on plasma OPN concentrations in preeclampsia.

We determined several markers of processes involved in the pathogenesis of preeclampsia, such as C-reactive protein (CRP), von Willebrand factor antigen (VWF:Ag), fibronectin, malondialdehyde (MDA) and cell-free fetal DNA. Increased CRP concentrations represent systemic inflammation characteristic of preeclampsia (27, 28). Plasma VWF:Ag concentrations indicate endothelial activation (29), while plasma fibronectin concentration is a reliable marker of endothelial injury in preeclampsia (30–33). The latter was shown to reflect the severity, organ involvement and complications of the disorder (34, 35). Additionally, fibronectin was found to predict the development of preeclampsia both in normotensive and in hypertensive pregnant women (34, 36, 37). Plasma concentrations of MDA also were demonstrated to be increased in preeclampsia as a consequence of placental and systemic oxidative stress (33, 38–40). Trophoblast particles can frequently be detected in the maternal peripheral circulation in preeclampsia. The mass of this trophoblast debris can be estimated by measurement of copies of cell-free fetal DNA in the maternal blood. Significant increases were observed in preeclampsia compared to normal pregnancies before and after the onset of clinical symptoms (41–44). We also examined whether these laboratory markers, as well as the clinical features of the study subjects, were related to OPN concentrations in our study groups.

Materials and methods

Study participants

Our study was designed using a case-control approach. Forty-four preeclamptic patients (19 with severe disease) and 44 normotensive

(blood pressure <140 mm Hg systolic and <90 mm Hg diastolic), healthy pregnant women with uncomplicated pregnancies matched for age and gestational age were involved in the study. The sample size provided sufficient statistical power (>80% at a Type I error rate of 0.05) to detect differences in plasma OPN concentrations between cases and controls which have been observed previously in coronary artery disease (CAD) (22). The study participants were enrolled in the First Department of Obstetrics and Gynecology and in the Department of Obstetrics and Gynecology of Kútvolgyi Clinical Center, at the Semmelweis University, Budapest, Hungary. All women were Caucasian and resided in the same geographic area in Hungary. Exclusion criteria were multifetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection and fetal congenital anomaly. The women were fasting, none were in active labor, and none had rupture of membranes.

Preeclampsia was defined as increased blood pressure (≥ 140 mm Hg systolic or ≥ 90 mm Hg diastolic on ≥ 2 occasions at least 6 h apart) that occurred after 20 weeks of gestation in a woman with previously normal blood pressure, accompanied by proteinuria (≥ 0.3 g/24 h). Blood pressure returned to normal by 12 weeks postpartum in each study patient with preeclampsia. Preeclampsia was regarded as severe if any of the following criteria was present: blood pressure ≥ 160 mm Hg systolic or ≥ 110 mm Hg diastolic, or proteinuria ≥ 5 g/24 h. Pregnant women with HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) were not enrolled in this study. 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 (45).

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the Semmelweis University, and written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki.

Biological samples

Maternal blood samples were obtained from the antecubital vein into plain tubes, EDTA or sodium citrate, and then centrifuged at room temperature with a relative centrifugal force of 3000 *g* for 10 min. The aliquots of serum and plasma were stored at -80°C until the analyses. To determine OPN, fibronectin, MDA and cell-free fetal DNA concentrations, we used EDTA-anticoagulated plasma samples, while VWF:Ag was measured in citrated plasma.

Laboratory methods

Quantitative detection of plasma OPN was performed using the Human Osteopontin ELISA assay (DRG International, Inc., Mountainside, NJ, USA, Cat. No. EIA-3116) according to the manufacturer's protocol. The sensitivity of the assay was 0.11 ng/mL, and the intra-/inter-assay coefficient of variation (CV) was <5%/<10%, respectively. Serum CRP concentrations were determined using the Cobas Integra 800 (Roche, Mannheim, Germany) and the manufacturer's kits. The lower detection limit was 0.07 mg/L. Plasma VWF:Ag concentrations were quantified using ELISA (Dakopatts, Glostrup, Denmark), while plasma fibronectin concentrations were measured by nephelometry (Dade Behring, Marburg, Germany) according to the manufacturer's instructions. The sensitivities of these assays were 6.16% and 0.01 g/L, respectively.

Concentrations of thiobarbituric acid reactive substances in blood plasma were estimated according to the method of Placer et al. (46). The assay procedure was based on the addition of 2-thiobarbituric

acid with MDA as the end-product of lipid peroxidation, under acidic conditions (pH = 2.0) and high temperature (100°C). The assay was calibrated using 1,1,3,3-tetraethoxy-propane (Fluka, Buchs, Switzerland) as a source of MDA. Concentrations of blood plasma MDA were calculated as nmol/mL of plasma. The limit of quantification was 0.82 nmol/mL.

The amount of cell-free fetal DNA in maternal plasma was determined in patients with male newborns by quantitative real-time PCR analysis of the sex determining region Y (SRY) gene, as we described previously (47). Briefly, DNA was extracted from 400 µL EDTA anticoagulated plasma with the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The DNA was eluted in 50 µL of elution buffer solution, and 1 µL was used as a template for the PCR reaction. For the SYBR Green real-time PCR analysis, we used a LightCycler 1.0 System (Roche Diagnostics, Mannheim, Germany). Circulating male fetal DNA was detected with the following primers for the SRY gene: forward 5'-GGC AAC GTC CAG GAT AGA GTG A-3', reverse 5'-TGC TGA TCT CTG AGT TTC GCA TT-3'. The PCR analysis was performed in 10 µL reaction volumes containing 1 µL DNA, 2.5 pmol/L of each amplification primer, 1 µL of DNA Master SYBR Green I mix (LightCycler FastStart DNA Master SYBR Green I kit: Taq polymerase, dNTP, MgCl₂), and 6 µL of nuclease free water. The PCR was performed in 40 cycles under the following conditions: initial denaturation 8 min at 95°C, 5 s denaturation at 95°C, 10 s annealing at 60°C, 15 s extension at 72°C, cooling to 4°C. To determine the number of copies of circulating DNA present in the plasma sample, a standard dilution curve with a known concentration of male genomic DNA was used. All samples were analyzed in duplicate and scored in blinded fashion. The detection limit was 1.5 pg/L.

Statistical analysis

Normal distribution of continuous variables was assessed using the Shapiro-Wilk's W-test. As the continuous variables were not normally distributed, non-parametric statistical methods were used. To compare continuous variables between two groups, the Mann-Whitney U-test was applied. Fisher's exact and Pearson's χ^2 -tests were performed to compare categorical variables between groups. The Spearman rank order correlation was performed to calculate correlation coefficients. As plasma OPN concentrations showed a skewed distribution, multiple linear regression analysis was performed and the scatterplot was created with logarithmically transformed data.

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). For all statistical analyses, $p < 0.05$ was considered statistically significant.

Data are reported as median (interquartile range) for continuous variables and as number (percent) for categorical variables.

Results

Patient characteristics

The clinical characteristics of the study participants are described in Table 1. There were no statistically significant differences in terms of maternal age and gestational age at blood collection and the percentage of smokers and primiparas between the two study groups. The body mass index (BMI) and systolic and diastolic blood pressures were significantly higher, while the gestational age at delivery and the fetal birth weight were significantly lower in the preeclamptic group compared with the control group. Fetal growth restriction was absent in control subjects, whereas the frequency of this condition was 25.0% in the preeclamptic group.

Laboratory parameters

As shown in Table 2, serum concentrations of CRP as well as of plasma concentrations of VWF antigen, fibronectin, MDA and cell-free fetal DNA were significantly higher in patients with preeclampsia compared with normotensive, healthy pregnant women. However, there was no significant difference in plasma OPN concentrations between the control and the preeclamptic group (Table 2).

We investigated whether clinical characteristics and laboratory parameters of the study participants were related to plasma OPN concentrations by calculating the Spearman rank order correlation coefficients (continuous variables) or by the Mann-Whitney U-test (categorical variables). As shown in Figure 1, in preeclamptic patients, plasma OPN

Table 1 Clinical characteristics of normotensive, healthy pregnant women and patients with preeclampsia.

	Controls (n = 44)	Preeclampsia (n = 44)	Statistical significance (p-value)
Age, years	30.5 (28–32.5)	29 (26–32.5)	NS
BMI at blood draw, kg/m ²	26.1 (24.3–28.2)	29.9 (25.7–34.0)	<0.05
Smokers	0 (0%)	3 (6.8%)	NS
Primiparas	27 (61.4%)	28 (63.6%)	NS
Systolic blood pressure, mm Hg	112.5 (110–120)	169.5 (160–180)	<0.001
Diastolic blood pressure, mm Hg	70 (60–80)	100 (100–110)	<0.001
Gestational age at blood draw, weeks	36 (36–37)	37 (35.5–38)	NS
Gestational age at delivery, weeks	39 (38–40)	38 (36–38)	<0.001
Fetal birth weight, g	3450 (3025–3550)	2900 (2225–3350)	<0.001
Fetal growth restriction	0 (0%)	11 (25.0%)	<0.001

Data are presented as median (25th–75th percentile) for continuous variables and as number (%) for categorical variables. NS, not significant; BMI, body mass index.

Table 2 Laboratory parameters of normotensive, healthy pregnant women and patients with preeclampsia.

	Controls (n=44)	Preeclampsia (n=44)	Statistical significance (p-value)
Serum CRP, mg/L	3.59 (1.68–7.40)	6.11 (1.92–12.12)	<0.05
Plasma VWF:Ag, %	148.4 (106.6–199.0)	183.0 (128.7–242.8)	<0.05
Plasma fibronectin, g/L	0.36 (0.32–0.47)	0.58 (0.39–0.82)	<0.001
Plasma malondialdehyde, nmol/mL	13.13 (8.38–18.61)	18.17 (14.98–20.31)	<0.05
Plasma fetal DNA, pg/ μ L	0.005 (0.0–0.178)	0.065 (0.034–0.267)	<0.05
Plasma osteopontin, ng/mL	7.40 (6.51–8.80)	7.77 (6.60–9.67)	NS

Data are presented as median (25th–75th percentile). NS, not significant; CRP, C-reactive protein; VWF:Ag, von Willebrand factor antigen.

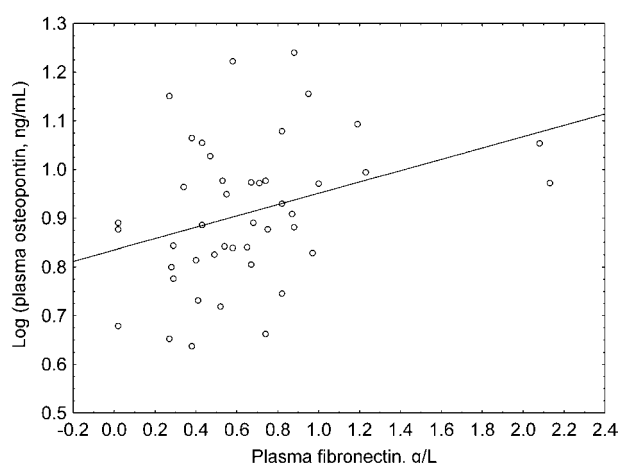


Figure 1 Scatterplot with linear fit and regression line of logarithmically transformed values of plasma osteopontin vs. plasma fibronectin concentrations in preeclamptic patients (Spearman $R=0.38$, standardized regression coefficient (β)=0.41, $p<0.05$ for both).

showed a significant positive linear association with plasma fibronectin concentrations (Spearman $R=0.38$, $p<0.05$), even after adjustment for BMI in multiple linear regression analysis (standardized regression coefficient (β)=0.41, $p<0.05$). However, no other relationship was found between clinical features and the measured laboratory parameters (serum CRP, plasma VWF:Ag, MDA and cell-free fetal DNA) of the study subjects and plasma OPN concentrations in normotensive, healthy pregnant women, or in preeclamptic patients (data not shown).

Given the association between plasma OPN and fibronectin concentrations in preeclampsia, we divided our preeclamptic group on the basis of plasma fibronectin 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 [median (interquartile range): 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; Figure 2]. 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%),

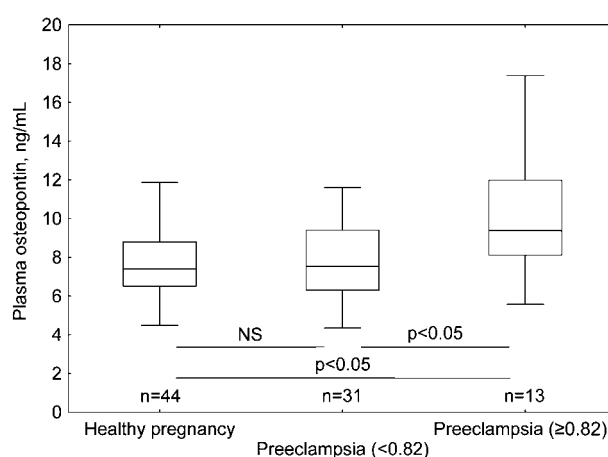


Figure 2 Plasma osteopontin concentrations in preeclamptic patients with plasma fibronectin in the upper quartile (≥ 0.82 g/L, $n=13$), below the 75th percentile (<0.82 g/L, $n=31$) and in healthy pregnant women ($n=44$).

Middle line: median; box: interquartile range; Whisker: range (excluding outliers); NS, not significant.

$p<0.05$], as well as by significantly higher diastolic blood pressure [median (interquartile range): 110 (100–120) vs. 100 (98–110) mm Hg, $p<0.05$], as compared to preeclamptic women with fibronectin concentrations in the lower three quartiles.

Discussion

In this study, we found that 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.

Several clinical surveys reported an association between altered OPN concentrations and cardiovascular diseases. Ohmori et al. observed that OPN plasma concentrations correlate with the extent of CAD (22). Coskun et al. found higher OPN concentrations in patients with acute coronary syndrome compared to patients with stable angina (48). Soe-

jima et al. showed that OPN production in peripheral T cells is increased in relation to the severity of heart failure (49). OPN presumably plays an important role in the development of atherosclerosis (25, 50), acting in two different ways. First, OPN participates in the maintenance of chronic systemic inflammation through its cytokine and chemokine effects (17, 51). Second, Georgiadou et al. showed that in blood samples of patients with CAD, OPN concentrations are independently associated with MDA concentrations; a well-known biomarker of lipid peroxidation and oxidative stress (23). Systemic inflammation with an excessive Th1 response and oxidative stress also are essential factors in the pathogenesis of preeclampsia (39, 52). However, despite these facts, we could not establish any association between OPN concentrations and the markers of inflammation (CRP) or oxidative stress (MDA) in preeclampsia. It is possible that overproduction of OPN by activated peripheral blood mononuclear cells was masked by its rapid consumption during the exaggerated inflammatory processes in preeclampsia.

The extravillous trophoblasts of the human placenta also express OPN, which regulates the invasiveness of these cells (12). Gabinskaya et al. studied human placentas with immunostaining. They showed that after the 30th gestational week, OPN is only present in cytotrophoblasts of preeclamptic placentas, but not in healthy ones (53). It was previously reported that a significantly higher amount of trophoblast debris can be found in the maternal circulation in preeclampsia compared to women with a normal pregnancy (41, 42, 54). Based on these observations, we examined the association between the concentrations of circulating fetal DNA as a marker of trophoblast debris, and OPN in maternal blood. However, we could not detect any relationship. Thus, we hypothesize that OPN can have only a local placental effect in preeclampsia.

Endothelial activation and injury play an important role in the pathogenesis of atherosclerosis, and OPN may participate in this process (13). Like atherosclerosis, preeclampsia is characterized by endothelial activation and injury. Therefore, we investigated whether plasma OPN concentrations are related to markers of endothelial activation (VWF:Ag), or endothelial damage (fibronectin) in preeclampsia. However, there was no relationship between VWF:Ag and OPN. Instead, we found a positive linear association between fibronectin and OPN concentrations. Furthermore, OPN concentrations were significantly higher in preeclamptic patients whose fibronectin concentrations were in the upper quartile compared to patients with preeclampsia with fibronectin below the 75th percentile, as well as to healthy pregnant women. In addition to having increased OPN concentrations, preeclamptic women with high fibronectin concentrations had a more severe form of the disease. OPN and fibronectin are components of the extracellular matrix and both contain an arginine-glycine-aspartic acid (RGD) sequence, which can bind integrin molecules. Interestingly, intermolecular cross-linking can be formed between these two proteins (55). Therefore, we suppose that OPN may be released from the vessel wall into the peripheral circulation together with fibro-

nectin in cases of extensive endothelial injury. This might explain the observed association between plasma OPN and fibronectin concentrations in preeclampsia. However, the relationship of OPN to other markers of endothelial dysfunction or injury in preeclampsia, such as circulating endothelial cells (56), thrombomodulin (57), endothelin-1 and E-selectin (33) should be investigated in future studies to confirm our findings.

Preeclampsia is a heterogeneous syndrome with various clinical manifestations. We identified a subgroup of preeclamptic patients with significantly increased plasma OPN concentrations. Defining more homogeneous study populations in this way may help to understand the complex etiology and pathogenesis of this multifactorial disorder.

The limitations of our study are its case-control design and the relatively low number of patients involved. Also, we did not compare OPN production of peripheral blood mononuclear cells between cases and controls.

In conclusion, plasma OPN concentrations are increased in preeclamptic patients with extensive endothelial injury. However, further studies are warranted to explore the association of OPN with endothelial damage.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. The funding sources played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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