Arch Gynecol Obstet (2017) 296:495–502
DOI 10.1007/s00404-017-4461-2

MATERNAL-FETAL MEDICINE

Is there any relationship between adipocytokines and angiogenesis factors to address endothelial dysfunction and platelet aggregation in untreated patients with preeclampsia?

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Received: 28 February 2017 / Accepted: 10 July 2017 / Published online: 13 July 2017
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Abstract
Purpose Preeclampsia is a multisystem disorder and its etiology remains still unclear. Recent hypotheses rely on imbalance between angiogenic and antiangiogenic factors and disruption of endothelial function of spiral arteries. In addition; increased VTE (venous thromboembolism) risk is still unclear in preeclampsia. Our aim was to investigate the relationship between endothelial dysfunction, adipocytokines, platelet function, and vasculogenesis in preeclampsia.
Methods Plasma angiogenic (PlGF, VEGF), antiangiogenic factors (sflt-1, endoglin) with adipocytokines (leptin, adiponectin, ghrelin), endothelial dysfunction markers (vWF, NO), and platelet function markers (ADP and collagen induced platelet aggregation, P-selectin) were examined in 30 early-onset, 22 late-onset preeclampsia, and 27 healthy pregnants. Enzyme-linked immunosorbent assay (ELISA) was used to determine the serum biomarker levels except NO. NO levels were determined using colorimetric method.
Results Endoglin, leptin, and vWF levels were increased in preeclampsia ($P < 0.001$), whereas PlGF, P-selectin ($P < 0.001$), and col-induced platelet aggregation slope ($P < 0.05$) were decreased in the same counterpart as compared to healthy pregnants. Endoglin also correlated with sflt-1 in preeclamptic patients.
Conclusion Increase in the levels of antiangiogenic factors and leptin herewith decline in the level of other angiogenic factor PlGF, did not affect nitric oxide and platelet aggregation markers significantly. Increased levels of vWF and endoglin might be result of endothelial dysfunction, so our findings suggest that an impaired angiogenesis may address endothelial dysfunction, but not platelet aggregation for preeclampsia.

Keywords Preeclampsia · Endoglin · Sflt-1 · Leptin · vWF · Endothelial dysfunction · Platelet aggregation

Introduction

Pre-eclampsia (PE) is a multisystem disorder of human pregnancy, characterised by hypertension and proteinuria, which is seen after the 20th week of gestation. According to the World Health Organization (WHO) and Confidential Enquires into Maternal Deaths in the United Kingdom [1], preeclampsia is the major causes of increased maternal and neonatal morbidity and mortality worldwide. As well as preeclampsia, its complications also affect the mother or fetus [2] and lead to further diseases in their later life. Unless the pathogenesis of PE is still unclear, defective placentation [3], inadequate trophoblast invasion [4–6], placental ischemia [7, 8], increased oxidative stress [9, 10, 16], release of antiangiogenic proteins into maternal plasma [9], excessive maternal inflammatory response [5], endothelial dysfunction [5, 9], and hypertension [5, 8, 9] included in the pathogenesis of the disease. In pregnancies, vascular endothelial cells proliferate and form a network of capillaries by angiogenesis and placental vasculogenesis is

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in balance among several factors and controlled by antiangiogenic factors [11]. The VEGF and Placental growth factor (PlGF) are angiogenic proteins which bind to antiangiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng). sFlt-1, binds VEGF and PlGF with high affinity and leads to reduce free VEGF levels and PlGF so, sFlt1 suppresses placental angiogenesis. On the other hand, hypoxia in the decidua in early phase of gestation increase HIF1-alpha, suppress normal trophoblast invasion for normal placenta by increasing TGFbeta3; this leads to increase s-eng in trophoblasts. S-eng is coreceptor, of TGF-β, promotes hypoxia, suppresses the vasorelaxion activity of TGF-β1, decreases NO level, and leads to endothelial dysfunction [12–14].

Recent studies show that adipokines; adiponectin, ghrelin, and leptin play a crucial role in the healthy pregnancy and preeclampsia. Leptin inhibits high apoptosis rate of trophoblastic cells, those seen during PE. Leptin, as an angiogenic factor, supports blood supply to the placenta together with other angiogenic factors such as VEGF and PIGF. Vitro studies show that leptin also promotes placental nutrient transporters and increases amino acid uptake by human syncytiotrophoblast cells [15–17]. Adiponectin might lessen the excessive inflammatory response in the vascular wall in PE, decrease CRP, and increase nitric oxide production [18, 19]. Ghrelin is a gastric mucosa-derived peptide having opposing role in the control of appetite. Beside this, ghrelin might also take place in blood pressure regulation in human [20].

Recently, endothelial dysfunction markers P-selectin and vWF have been paid more attention in the pathogenesis of preeclampsia. Both are stored by platelets and endothelial cells and can be released as a response to prothrombotic and inflammatory factors. P-selectin and vWF can induce accumulation of platelets, leukocytes, and pro-coagulant factors which will promote wall injury and thrombus formation [21, 22].

Emerging data suggest that disruption in vasculogenesis, abnormal haemostatic activation, and inflammation might have impact on pathogenesis of preeclampsia. The present study was undertaken to evaluate the relationship between endothelial dysfunction, adipocytokines, platelet function, and vasculogenesis among the stages of preeclampsia.

Materials and methods

Patients

A Total of 52 preeclamptic pregnant were stratified as 30 early-onsets (symptoms occur <32 gestational weeks) and 22 late-onset preeclamptic (symptoms occur >32 gestational weeks), and 27 healthy pregnant women as a control group were included into the study. The control group was matched for maternal and gestational age and body mass index (BMI) at time point of sampling with preeclamptic counterparts. All patients and controls were Caucasian race of Turkish descent. All participants were informed about the survey and freely signed and dated the consent form. The protocol was approved by the Ethics Committee of Cerrahpasa Medical Faculty and was conducted in accordance with the Declaration of Helsinki. Peripheral blood samples from patients with preeclampsia and controls were obtained from the department of Obstetrics and Gynecology in Cerrahpasa Medical School. Preeclampsia was defined as a new-onset hypertension SBP >140 mmHg or DBP >90 mmHg, observed on at least two separate measurements of 6 h or more apart, combined with proteinuria >300 mg per 24 h or >2 on a dipstick [23]. Exclusion criteria were women with infection, multiple pregnancies, chronic hypertension, diabetes mellitus, having previous history of venous or arterial thromboembolic disease, chronic kidney disease, systemic inflammatory disorders (such as systemic lupus erythematosus), anti-phospholipid syndrome or current exposure to antiplatelet and/or anticoagulant therapy, nonsteroidal anti-inflammatory drugs, antidepressants or oral contraceptives.

Samples

Plasma samples for PLGF, VEGF, sflt-1, TGFβ1, HIF-1, endoglin, adiponectin, vWF, leptin, ghrelin, NO, P-selectin, and platelet aggregation determination were collected from venous blood after a −12 h fasting on 0, 5 ml 3, 8% citrate/5 ml of blood. It was centrifuged 3500*g* for 15 min. The separated plasma was stored in several small aliquots at −70 C until being assayed.

Laboratory methods

Enzyme-linked immunosorbent assay (ELISA) procedure was used to determine the plasma PIGF (DRG, catalog no EIA-4529), VEGF (Aviscera Bioscience catalog no SK00122-01), sflt-1 (Aviscera Bioscience CA, USA catalog no SK00114-06), TGFβ1 (Aviscera Bioscience CA, USA catalog no SK00033-02), HIF-1 (USCN, China catalog no E90798Ra), endoglin (Aviscera Bioscience, CA, USA catalog no SK00697-01), adiponectin (Assay Pro, MO, USA catalog no EA2500-1), vWF (Assay Pro, MO, USA catalog no EV2030-1), leptin (Assay Pro, MO, USA catalog no EL2001-1), ghrelin (Millipore, MS, USA catalog no: EZGRT-89K), P-selectin (Aviscera Bioscience catalog no SK00093-02), NO levels (MBL, MA, USA catalog no JM-K262-200) were determined by colorimetric method.

Platelet function was evaluated by an ADP-induced platelet aggregation method conventionally measured by optical density [24].

Platelet reactivity ex vivo was assessed by PRP (platelet rich plasma) aggregometer using a ChronoLog after stimulating samples, collagen (1 mg/mL), and adenosine di-phosphate (ADP) (1 mmol/L). Peak aggregation within 5 min of agonist stimulation was recorded in ohms and 5 µL of ADP was added to 495 µL sample for a final concentration of 10 µM. Then, the tube was transferred to the sample containers of the aggregometer and kept at 37 °C for 3 min, after which it was treated with ADP (Chrono log) for 3 min. Platelet aggregation was observed and the aggregation curve taken from the aggregometer (Chronolog 500, USA) was evaluated in terms of slope and amplitude percentage (slope *W*, amplitude activity %). Collagen was used instead of ADP for collagen-induced platelet aggregation [25, 26].

Statistical analysis

Analysis of data was done with SPSS statistical analysis software (version 20.0; SPSS Inc, Chicago, IL, USA). Results were expressed as mean ± SD or median- interquartile range. VEGF, PLGF levels were logarithmically transformed, to achieve normal distributions. General linear measurement analyses, used for anthropometric and metabolic parameters or group means, were compared using Chi square or student's *t* test. Correlation analysis was done by Pearson correlation analysis. Multiple stepwise regression analysis was applied to predict the variables that independently and significantly contributed to the dependent variable (preeclampsia). All analyses were two-tailed, and *P* values less than 0.05 were considered statistically significant.

Results

Characteristics of study population are represented in Table 1. Preeclamptic and control pregnants were at same age and their gestational age, BMI, and blood sampling weeks were also similar ($P > 0.05$). Systolic/diastolic blood pressures were significantly higher in preeclampsia than control pregnants ($P = 0.000$).

In Table 2, we examined angiogenic, antiangiogenic factors, adipocytes, and platelet aggregation markers. Compared with control pregnants, preeclamptic patients had higher leptin ($P = 0.000$), vWF ($P = 0.000$), endoglin levels ($P = 0.000$), significantly lower PlGF ($P = 0.000$), P-selectin ($P = 0.003$), and collagen S (slope) ($P = 0.020$) levels.

We next analyzed the association of plgf/sflt-1 ratio, endoglin and vWF among early-onset (EOP), late-onset (LOP), and control pregnants (Fig. 1). There was a stepwise decrease in plgf/sflt-1 ratio across control group, EOP, LOP (Fig. 1a). There was a proportional decline seen for early-onset subjects. There was a stepwise increase in endoglin tertiles with highest levels for the third tertile in EOP (Fig. 1b). The second tertile of endoglin was higher for late-onset pregnants. There was also a stepwise increase among vWF tertiles with highest levels for the third tertile in EOP; the second and third tertiles of vWF also had higher subjects in late onset of preeclampsia (Figure 1c). For healthy pregnants, the picture was the same for endoglin and vWF with stepwise decline among tertiles.

Then we examined the correlation of parameters, related with impaired endothelial function in control pregnants and preeclampsia (Table 3). There were positive correlations between sflt-1 and endoglin ($r = 0.392$, $P < 0.01$), only in preeclampsia. Nitric oxide was positively associated with endoglin ($r = 0.373$, $P < 0.01$) in preeclamptic subjects. In control group PLGF was only positively associated with NO ($r = 0.535$, $P < 0.01$) and negatively associated with vWF ($r = 0.459$, $P < 0.05$). We did not find any association between platelet aggregation and any other parameters we tested.

Finally, we performed a stepwise multiple logistic regression analysis to identify variables that independently and significantly contributed to the presence of preeclampsia. In univariate analysis, incline in endoglin level was

Table 1 Characteristics of study population (mean ± SD)

	Preeclampsia ($n = 52$)	Control pregnants ($n = 27$)	*P* value
Age (years)	30.02 ± 5.48	30.75 ± 5.04	NS
Systolic BP (mmhg)	155.74 ± 19.09	109.38 ± 9.13	0.000
Diastolic BP (mmhg)	100.93 ± 14.31	68.44 ± 8.46	0.000
BMI (kg/m^2)	30.94 ± 5.54	29.61 ± 3.23	NS
Gestational age at birth	33.22 ± 3.63	39.19 ± 1.42	0.000
Gestational age at blood sampling	31.9 ± 4.2	32.3 ± 4.1	NS

Data are expressed as mean ± SD; group means were compared by student's *t* test

NS not significant

Table 2 Angiogenic, antiangiogenic factors, and platelet aggregation markers in preeclampsia and control pregnants

	Preeclampsia ($n = 52$)	Control pregnants ($n = 27$)	P value
Ghrelin (pg/mL)	321.54 ± 42.18	322.28 ± 8.71	NS
Leptin (ng/mL)	126.71 ± 41.67	75.38 ± 28.76	0.000
Adiponectin (μg/mL)	17.33 ± 10.75	17.93 ± 7.67	NS
TGFβ1 (ng/mL)	9.11 ± 6.03	8.84 ± 5.56	NS
vWF (U/mL)	5.89 ± 2.52	3 ± 1.4	0.000
Sflt-1 (ng/ml)	9.99 ± 1.97	10.12 ± 5.03	NS
VEGF (pg/mL)	20.94 (18.13–32.84)	33.07 (9.05–48.02)	NS
Endoglin (ng/mL)	10.22 ± 1.13	8.78 ± 1.23	0.000
PLGF (pg/mL)	59.28 (22.57–101.27)	629.97 (238.63–825.90)	0.000
Plgf/sflt-1	6.46 (2.54–10.39)	61.57 (15.88–128.92)	0.000
p-Selektin (ng/mL)	114.25 ± 64.85	178.7 ± 62.03	0.003
HIF-1 (ng/mL)	1.20 ± 0.33	1.39 ± 0.52	NS
Nitric Oxide (μM)	15.08 ± 11.7	13.56 ± 6.42	NS
ADP-S (ohms)	66.92 ± 12.65	66.47 ± 15.71	NS
ADP-A (%)	77.78 ± 17.61	84.82 ± 23.32	NS
Collagen-S (ohms)	74.4 ± 19.36	85.8 ± 18.85	0.020
Collagen-A (%)	89.73 ± 13.63	96.67 ± 18.12	NS

Data are expressed as mean ± SD, or median (interquartile range) for non-normally distributed variables. Values for VEGF, PlGF, PlGF/sflt-1 were logarithmically transformed before inference analyses. Nontransformed values are shown. Group means were compared by student's *t* test

NS not significant

associated with moderate odds ratio for preeclampsia [odds ratio (OR) 1.001, $P = 0.000$] (Table 4, model 1A). Further, in a multivariate logistic regression model (model 2A) after adjusting for other endothelial dysfunction markers, endoglin [odds ratio (OR) 1.001, $P = 0.023$] was independently associated with preeclampsia.

The test performance for the most promising individual biomarkers are defined by ROC areas. Endoglin had the highest ROC area (0.78; 95% CI 0.64–0.91) for determining early preeclampsia; further, integration of endoglin with sflt-1 (as a ratio) did not change the ROC (0.78 vs 0.77) for EOP. PLGF had the second higher ROC area (0.74; 95% CI 0.61–0.89) and combination with sflt-1 (as a ratio) did not improve the predictive power for EOP. For late onset of preeclampsia, vWF had the highest ROC area (0.68; 95% CI 0.56–0.83).

Discussion

In the present study, we investigated the relationship between adipocytokines, angiogenesis, and endothelial dysfunction. We report that vWF is a more accurate endothelial dysfunction marker for preeclampsia. Hyperleptinemia and increased vWF levels in preeclampsia might partially increase platelet aggregation; however, these changes did not significantly affect platelet aggregation.

The vascular endothelium synthesizes certain biomolecules for regular vascular function. The VonWillebrand factor (vWF) is an important glycoprotein in the regulation of haemostasis and its level increases in plasma as a result of endothelial damage. vWF acts on the platelets, increases their adhesion to sub endothelial layer, and contributes to thrombus formation [27]. Platelet-derived microparticles (PMPs) are released from platelets as a consequence of vascular injury. We found an increase in the level of vWF in preeclampsia compared to healthy pregnants. Our finding was supported by other recent studies [28–30]. We found decreased P-selectin levels in preeclamptic pregnants compared to control group. P-selectin is a cell-adhesion molecule and responsible for the adhesion of certain leukocytes and platelets to the endothelium. We also did not find any significant change in platelet aggregation markers between groups, except for collagen E. Decreased levels of P-selectin and collagen E further the adverse relationship between vWF and P-selectin which revealed that platelet activation was marked somehow by other factors. Circulating levels of sFlt-1 are elevated in the serum of women with preeclampsia and the source was thought to be placental. Recently, it has been shown that sFlt-1 also released from monocytes; however, the stimulus has not been defined yet [31, 32]. We could not find any difference in sflt-1 levels between groups. Nitric oxide is another important molecule which contributes vasodilation and inhibition of platelet activation [33]. We found a significant association between P-selectin

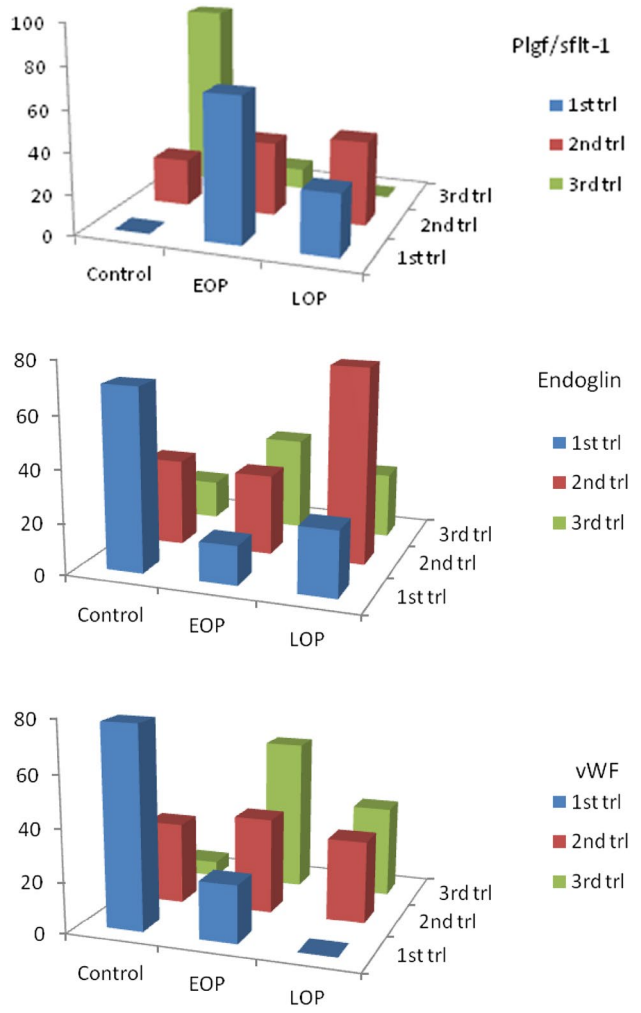

Fig. 1 Association of plgf/sflt-1, endogiln and vWF among EOP, LOP and control pregnants. PlGF/sflt-1 ratio, endoglin, vWF tertiles across EOP,LOP, control pregnants. Data are mean ± SD. *P* values calculated using one-way ANOVA test. Values for PlGF/sflt-1 were logarithmically transformed before analysis. Nontransformed values are shown in the *graphs*

and NO levels for preeclampsia. Again, we could not find any difference in NO levels between groups. Yoneyama et al. have shown that, in vitro blockade of NO synthesis, increase P-selectin on platelets in normal pregnancy and preeclampsia; however, the extent of increase was more profound in healthy pregnants than in preeclampsia [34]. The results are consistent with the concept that NO may act in a compensatory manner to counteract platelet activation in preeclampsia and the inhibitory effects in preeclampsia may be a possible contributor factor to the disease pathogenesis.

Another possible explanation for intriguing alterations between NO, P-selectin, vWF, and platelet aggregation might be the increased level of endoglin. Incline in plasma sEng concentration was shown in various metabolic and cardiovascular diseases such as hypercholesterolemia/atherosclerosis [35], preeclampsia [36], hypertension, and type II diabetes mellitus [37]. In our study group, we showed that s-endoglin levels were increased in preeclampsia. Interestingly, there was a significant correlation between NO and endoglin for preeclamptic subjects. Some researchers suggested that s-eng contribute endothelial dysfunction via alteration of NO-dependent vasodilatation [38, 39]. Increased endoglin level and decreased PlGF might be the reason for impaired NO level which might mask platelet activation, represented by low P-selectin levels in the preeclamptic group. As discussed above, and consensus with other researchers, our results supported the importance of endoglin as an endothelial dysfunction marker. We noted decreased PLGF levels in preeclampsia and no significant change for VEGF levels in the same counterpart. Our results are in agreement with others [8].

We also checked the levels of adipocytokines (ghrelin, adiponectin, leptin) in preeclampsia and control pregnants, except for leptin, we could not find any significant difference between them. Leptin levels were increased in preeclamptic pregnants when compared to controls. We could not find any correlation between either adipocytokines or any other parameters. Possible mechanism suggested for increased leptin levels in preeclampsia is explained by increased nutrient delivery for the fetus to maintain optimal growth. To the best of our knowledge, leptin plays a role in angiogenesis, immunomodulation, and fatty acid metabolism [40, 41]. Murayama et al. stated that platelets have long form of leptin receptor on their surfaces and contribute to the phosphorylation of specific platelet proteins [42, 43]. Nakata et al. also suggested that leptin at high concentrations has an impact on platelet activation [44]. Controversial results have been published regarding the circulating levels of adiponectin and leptin in PE. Most studies, like our results, have demonstrated increased leptin levels in PE [45, 46]. Some studies reported increased adiponectin levels in PE [47, 48]; we did not find any difference; however, others have found no differences [49, 50] or even decreased [51, 52] levels for PE. Adiponectin stimulates the production of NO in endothelial cells directly via phosphorylation of endothelial NO synthesa (eNOS) by AMPK [53]. Besides the role in regulating apatite, ghrelin might also play a role in blood pressure regulation in human and in rats [54, 55]. Some studies showed that patients with pregnancy-induced hypertension have significantly lower levels of ghrelin compared to normal pregnants, and a negative correlation was seen between plasma ghrelin concentration and systemic blood pressure [54, 55]. In our study, there were no significant differences in ghrelin levels between preeclamptic and healthy pregnant.

We acknowledge limitations and strengths of the study. The main limitation of our study is the small number of

Table 3 Correlation of parameters in preeclampsia and control pregnants

Control	PLGF	Sflt-1	P-selectin	vWF	Endoglin	NO	Leptin	VEGF
PLGF	1	−0.083	0.102	−0.459*	−0.219	0.535**	−0.325	−0.008
Sflt-1	−0.083	1	0.697**	−0.089	0.079	−0.072	0.079	0.035
P-selectin	−0.113	0.697**	1	−0.091	−0.068	−0.135	0.215	−0.004
vWF	−0.459*	−0.089	−0.038	1	−0.091	−0.282	0.003	0.042
Endoglin	−0.219	0.079	0.119	−0.091	1	−0.224	0.155	0.015
NO	0.535**	−0.072	−0.282	−0.282	−0.224	1	−0.306	0.231
Leptin	−0.325	0.079	0.381	0.003	0.155	−0.306	1	−0.221
VEGF	−0.008	0.035	−0.192	0.042	0.015	0.231	−0.221	1
Preeclampsia								
PLGF	1	0.358**	0.209	−0.191	0.208	−0.037	0.194	0.066
Sflt-1	0.358**	1	−0.246	−0.004	0.392**	0.169	0.154	0.511**
P-selectin	−0.141	−0.033	1	0.489**	0.001	0.308*	−0.045	−0.040
vWF	−0.191	−0.004	0.0.074	1	0.060	0.152	−0.138	0.125
Endoglin	0.208	0.392**	−0.029	0.060	1	0.373**	0.216	0.149
NO	−0.037	0.169	−0.035	0.152	0.373**	1	0.221	−0.123
Leptin	0.194	0.154	0.001	−0.138	0.216	0.221	1	0.014
VEGF	0.066	0.511**	0.033	0.125	0.149	−0.123	0.014	1

* Correlation is significant at the $P < 0.05$ level

** Correlation is significant at the $P < 0.01$

Table 4 Multiple stepwise logistic regression analysis relating endoglin level to the presence of preeclampsia

Models	Preeclampsia	
	OR (%95 CI)	*P* value
A. Endoglin level		
Model 1A endoglin level only		
Endoglin level	1.001 (1.000–1.002)	0.000
Model 2A other endothelial dysfunction factors		
Endoglin level	1.001 (1.000–1.002)	0.027
PLGF	1.000 (0.980–1.021)	NS
P–Selectin	1.000 (1.000–1.000)	NS
Nitric oxide	0.975 (0.848–1.120)	NS
Sflt-1	0.883 (0.195–3.994)	NS
Plgf/sflt-1 ratio	0.974 (0.821–1.155)	NS
vWF	1.001 (1.000–1.002)	NS
VEGF	0.997(0.985–1.010)	NS

CI confidence interval, *NS* not significant

patients. We found positive correlations between sflt-1 and plgf, VEGF. These unexpected correlations might be the result of limited patient number. We do not have pre-pregnancy BMI of patients and controls. As for the strengths of the study, none of the patients had any treatments for preeclampsia. In this study, our aim was detecting the crucial key molecules of the platelet aggregation, angiogenic and antiangiogenic factors, and endothelial dysfunction parameters in preeclamptic patients. This is the first study reporting the relationship of platelet aggregation, endothelial dysfunction, angiogenesis, and the role of adipocytokines in preeclamptic patients, and having highly broad exclusion criteria of the study is the another strong point.

As a conclusion, despite the increase in the levels of antiangiogenic factors and leptin, herewith decline in the level of other angiogenic factor Plgf did not affect related molecules such as nitric oxide and platelet aggregation markers significantly. On the other hand, incline in vWF and endoglin levels might be result of endothelial dysfunction. Novel associations between angiogenic factors, leptin, Plgf, NO, and platelet aggregation could be the reasons for compensatory mechanisms against endothelial dysfunction, which results in decrease of P-selectin expression that induces platelet aggregation. Additional molecular studies are required to explain increased thrombosis risk in preeclampsia.

Acknowledgements This work was supported by the grant of University of Istanbul Research Foundation. Project No. is 19383.

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Ethical approval The study protocol was previously reviewed and approved by the ethics committee of the University of Istanbul, Cerrahpasa Medical School (Issue No. 13413, 08/05/2012). All procedures performed in study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest statement All authors declare that they have no conflict of interest.

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