 doi:10.20944/preprints201909.0252.v1

Effect of Serum Copper on Circulating Angiogenesis-related Factors in Women with Preeclampsia

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 doi:10.20944/preprints201909.0252.v1

Abstract

Preeclampsia (PE) is characterized by a series of clinical features such as hypertension and proteinuria associated with endothelial dysfunction and the impairment of placenta vascular endothelial integrity. This study aimed to investigate the effect of serum copper (Cu) level on some angiogenesis-related factors including vascular endothelial growth factor-A (VEGF-A), soluble Fms-like tyrosine kinase-1 (sVEGF-R1), soluble endoglin (sEng) and cerruloplasmin (Cp) in Iraqi women with preeclampsia (PE) and control pregnant women. Therefore, 60 women with PE in addition to 30 healthy pregnant women were enrolled in the study. Serum concentration of sEng, VEGF-A, sVEGF-R1, and Cu in PE group significantly increased ($p<0.05$) in the PE group compared with that in the control group. Increased production of antiangiogenic factors, soluble VEGF-A and sEng contribute to the pathophysiology of PE, indicating the involvement of these parameters in the angiogenic balance in patients with PE. Tests for between-subject effects showed that the circulating angiogenesis factors and Cu were significantly associated with the presence of PE. Serum Cu level was significantly correlated with VEGF- A and VEGF-R1 levels but not with sEng. Multiple regression analysis revealed that only Cp and BP can significantly predict the complications in women with PE. In conclusion, serum Cu has a role in the angiogenesis in women with PE and may be a new drug target in the prevention or treatment of PE.

Keywords: Copper; Endoglin; Preeclampsia; VEGF-A; sVEGF-R1.

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1. Introduction

Preeclampsia (PE) is a pregnancy disorder characterized by maternal hypertension, proteinuria and oedema and is a major cause of maternal and foetal morbidity and mortality. Its incidence rate generally varies from 3% to 7% of all pregnancies [1]. PE is a complex disorder of pregnancy with the involvement of multisystem and many genetic factors [2, 3].

Vascular endothelial growth factor-A (VEGF-A) is a glycoprotein that induces the growth of blood vessels through its interaction with sVEGF-R1 and -R2 receptors. Soluble Fms-like tyrosine kinase-1 (sFlt-1) is a tyrosine kinase protein that is produced by the splicing of VEGF receptor 1 (VEGFR-1). A non-membrane associated protein, sVEGF-R1, has antiangiogenic properties, binds the angiogenic factors VEGF and placental growth factor (PlGF) and reduces blood vessel growth by decreasing free VEGF and PlGF concentrations. Abnormally high levels of VEGFR-1 have been reported in patients with PE [4, 5]. VEGF regulates endothelial cell proliferation, angiogenesis and vascular permeability [6]. Imbalance of VEGF and sVEGFR-1 levels can cause pathological conditions, such as tumour growth or PE [7].

Alterations in the circulating concentrations of VEGF and VEGF-R1 in preeclamptic pregnancies affect foetal cerebrovascular function neurodevelopment and may cause cognitive alterations in post-natal life [8]. Anti-angiogenic factors, including sVEGFR-1 and soluble endoglin (sEng), are proposed to be responsible for the endothelial dysfunction in PE [9]. Endoglin is a transmembrane receptor that transforms growth factors β1 and regulates endothelium [10]. Soluble Eng is a proteolytic cleavage product of membrane endoglin, and its plasma levels were reportedly increased in various diseases, including type II diabetes mellitus and PE. Soluble Eng might damage vascular endothelium, but detailed information about the potential mechanisms involved remains scarce [11, 12].

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Copper (Cu) is one of the essential trace elements in human body, and its deficiency leads to growth and developmental failure and/or neurological dysfunction. The angiogenesis-promoting ability of Cu with different suggested mechanisms has been known for more than two decades. Cu stimulates the factors involved in vessel formation and maturation, such as VEGF [13] and endothelial cell proliferation in cultures [14]. However, the direct effect of serum Cu on angiogenesis-related biomarkers is poorly understood.

Ceruloplasmin (Cp) is Cu-binding protein in human plasma, has antioxidant function through its ferroxidase activity and has recently been proposed as a physiological defence mechanism [15]. Other known functions include involvement in angiogenesis, coagulation and nitric oxide homeostasis [16, 17]. Furthermore, PE is associated with the upregulation of placental ceruloplasmin expression, which possibly originates from syncitiotrophoblast [18]. Although elevated serum ceruloplasmin levels have been reported in pregnancies complicated with PE, a consensus in this field is lacking. This study aimed to determine the effect of serum Cu on the serum levels of VEGF-A, sVEGF-R1and sEng in patients with PE to provide further insights into the pathological changes associated with PE.

2. Materials and Methods

2.1. Subjects

A- Patients: Sixty women with PE with age range of 21–40 years participated in this study. Samples were collected from the participants in the Labor Hall and Operation Hall in Maternity & Children Hospital in Muthanna Governorate, South of Iraq from August until December 2016. PE was diagnosed by specialist gynaecologist doctors according to the International Society of the Study of Hypertension in Pregnancy, which is recognised by the International Federation of Gynecology and Obstetrics and World Health Organization. PE is defined as hypertension (systolic blood pressure

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≥140 mmHg and/or diastolic blood pressure ≥90 mmHg measured twice after 20 weeks of gestation) in combination with proteinuria (≥1+ dipstick proteinuria). Self-voiding was used for urine sampling. A single blood sample was obtained from women with PE (n = 60) and control pregnant women (n=30) with corresponding age and gestational age. Informed written consent was obtained from all participants. This study was approved by the local ethics committee, number 238 in 2016. Exclusion criteria included having chronic hypertension, maternal diabetes, renal disease, multiple pregnancy, foetal anomaly, vascular disease, intrauterine foetal death and history of drug use and smoking.

B- Controls: Thirty healthy pregnant women at gestational age of 34±1.71 weeks were selected as the control group. Their age range was comparable with that of the patients. None of these subjects suffered from hypertension.

C-Exclusion Criteria: Exclusion criteria also included multifoetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, renal disorder, maternal or foetal infection and foetal congenital anomaly.

2.2. Measurements

Maternal blood samples were obtained from an antecubital vein into plain tubes, allowed to clot for 15 min and then centrifuged at 3000 Xg for 5 min. The aliquots of serum were stored at -18 °C until analysis. Following blood sampling, the serum samples were analysed for the levels of sEng, sVEGF-R1, VEGF and Cp by using quantitative sandwich enzyme immunoassay.

Serum sEng, sVEGF-R1, VEGF and Cp levels were measured using ELISA kits supplied by Elabscience®, China following the sandwich-ELISA principle. The microplate was pre-coated with an antibody specific to the analyte. Standards or samples were added to the wells and combined with the specific antibody. A biotinylated detection antibody specific for the analyte and avidin-

 doi:10.20944/preprints201909.0252.v1

horseradish peroxidase conjugate were added to each micro plate well and then incubated. Free components were washed away. The TMB substrate solution was added to each well. Only those wells that contain immobilised analyte will appear blue in colour. The enzymatic reaction was terminated by adding acidic stop solution, and the colour turns yellow. Optical density was measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of the analyte to be measured, which can be obtained from the standard curve. Serum Cu concentration was examined by an atomic absorption spectrophotometer (PG Instruments Ltd., AA990).

2.3. Statistical Analysis

The distribution types of the variable results were examined by using the Kolmogorov-Smirnov test. For normally distributed variables, the results were expressed as mean±standard deviation. The Chi Square (χ^2) test was used to check associations between categorical variables and analysis of variance (ANOVAs) to check differences in continuous variables between groups. Protected pairwise post-hoc analyses were employed to examine the differences between group means. Multivariate general linear model (GLM) analysis was used to examine the effects of diagnosis (PE versus controls) on the different parameters, which were entered as dependent variables. Pearson's correlation coefficients (r) were used to estimate the correlation between parameters. Power analysis showed that using an effect size of 0.3, α=0.05, power=0.8 and 2 groups the total sample size should be 90. All statistical analyses were performed by using the SPSS Statistics Version 25 (2017) by IBM-USA.

3. Results

3.1. Comparison between Patients and Control groups

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The characteristics, clinical and biomarker data of PE and healthy control women are presented in Table 1.

Table 1: Characteristic, clinical and biomarker data in PE and healthy control women.

Parameter	Patients	Control	F/χ^2	df	p-value
Age yrs.	31.87±5.17	33.6±3.56	2.72	1	0.103
BMI kg/m^2	34.77±4.49	30.65±3.33	19.803	1	0.008
WHR	0.89±0.05	0.88±0.07	1.325	1	0.323
Gestational age Wks.	32.18±1.75	32.23±1.93	1.838	1	0.179
No. of pregnancies	2.50±1.16	2.60±0.97	2.753	1	0.602
Miscarriage Y(N)	17(43)	2(28)	3.996	1	0.036
Complications Y(N)	13(47)	0(30)	15.448	2	<0.001
SysBP mmHg	155.17±19.18	111±10.62	137.503	1	<0.001
DiasBP mmHg	98.5±8.99	79.67±1.83	128.405	1	<0.001
VEGF-A pg/ml	55.4±19.83	40.56±16.12	12.604	1	0.004
VEGF-R1 pg/ml	3015.86±891.60	2385.55±738.39	11.15	1	0.002
VEGF- (R1/A)	61.53±30.35	70.14±38.21	1.35	1	0.248
Eng pg/ml	4086.26±1073.51	2619.54±945.41	20.917	1	<0.001
Cp pg/ml	316.86±115.72	268.25±96.72	3.311	1	0.072
Cu µM	24.29±9.63	20.35±5.92	4.208	1	0.043

This table shows the differences in the variables between women with PE and controls. The data of this table and the inter-correlation matrix among the variables were used to determine which ones to use as extraneous variables in the ultimate multivariate analyses. Biomarkers were set as dependent variables. Nevertheless, this table shows no significant differences in age, WHR, weeks of pregnancy

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and number of previous pregnancies between the groups. Women with PE have higher blood pressure, miscarriage rate and complications than healthy control women. The serum level of biomarkers (VEGF-A, VEGF-R1, VEGF-R1/A ratio, sEng and Cu) was higher in women with PE than in the controls. No significant differences in the serum Cp level were found between the groups.

3.2. Association between characteristics and clinical parameters on the circulating angiogenesis biomarkers

Table 2 shows the outcome of a multivariate GLM analysis with the six biomarker values as the dependent variables and diagnosis (PE and control groups) as the primary outcome variable with adjustments for BP, complications, BMI, WHR and weeks of pregnancy.

Table 2: Results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as explanatory variable while adjusting for extraneous variables.

Tests	Dependent Variable	Explanatory variables	F	df	p-value	Partial η^2
Multivariate	All biomarkers	Diagnosis	2.198	6/76	0.048	0.168
		Gestational age	1.428	6/76	0.215	0.101

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		SysBP	1.477	6/76	0.197	0.104
		DiasBP	0.848	6/76	0.537	0.063
		Complications	1.087	6/76	0.378	0.079
		WHR	1.134	6/76	0.351	0.082
		BMI	1.687	6/76	0.478	0.074
Between-Subjects Effects	VEGF-A	Diagnosis	2.977	1/81	0.036	0.125
	VEGF-R1	Diagnosis	3.508	1/81	0.022	0.182
	R1/A	Diagnosis	0.093	1/81	0.761	0.001
	sEng	Diagnosis	10.338	1/81	0.039	0.113
	Cp	Diagnosis	0.053	1/81	0.819	0.001
	Cu	Diagnosis	1.777	1/81	0.049	0.084

These parameters had no significant effect on the circulating angiogenesis biomarkers and Cu. A significant association was found between the biomarkers and diagnosis (partial $\eta 2=0.168$, $p=0.048$). Tests for between-subjects effects showed that the circulating angiogenesis factors and Cu, but not Cp and VEGF-(R1/A), were significantly associated with diagnosis with a particularly strong effect on VEGF-R1 (partial $\eta 2=0.182$, $p=0.022$), VEGF-A (partial $\eta 2=0.125$, $p=0.036$) and sEng (partial $\eta 2=0.113$. $p=0.039$).

3.3. Effect of Cu on the angiogenesis factors

After p-correction, the associations between the biomarkers were obtained. Serum Cu level was significantly correlated with the levels of VEGF-A ($r=0.292$, $p=0.022$) and VEGF-R1 ($r=0.308$, $p=0.008$) as plotted in Figure 1.

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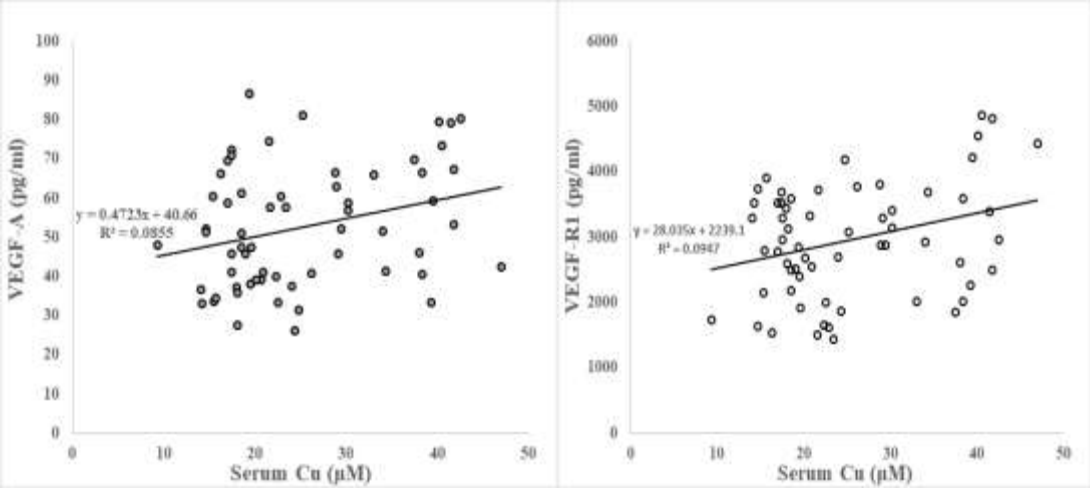

Figure 1. Correlation of serum Cu with VEGF-A (left) and with VEGF-R1 (right) in PE women

The correlation between serum Cu and Cp was expected (r=0.739, p<0.001). Serum Cu was not significantly correlated with Eng. However, a significant correlation was found between endoglin and VEGF-A (r=0.287, p=0.013).

3.4. Multiple regression analysis

Multiple regression analysis revealed that only Cp and BP can significantly predict the complications in women with PE. Increase in the serum level of these parameters by 10 units also increases the likelihood of having complications by 8% for Cp (exp(B)=1.008), 11% by SysBP (exp(B)=1.011) and 18% by DiasBP (exp(B)=1.021). sEng and BP are the best factors for predicting the disease. A 10 unit increase in the level of these parameters also increases the predictability of disease by 3% for sEng (exp(B)=1.003), 4% for SysBP (exp(B)=1.004) and 11% for DiasBP (exp(B)=1.011). Cp is the only predicting factor for serum Cu (exp (B)=1.028).

4. Discussion

 doi:10.20944/preprints201909.0252.v1

4.1. Characteristics, clinical and circulating angiogenesis biomarkers

The major finding of Table 1 is the increase in the circulating angiogenesis biomarkers in women with PE compared with those in control women. BMI and WHR are significantly high in women with PE [19, 20]. The increase in BMI may be due to the oedema in the patient group that is associated with proteinuria [20]. Increases in BMI in the normal range are also associated with an increased risk of PE [21]. Therefore, our patients may have a high BMI even in the pre-pregnancy state. Miscarriage was recorded more frequently in women with PE than in the controls because PE is a common cause of maternal and foetal morbidity and mortality [22].

The VEGF-A levels of the PE group were higher than those of the control group as shown in Table 1. Many researchers reported similar results and showed an increase in VEGF in PE compared with that in healthy pregnant women [23]. The increased oxidative stress and VEGF might have enhanced the impairment of placental perfusion and increased the peroxynitrite formation, product of NO and superoxide reaction, thereby contributing to the pathophysiology of this disease [24]. Extensive human epidemiological and experimental studies suggest that excessive placental soluble VEGF-A, a potent anti-angiogenic factor, leads to maternal hypertension, proteinuria and other systemic complications of PE. This phenomenon occurs because syncytial mitochondrial dysfunction in PE leads to the abnormal amplification of angiogenesis factors such as VEGF-A and sEng [25]. Some studies showed an increase in circulating VEGF in PE [26]. Villous explants from PE produce greater amounts of VEGF than those from normal pregnant women [27]. The severe vasoconstriction in PE increases vascular shear-stress, which in turn increases the circulating VEGF [28].

As depicted in Table 1, sVEGF-R1 levels were higher in the PE group than those in the control group. Significantly high sVEGF-R1 levels were observed for all types of PE, indicating that VEGF is involved in the angiogenic balance in mothers with PE and their pregnancy outcome [29, 30].

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sVEGF-R1 was increased in women with PE [29] and may have caused some complications associated with PE, such as proteinuria and hypertension [31]. Administering sVEGF-R1 to pregnant rats induces hypertension, proteinuria and glomerular endotheliosis, the classic lesion of PE. These observations suggest that excessive plasma sVEGF-R1 contributes to the pathogenesis of PE. This increased level could be attributed to the maximum intensity of Eng and sVEGF-R1 expression in the syncytiotrophoblast and extravillous cytotrophoblast cells in severe PE [32].

No significant difference on the sVEGF-(R1/A) ratio was found in patients with PE and control groups. This insignificance result is due to the parallel increase in the ratio's numerator and denominator that represents sVEGF-R1 and VEGF, respectively. Although the groups had varied ratios, the change was not significantly different. Here, this ratio was introduced in PE for the first time. VEGF-A/R1 ratio is suggested to be an independent predictor of acute myocardial infarction patient outcome, and its significance should be assessed when considering antiangiogenic therapy [33]. However, this ratio has not been considered in PE disorder. VEGF and sVEGF-R1 have received great interest due to their vital role in neovascularisation (vasculogenesis and angiogenesis) and in various physical and pathological processes such as PE. VEGF is secreted in response to tissue hypoxia and endothelial cell damage. Alterations in the circulating levels of this factor may therefore distinguish pregnancies with a high possibility of developing PE [34]. This effect depends on the response of sVEGF-R1. Therefore, the ratio in PE should be further studied to understand the role of these factors in PE.

The increase in sEng in PE patients was previously reported [35, 36]. Serum sEng was high from 18 weeks onwards, and the difference increases with gestational age. Khalil et al., (2014) suggested that the maternal plasma sEng may be a useful mid- and late-gestation biomarker for the development of PE. Increased production of antiangiogenic factors sVEGF-R1 and sEng contribute to the

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pathophysiology of PE [37]. Soluble Eng levels are independently associated with the development and severity of PE [38]. Serum levels of sEng may be increased in both early and late PE [39, 40]. The increase in sEng is due to the stimulated expression of the gene that is responsible for the synthesis of this protein. Different Eng pathway genes may be involved in PE development [41]. The anti-angiogenic factors (sEng and sVEGF-R1) produce systemic endothelial dysfunction, thereby resulting in hypertension, proteinuria and other systemic manifestations of PE [42].

The increased Cp level in patients with PE compared with that in the control women has been reported [43, 44]. Patients with PE had increased Cp levels, which seem to be a consequence of oxidative stress [45]. The serum level and antioxidant activity of Cp are significantly lower in normal pregnancy than mild and severe PE. [46]. Cp and its associated ferroxidase activity, which is induced by the hypoxia accompanying severe PE, are important in an endogenous cellular program to mitigate the damaging effects of subsequent reperfusion injury in this site [18]. Loss of ferroxidase activity of Cp and reduced total iron binding capacity were reported in women with PE. Thus, the plasma of women with PE lacks the protective anti-oxidative action of these substances [47].

Patients with PE had higher serum Cu level than healthy pregnancy controls [48, 49]. The increase in Cu can be explained by the parallel increase in Cp, which carries the Cu atoms. The modest elevation in Cu may contribute to oxidative stress, later in pregnancy, in the women that have developed PE [50].

4.2. *Association between characteristics and clinical parameters on the circulating angiogenesis biomarkers*

Table 2 shows the lack of significant effects of the characteristic parameters on the circulating angiogenesis biomarkers and Cu and any change in these parameters due to the disease itself. A significant association was found between the angiogenic related biomarkers and PE (partial

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η^2=0.168, p=0.048). Tests for between-subjects effects showed that the circulating angiogenesis factors and Cu were significantly associated with PE with a particularly strong effect on VEGF-R1 (partial η^2=0.182, p=0.022), VEGF-A (partial η^2=0.125, p=0.036) and sEng (partial η^2=0.113. p=0.039). sVEGF-R1 and sEng, an endothelial receptor that transforms growth factor beta, were directly involved in the systemic endothelial dysfunction of the mother [51].

The results also showed that VEGF-A and sVEGF-R1 were significantly correlated with Cu. VEGF-A and its receptor sVEGF-R1 play a major role in physiological and pathological angiogenesis i.e., VEGF-A regulates angiogenesis and vascular permeability by activating sVEGF-R1 [52].

4.3. Effect of Cu on angiogenesis factors

Figure 1 shows that serum Cu level was significantly correlated with the levels of VEGF-A (r=0.292, p=0.022) and VEGF-R1 (r=0.308, p=0.008). The correlation between serum Cu and Cp was expected (r=0.739, p<0.001) because ceruloplasmin is the main Cu-carrying protein, and the change in Cp causes a parallel change in serum Cu. However, serum Cu was not significantly correlated with sEng. Cu can produce a highly reactive hydroxyl radical. The generation of this radical can begin lipid peroxidation which may in turn cause endothelial cell damage [53]. Cu concentration increases in patients with PE [48]. The parallel increase in VEGF-A, VEGFR-1 and serum Cu indicates a correlation among these parameters as a mechanism related to the angiogenesis in PE.

Another interesting result is the significant correlation between sEng and VEGF-A (r=0.287, p=0.013). Soluble Eng performs better than standard clinical evaluation in detecting adverse maternal and foetal outcomes that occur within 2 weeks of presentation. Serum sEng was strongly correlated with sVEGF-R1 level, thereby suggesting common pathogenic pathways leading to PE [54].

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The angiogenesis-promoting ability of Cu has been established [13]. Cu stimulates endothelial cell proliferation in cultures [14], and this stimulation is accompanied by the enhanced production of VEGF [55]. Several factors are involved in the regulation of endothelial cell growth and function, and the most studied is VEGF [56]. VEGF plays a pivotal role in the initiation and formation of blood vessels which involve endothelial cell growth and differentiation [56].

Many research efforts have been focused on how Cu regulates VEGF expression [55, 57]. Sufficient evidence shows that Cu stimulates and is required for VEGF expression [55, 57]. However, this regulating activity is not the only action of Cu in angiogenesis; Cu also affects the expression and activation of other factors involved in blood vessel formation and maturation [58]. Therefore, Cu promoting blood vessel formation has a significant advantage over the effect of VEGF alone because Cu promotes the formation and maturation of new blood vessels. The regulating effect of Cu for a group of factors involved in angiogenesis suggests that this element would interact with a critical regulator, or a group of such regulators that work symphonically to control the expression of VEGF and related factors in blood vessel formation and maturation. Cu is required for VEGF expression through its regulation of hypoxia-inducible factor activity [59], a transcription factor that is required for VEGF expression.

4.4. Multiple regression analysis

Multiple regression analysis revealed that the complication in women with PE can be significantly predicted by the increase in Cp and BP due to the adverse effect of the increase in BP on the health of blood vessels [60]. Cp is important in complications because it is an acute phase reactant protein, and its increased level reflects the inflammatory response in women with PE. Inflammation in PE is the initial complication in PE; therefore, Cp is an important indicator for complications in PE [44, 61], whereas sEng and BP are the best factors for predicting this disease. sEng level is lower in normal

women than in healthy pregnant women. The sEng level was high in women with PE and can also be used to differentiate between severe and mild PE [62].

5. Conclusions

High serum levels of biomarkers (VEGF-A, VEGF-R1, sEng and Cu) were observed in women with PE, indicating the involvement of these parameters in the angiogenic balance in these patients. Tests for between-subjects effects showed that the circulating angiogenesis factors and Cu were significantly associated with the presence of PE. Serum Cu level was significantly correlated with VEGF-A and VEGF-R1 levels but not with sEng. A significant correlation between endoglin and VEGF-A was also noticed in the PE group. Multiple regression analysis revealed that only Cp and BP can significantly predict the complications in women with PE. Serum Cu has a role in the angiogenesis in women with PE and thus could be a new drug target in the prevention or treatment of PE.

Conflict of interest

The authors declared no conflict of interest.

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