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Diagnostic Value of Homocysteine and Other Preeclampsia Markers: Relationship with Severity

[1]Amal Mansour, [2]Hisham Harb and [2]Mohamed Abdelhafeez
[1]Department of Biochemistry, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[2]Department of Obstetrics and Gynecology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Corresponding Author: Amal Mansour, Department of Medical Biochemistry, Faculty of Medicine, Ain Shams University, Abbassia, Cairo, Egypt, 11381 Tel: 002 01111192610 Fax: 00 202 26859928

ABSTRACT

Homocysteine and some related cytokines are involved in Preeclampsia (PE) pathogenesis but their role in progression of the disease is controversial, accordingly the aim of this study was to evaluate maternal serum Homocysteine levels and relate them to tumor necrosis factor-alpha (TNF-α) and Transforming Growth Factor-beta 1 (TGF-β) as predictive tests for the development and progression of preeclampsia. Seventy five pregnant women (30 mild-PE and 30 sever-PE and 15 normotensive pregnant women) were included in this case-control study. Maternal serum Homocysteine, TNF-α and TGF-β 1 levels were measured in the third trimester by immunosorbent assay (ELISA). Homocysteine and TNF-α serum levels were discriminating between sever and mild-PE which suggests them as PE severity biomarker. Homocysteine, TNF-α and TGF-β increased in the PE group than normotensive pregnant group. The combination between them increased their significance. In conclusion, Women with preeclampsia have high serum homocysteine levels that are directly related to TNF-α and TGF-β and can be considered to play important role in preeclampsia progression.

Key words: Preeclampsia, homocysteine, tumor necrosis factor alpha, transforming growth factor beta, enzyme linked immune sorbent assay

INTRODUCTION

Pre-eclampsia (PE) is a potentially serious condition and can be life threatening for both mother and child (Sibai *et al*., 2005). Intrauterine growth retardation, pre-term delivery, low birth weight, fetal death and neonatal death due to complication of pre-term delivery are common perinatal outcomes associated with pre-eclampsia, so early detection, careful monitoring and treatment of pre-eclampsia by appropriate prenatal care is crucial in preventing mortality related to PE (Hoque *et al*., 2008).

Although it is likely that the causes of PE are multi-factorial and may involve genetic, immune, placental and other factors (Redman and Sargent, 2005). However, it is proposed that C-reactive Protein-a (CRP-a) play an important role in eliciting inflammatory response characteristic of preeclampsia and its level is highly increased with severe preeclampsia than mild preeclampsia (Ghazavi *et al*., 2008), moreover, angiogenic factors released from the placenta and hyperhomocysteinemia trigger the maternal symptoms (Wang *et al*., 2010; Hasanzadeh *et al*., 2008). The Homocysteine is sulfur containing compound that is derived from demethylation of the dietary essential amino acid "Methionine" to produce compounds required for the growth of cells

and tissues in the human body (Hoque *et al*., 2008). However, in hyperhomocysteinemia, homocysteine interferes with fibrinolytic system and undergoes auto-oxidation to produce Reactive Oxygen Species (ROS), which inactivates nitric oxide and thrombomodulin leading to endothelial dysfunction which is associated with a number of pregnancy associated diseases such as preeclampsia, placental abruption, recurrent pregnancy loss and neural tube defect in newborn (Hoque *et al*., 2008). Low antioxidant activity, HDL-C (Howlader *et al*., 2007) and copper (Ugwuja *et al*., 2010) concentration also may contribute to the promotion of oxidative stress and vascular dysfunction seen in PE that may play a significant role in its pathophysiology. Another possible triggering factor of PE is dead trophoblasts. They are hypothesized to die by apoptosis in normal pregnancy, but by necrosis in PE. They are discarded from the placenta then expelled to be trapped in the maternal pulmonary capillaries (Lee *et al*., 2010). The trapped trophoblasts may be phagocytosed by the pulmonary endothelial cells and the phagocytosis of these necrotic cells leads to the activation of endothelial cells to secrete Transforming growth factor beta-1 (TGF-β 1) (Walshe, 2010). The transforming growth factor beta (TGF-beta) is an essential regulator of placental development and functions; it exerts several regulatory effects on trophoblasts, such as inhibition of proliferation, invasiveness and stimulation of differentiation (Germain *et al*., 2007). However, it also exerts an antiangiogenic effect (Sahib *et al*., 2009) leading to abnormal placentation. Its signaling pathway through Endoglin (part of the TGF beta receptor complex) plays an important role in the pathogenesis and progression of gestational trophoblastic disease (Young *et al*., 2010) and thus may be considered as a potential therapeutic target and a diagnostic biomarker (Xuan *et al*., 2007).

However, it was reported that women who experience PE with severe maternal and/or fetal complications are more likely to have a genetic predisposition to produce high levels of TGF-β 1 as defined by polymorphisms at codon 10 (Thornburg *et al*., 2010). In clinically established PE, maternal circulating levels of cytokines, such as TGF-β, IL-6 and TNF-α, are reported to be elevated as well (Wang *et al*., 2009a; Irani *et al*., 2010). However, it was reported lower level of TGF-β in PE than in normal pregnancy and even it was less in sever PE than in mild PE (Wang *et al*., 2009b). Many studies have helped elucidate the complex and multiple roles of this ubiquitously expressed growth factor which was said to down regulate the TNF-α expression. However, maternal TNF-α was reported to be increased in PE (Seriolo *et al*., 2006).

This study designed to explore the association between hyperhomocysteinemia, serum concentrations of TNF-α, TGF-β1 and PE severity and to the correlate between the concentrations of these molecules and the severity of PE. The knowledge of which expected to be used for prevention of pre-eclampsia.

MATERIALS AND METHODS

Subjects: In a case-control study serum homocysteine, TNF-α and TGF-β1 were measured in 75 Egyptian women in the third trimester of pregnancy. They were classified into two groups of PE patients (based on the criteria of the International Society of the Study of Hypertension in Pregnancy) (Brown *et al*., 2001): mild-PE group (30 women, mean age: 26.7±5.8, with systolic/diastolic blood pressure equal to or more than 140/90 mMHg and more than +1 proteinuria on a urine dipstick), severe-PE group (30 women, mean age: 27.2±5.93, with systolic/diastolic blood pressure equal to or more than 160/110 mMHg and +2 or more proteinuria on a urine dipstick). In addition to age-matched 15 normotensive healthy pregnant women volunteers (control) their mean age was 26.1±5.8, they shared the same socio-economic status of the PE groups. All subjects

were chosen during their routine outpatient checkup at Ain shams obstetrics hospital clinic, from March 2009 to November 2009. All groups are not under any therapeutic regimen, they didn't have any clinical or laboratory renal insufficiency, chronic hypertension, diabetes mellitus, multiple gestation, neoplasia or neurological disorders.

Samples and methods: Informed consent was obtained from patients and normotensive volunteers prior to the study. The study was approved by Ethics Committees of the Ain Shams University Hospitals. Five milliliters Fasting blood samples were collected from them soon after the disease became manifest. None was in labor when the samples were collected. To obtain and clarify serum, samples were left to stand at room temperature for at least 30 min to allow the blood to clot and then centrifuged at 2000 RPM for 15 min and aliquoted. All samples were stored at -80°C until assay.

Serum Homocysteine measurements were conducted according to manufacturer's protocols of FHCY200 Axis® Homocysteine EIA control Kit ELISA kits supplied by FHCY200A for Indian. Assay range was from 2 to 50 μmol L^{-1} (Germain *et al*., 2007).

Serum TNF-α measurements were conducted according to manufacturer's protocols of AviBion Human TNF-α ELISA kits supplied by Orgenium Laboratories, FIN-07120 Vantaa, Finland, Assay range was from 1 to 4000 pg mL^{-1} (Bienvenu *et al*., 1993).

Serum TGF-β1 was assayed according to manufacturer's protocols of DRG® TGF-β1 ELISA (EIA-1864) supplied from Diagnostic Biochem Canada Incorporation that used for the measurement of human TGF-β1, The minimum detectable concentration of TGF-β1 by this assay is estimated to be 1.9 pg mL^{-1} (Kropf *et al*., 1997).

The samples that exceeded the reading of highest standard were further diluted 2 times; absorbance value was read at 450 nm for all.

Statistical analysis: The data were expressed as Mean±SD deviation. Two-tailed unpaired t test was used for continuous variables where $p<0.05$ was considered statistically significant. Pearson's correlation was used to explore the relationship between Homocysteine, TNF-α, TGF-β1 and different variables among PE groups. All statistical analysis were performed with the Statistical Package for Social Science version 15.0 (SPSS Inc.,Chicago, Illinois).

Receiver Operating Characteristics (ROC) curves: ROC curves were used to discriminate positive from negative results. The diagnostic tests that approach 1 indicate a perfect discriminator. ROC curves also determined the threshold value for optimal sensitivity and specificity which was constructed by calculating the true positive fraction (sensitivity percent) and the false positive fraction (100-specificity) of markers at several cutoff points (Henderson, 1993).

RESULTS

Serum Homocysteine in severe-PE was about 3 folds higher than those in control ($p = 0.00$) and about 2 folds higher in severe PE than those in mild-PE ($p = 0.00$). Serum TNF-α in severe-PE was 8 fold higher than those in control ($p = 0.00$) and about 2 folds higher in severe PE than those in mild-PE ($p = 0.01$). Serum TGF-β1 in severe-PE was highly significantly elevated in mild ($p = 0.025$) and severe ($p = 0.045$) PE than those in control (Table 1).

Statistically significant positive correlations were obtained between serum Homocysteine and other two markers ($p = 0.00$ for both) and between TNF-α levels and TGF-β1 ($p = 0.035$).

Table 1: Demographic data, serum level of tumor necrosis factor- alpha (TNF-α) and transforming growth factor- beta (TGF-β) of patients of mild and sever PE compared to normotensive pregnant women

	Mean±SD			
Variables	N (15)	MPE (30)	SPE (30)	p
Maternal Age (Mean±SD)	26.1±5.8	26.7±5.8	27.2±5.925	[1]0.699
				[2]0.53
				[3]0.826
Gestetional age (Mean±SD)	37.2±2.38	37.2±2.9	36.8±4.28	[1]0.42
				[2]0.953
				[3]0.31
Primigravida % (no.)	30% (6/20)	43.3% (13/30)	60% (12/20)	[1]0.001**
				[2]0.001**
				[3]0.001**
BMI	23.2±2.8	23.5±4.9	24.2±4.2	[1]0.72
				[2]0.516
				[3]0.24
Homocysteine (μmol L^{-1})	6.24±2.95	9.9±2.5	17.42±4.12	[1]0.000**
				[2]0.000**
				[3]0.000**
TNF-α (ng mL^{-1})	0.31±0.12	1.3±0.75	2.4±0.56	[1]0.001**
				[2]0.00**
				[3]0.00**
TGF-β (ng mL^{-1})	36±3.3	48.2±1.9	48.4±3.4	[1]0.83
				[2]0.025*
				[3]0.045*

*Significant (<0.05), ** Highly significant (<0.01), [1]Statistical analysis of mild versus sever PE, [2]Statistical analysis of Control versus mild PE. [3]Statistical analysis of Control versus sever PE, C: Normotensive, MPE: Mild preeclampsia, SPE: Severe preeclampsia, BMI: Body mass index, TGF-β: Transforming factor beta, TNF-α: tumor necrosis factor alpha

Table 2: Correlation Between Serum Homocysteine, TNF-α and TGF-β And Different Variables Among PE Groups

	Homocysteine (μmol L^{-1})		Serum TNF-α (ng mL^{-1})		Serum TGF-β (ng mL^{-1})	
Variables	R	P	R	P	R	P
Maternal age	0.044	0.7	0.206	0.221	0.115	0.392
G. A	0.039	0.73	-0.319	0.054	-0.214	0.107
SBP	0.757	0.00**	0.664	0.00**	0.491	0.00*
DBP	0.793	0.00**	0.707	0.00**	0.507	0.00*
Albumin	0.843	0.00**	0.626	0.00**	0.480	0.00*
Homocysteine (μmol L^{-1})	-					
TNF-α (ng mL^{-1})	0.756	0.00**	-			
TGF-β (ng mL^{-1})	0.348	0.00**	0.348	0.035*	-	

*Significant (< 0.05), **Highly significant (<0.01), SBP: Systolic blood pressure, DBP: Diastolic blood pressure, G.A: gestational age. TGF-β: Transforming factor beta, TNF-α: Tumor necrosis factor alpha

Statistically significant positive correlations were obtained between the three markers and Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) and albumin and Serum TGF-β1 in PE group ($p = 0.00$ for all) (Table 2).

The best cutoff value that maximizes the sum of sensitivity and specificity of serum Homocysteine in normotensives versus PE was 8.04 μmol L^{-1}, at which it had 90% sensitivity and

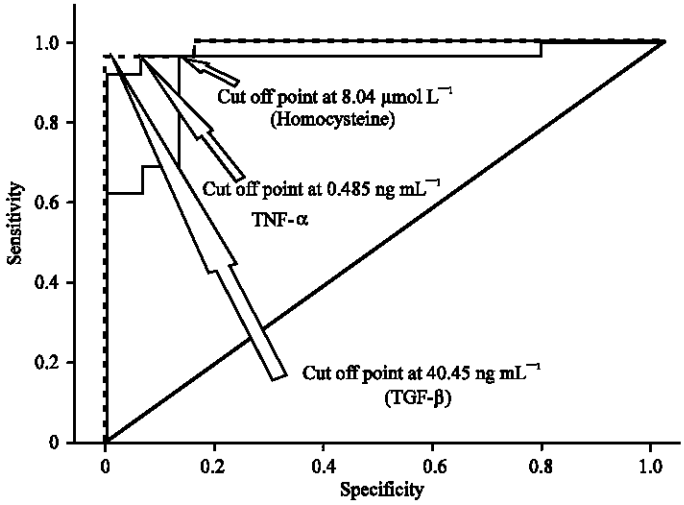

Fig. 1: ROC Curves analysis of Homocysteine, TNF-α and TGF-β of normal versus Preeclampsia. Arrows denote cut off points at 8.04 µmol L^{-1} (with 90 % sensitivity), 0.485 ng mL^{-1} (with 95.65% sensitivity) and 40.45 ng mL^{-1} (with absolute sensitivity). Areas under the curves were 0.989, 0.96 and 0.992, respectively

Table 3: Combined Sensitivity, Specificity, Accuracy, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) For TNF-α and TGF-β in normal pregnant women versus preeclampsia

Parameters	Sensitivity%	Specificity%	PPV%	NPV%	Accuracy%
Homocysteine (H)	90	80	94.74	66.67	88
TNF-α (TNF)	95.65	94	98	88.24	95.16
TGF-β (TGF)	100	87	96.7	100	97.3
H+ TNF	96.67	73.3	93.6	84.62	92
TNF+TGF	100	80	95.2	100	96
H+TGF	098.3	87	96.7	92.86	96
H+ TNF+TGF	100	80	95.2	100	96

only 80% specificity. The best cutoff value of serum TNF-α was 0.485 ng mL^{-1}, at which it had 95.65%, sensitivity and 94% specificity and at 40.45 ng mL^{-1} serum TGF-β it had 100% sensitivity and 87% specificity. (Fig.1 and Table 3), absolute sensitivity was obtained by combination between TNF-α and TGF-β but with less specificity (80%) than if we used each marker alone.

Homocysteine at cut off value 12.99 µmol L^{-1} was more sensitive (97.1%) in differentiation between mild and severe-PE than its differentiation between normotensives and PE groups with 86.7% specificity. The best cutoff value that maximize the sum of sensitivity and specificity of serum TNF-α in mild versus severe-PE was 1.7 ng mL^{-1}. TNF-α was more sensitive (100%) in differentiation between mild and severe-PE but less specific (90.9%) than its differentiation between normotensives and PE groups. The best cutoff value of serum TGF-β1 in mild versus sever PE was 45.6 ng mL^{-1}. TGF-β1 was less sensitive (93.8%) and less specific (50%) in differentiation between mild and severe-PE than its differentiation between normotensives and PE groups. Absolute sensitivity was abstained by combination between Homocysteine and TNF-α with the same specificity (86.7%) (Fig. 2, Table 4).

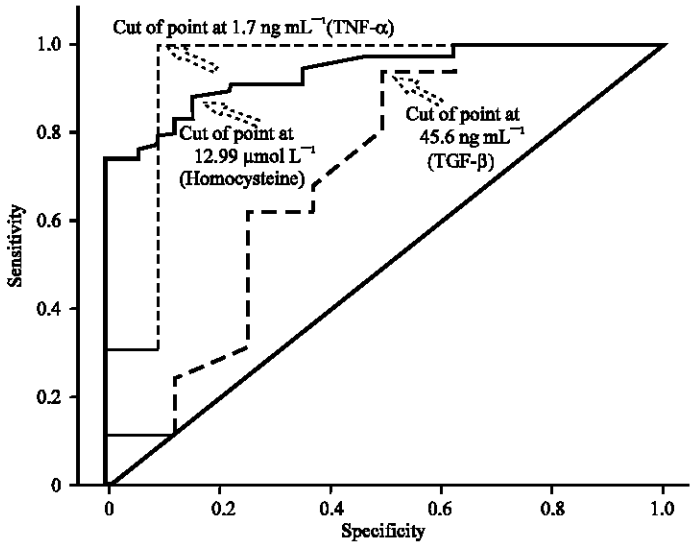

Fig. 2: ROC Curves analysis of Homocysteine, TNF-α and TGF-β of mild versus severe Preeclampsia. Arrows denote cut off points at 12.99 µmol L^{-1} (with 97.1% sensitivity), 1.7 ng mL^{-1} (with absolute sensitivity) and at 45.6 ng mL^{-1} (with 93.8% sensitivity). Areas under the curves were 0.97, 0.94 and 0.715, respectively

Table 4: Combined Sensitivity, Specificity, Accuracy, Positive Predictive Value (PPV) And Negative Predictive Value (NPV) For TNF-α and TGF-β in Mild versus sever preeclampsia

Parameters	Sensitivity%	Specificity%	PPV%	NPV%	Accuracy%
Homocysteine (H)	97.1	86.7	89.19	96.3	92.19
TNF-α (TNF)	100	90.9	92	100	95.31
TGF-β (TGF)	93.8	50	68.1	88.24	73.44
H+TNF	100	86.7	96.7	100	97.3
TNF+TGF	100	46.7	66.39	100	76.6
H+TGF	100	50	87.18	100	92.19
H+TGF+TNF	100	43.3	66.67	100	73.4

DISCUSSION

The maternal syndrome of preeclampsia has previously been recognized to be a generalized maternal endothelial cell dysfunction (Wang *et al*., 2009b). It starts with inadequate cytotrophoblast invasion and ending with widespread maternal endothelial dysfunction (Chen *et al*., 2010b).

Placental oxidative stress due to Reactive Oxygen Species (ROS) (Hoque *et al*., 2008) and deficiency of antioxidants activity in the serum of women with preeclampsia (Shakour-Shahabi *et al*., 2010) are reported to be the promoter for the endothelial cell dysfunction, which is considered as the main cause of preeclampsia. This leads to abnormal placentation and reduced perfusion which show the way to ischemia reperfusion injury of the placenta. (Hoque *et al*., 2008). Homocysteine is an important factor of oxidative stress production (Hoque *et al*., 2008; Khosrowbeygi *et al*., 2011) as it undergoes auto-oxidation that increases the insult of the oxidative stress leading to endothelial damage, endothelial dysfunction and necrosis of the trophoblasts (Walshe, 2010), the phagocytosis of these necrotic trophoblasts leads to the activation of endothelial cells to secrete Transforming growth factor beta-1 (TGF-β 1) (Wang *et al*.,

2010) which has antiangiogenic effect (Sahib *et al*., 2009) leading to abnormal placentation. The presences of the oxidative stress in this disease enhance the inflammatory responses (Peracoli *et al*., 2011) and the secretion of pro- inflammatory cytokines (Mori *et al*., 2011). TNF-α is considered as one of the most important pro-inflammatory cytokines (Stanczuk *et al*., 2007, Mori *et al*., 2011) that found to be upregulated and disrupt the maternal endothelium, this change in the normal angiogenic balance toward an anti-angiogenic state result in hypertension, proteinuria, glomerular endotheliosis, HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome and cerebral edema-the clinical signs of PE and eclampsia (Germain *et al*., 2007). A clue of its role in the inflammatory response is the administration TNF-α neutralizing antibodies has significantly attenuate the inflammatory response in endothelial cells and calm the key features of PE (Seriolo *et al*., 2006). In clinically established PE, maternal circulating levels of cytokines, such as TGF-β, IL-6 and TNF-α, are reported to be elevated, suggesting their possible role in the excess trophoblast death. If reflected in vivo this might explain, at least in part, how some cytokines could affect trophoblast shedding/deportation and contribute to the pathogenesis of PE (Chen *et al*., 2010a). Our study agreed with those previous studies and reported direct correlation between homocystein (ROS producer) with TNF-α ($p< 0.00$) and one of the placental anti-angiogenic factors (Transforming growth factor-β (TGF-β)) which is considered as a product of endothelial dysfunction ($p<0.00$).

Many studies abroad have demonstrated the relationship between hyperhomocysteinemia and severity of pre-eclampsia (Zeeman *et al*., 2003) while others have refuted an association (Mignini *et al*., 2005). This relationship has been shown in early pregnancy (Cotter *et al*., 2001), in second trimester (Sorensen *et al*., 1999) and in the third trimester of pregnancy (Sanchez *et al*., 2001). Cotter *et al*. (2001) in their study concluded that in early pregnancy increased Homocysteine may be associated with a 4-fold increased risk for development of mild pre-eclampsia. In consistent with our findings (Hasanzadeh *et al*., 2008) also found that homocysteine level increase with sever Pre-eclampsia more than mild Pre-eclampsia. However, some studies showed no significant difference of homocysteine concentration between mild and sever pre-eclampsia (Middledrop *et al*., 2004), which might be due to smaller sample size. Vitamin B12, vitamin B6 and riboflavin are involved in the metabolism of Homocysteine (Strain *et al*., 2004) and folic acid regulates its levels (El-Gindi and Hussien, 2007), elevated homocysteine is a marker of decreased methylation capacity of cells and low vitamin B complex (Patrick *et al*., 2004) especially, vitamin B12 and folic acid (Alshatwi, 2007). So, vitamin B complex (Strain *et al*., 2004) and folic acid (Shakour-Shahabi *et al*., 2010) supplementations could have a role in preventing the elevation of homocysteine in pregnant women. Moreover, antioxidants, as vit C, A and E have the ability to counter act the oxidative stress produced by homocystein and can down regulate the TGF-β expression, this provide protective effect against abnormal placentation due to antiangiogenic affect of TGF-β (Sahib *et al*., 2009).

Present study showed that elevated Homocysteine level is directly correlated with key features of pre-eclampsia ($p<0.00$ for systolic, diastolic blood pressure and proteinuria) and its levels were higher in sever than mild preeclampsia. So, high maternal Homocysteine levels seem to have causal role in the etiopathogenesis and severity of pre-eclampsia. In fact our study suggests the measurement of serum homocysteine in all pregnant women as a part of routine antenatal check-up and thereby monitoring and management of hyperhomocysteinemia in antenatal period taking into account the B-vitamin supplementation might help substantially to reduce the adverse pregnancy outcome.

It has been found also that serum TNF-α levels were higher in patients with PE than the normotensive ones and it is highly related to the severity of PE ($p = 0.001$). This result considers the consequence of increased inflammatory response and endothelial damage in the pathophysiology of these patients. Serum TNF-α concentrations in patients with PE were also found to be increased in many other studies (Serin *et al*., 2002) that reported determination of TNF-α may be useful for the prediction in the early third trimester.-Exploration of TNF-α role in PE by studying the opposing effects of other molecules such as Digibind (a polyclonal sheep digoxin binding Fab fragment) that reported to have the ability to attenuate vasoconstriction and other clinical symptoms of PE; by blocking TNF-α-induced down-regulation of Na+/K+-ATPase β1 expression, consequently to offset increased inflammatory response in endothelial cells (Wang *et al*., 2009a). Moreover, injection of angiotensin II type I (AT1) receptor agonistic autoantibody (AA) (AT1-AA) induced PE and Synchronized with increased in the pro-inflammatory cytokine TNF-α in the circulation of AT1-AA-injected pregnant mice but not in nonpregnant mice (Irani *et al*., 2010).

Serum TGF-β levels were reported to increased in PE and moreover to be correlated with the severity of this disease (Lim *et al*., 2008), as some findings suggested that women who experience eclampsia/PE with severe maternal and/or fetal complications are more likely to have a genetic predisposition to produce high levels of transforming growth factor-beta 1 as defined by polymorphisms at codon 10 (Stanczuk *et al*., 2007). There was association between impairment in platelet responsiveness and higher levels of TGF-β1 in the plasma of patients with PE suggestd that this cytokine could play a role in the pathophysiological events of PE that are dependent on platelet activation (Peracoli *et al*., 2008).

In the present study, although the levels of serum TGF-β were higher in sever than mild-PE patients, the difference between both groups was not significant, thus its value as a prognostic marker is uncertain. However, Chen *et al*. (2010a) suggested that serum TGF-β has an important role in development of PE as its Inhibition prevented vasoconstriction by inhibiting endothelial cell activation in response to phagocytosing necrotic trophoblasts in PE (Chen *et al*., 2010a).

The best sensitivity and specificity found to differentiate between normal pregnant women PE was found in TNF-α TGF-β. The combination between them increases the sensitivity to 100%, However it decreases the specificity of to (80%).

The best sensitivity and specificity found to differentiate between severe and mild PE was found in TNF α followed by homocysteine. The combination between them increase the sensitivity to 100% with the same specificity of Homocysteine (86.7%).

This study was among the first to evaluate correlation and combined sensitivity of these three markers and their relation with the severity of PE. It includes strict inclusion criteria of the study and control subjects. In addition, it is unique in the comparisons between cutoff values of these parameters for confirmation of the possible roles of these markers in prediction of progression of PE.

In conclusion the increased level of the homocysteine, TGF-β and TNF-α may play a role in the pathogenesis of PE and its progression. Moreover, these markers can be considered as therapeutic targets to ameliorate the clinical disease and morbidity of PE that need larger clinical study to evaluate this role.

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