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Asymmetric Dimethyl Arginine and Cell Free Total DNA as Diagnostic Markers in Preeclampsia

Zakia A. Z. Mohamed[1], Sabah E. Abd-Elraheem[1,*], Neven Abdelshafy Aly Ebrahim[2], Hayam Hamza Mansour[3], Niveen M. Gad[4]

[1]Clinical Pathology Department, Faculty of Medicine (for girls), Al-Azhar University, Cairo, Egypt
[2]Obstetrics and Gynecology, Faculty of Medicine (for girls), Al-Azhar University, Cairo, Egypt
[3]Internal Medicine, Faculty of Medicine (for Girls) Al-Azhar University, Cairo, Egypt
[4]Microbiology and Immunology Department, Banha Faculty of Medicine, Egypt

Abstract This study was conducted to evaluate the predictive value of cell-free total DNA (cfDNA), asymmetric dimethyl arginine (ADMA) and compare between them in early diagnosis of preeclampsia (PE). This study was carried on twenty-five females with multiple pregnancies and had preeclampsia (preeclampsia group); they were admitted to obstetrics & gynecology department and followed by nephrologist or seen in nephrology & gynecology outpatient clinics of Al-Zahra University Hospital. Preeclampsia was diagnosed by the increased blood pressure ≥140 mm Hg systolic and ≥90 mm Hg diastolic measured on two occasions at least four hours apart accompanied by proteinuria—urinary excretion of more than 0.3g protein in a 24hr specimen. The control group consisted of age-matched twenty healthy pregnant women who had no medical problems, were normotensive throughout pregnancy and had neither proteinuria nor hyperuricemia (control group). All studied groups were subjected to full medical history, clinical examination, and laboratory investigations that include blood urea, serum creatinine, serum uric acid, 24 hr urinary protein, Cell-free DNA and ADMA. **Results:** there was highly significant increase in the level of ADMA, systolic blood pressure, and diastolic blood pressure in patient group compared with control group p value = (0.000) group. There was significant increase in total DNA levels in patient group than control group p value = (0.043). In our study we found significantly elevated level of serum creatinine and uric acid and urinary protein in preeclampsia group compared with control group. Also there was significant correlation between ADMA, total DNA levels and serum creatinine and proteinuria Value (<0.05). No correlation between levels of total DNA and ADMA, systolic blood pressure, and diastolic blood pressure in patient group Value (>0.291). **Conclusions:** cf DNA and ADMA are good predictor of diagnosis of PE and further studies for value of combining them may be promising.

Keywords Preeclampsia, Cell-free total DNA, ADMA

1. Introduction

Pre-eclampsia is a disorder of pregnancy, characterized by the onset of high blood pressure and either proteinuria or end-organ dysfunction. This condition begins after 20 weeks of pregnancy. Other clinical manifestations include visual disorders, headaches, epigastric pain, thrombocytopenia, renal failure, abnormal liver function, pulmonary edema, brain hemorrhage, hepatic failure and death. Moderate to severe microangiopathy of target organs, including the brain, liver, kidney, and placenta are responsible for these clinical manifestations [1].

The maternal and fetal/placental factors are the main causes of pathophysiology of PE. As abnormalities in the development of the placental blood vessels early in pregnancy may lead to relative placental under-perfusion, hypoxia and ischemia, antiangiogenic factors are released in the mother's circulation. These factors change the function of maternal systemic endothelial function causing high blood pressure and other manifestations of the disease [2].

Cell-free total DNA is both maternal and fetal in origin, and the cell-free fetal DNA fraction represents approximately 3 – 6% of the total DNA present in maternal plasma. Fetal DNA and RNA enter the maternal system after apoptosis and necrosis of the placenta. Since PE is associated with placental impairment or abnormal development and increased trophoblast migration, it is biologically reasonable that increased levels of cell-free fetal DNA are associated with the risk of PE [3].

Human fetal DNA triggers *in vitro* activation of NF-κB with production of pro-inflammatory IL-6 in both human B-cell line and peripheral blood mononuclear cells from both pregnant and non-pregnant donors [4]. Therefore, elevation

* Corresponding author:
drsabahibrahim@yahoo.com (Sabah E. Abd-Elraheem)
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in cell-free fetal DNA has been suggested to be the result of a placental lesion, whilst elevations in maternal cell-free DNA could result from the generation of network of neutrophil extracellular traps (NETs), primarily composed of DNA and chromatin from polymorph-nuclear neutrophils (PMNs). PMNs have the ability to extrude their DNA into the extracellular environment. These NETs appear to be caused by the high release of highly inflammatory placental micro-debris [3].

A number of studies have mentioned that changes in cell-free fetal DNA levels occur early in pregnancy before symptoms of preeclampsia appear. However, cell-free DNA is increased only when the patient becomes symptomatic. Thus, there are two phases present in preeclampsia: the pre-clinical stage involving placental lesion and the second clinical stage involving a systemic inflammatory response by the mother [5, 6].

Dimethylarginines are the degradation products of methylated proteins. The methyl groups are derived from S-adenosylmethionine, with the participation of the enzymes protein arginine methyltransferase type 1 (PRMT1). The asymmetrically methylated arginine and ADMA are competitive inhibitors of the nitric oxide synthases (NOS) [7]. Nitric oxide (NO) is one of the major endothelium derived vasoactive substances who has a major role in maintaining endothelial homeostasis. NO contributes to vasodilation, decreasing the vascular resistance observed during normal pregnancy [8]. Elevated levels of ADMA inhibit NO synthesis and therefore impair endothelial function, leading to increased blood pressure and systemic vascular resistance [9]. However, plasma ADMA concentrations are more than tenfold higher than those of NMMA and therefore are biologically considered more relevant [10].

Maternal plasma ADMA has been reported to be higher in women with preeclampsia and in women who later had a pregnancy with a growth-restricted infant in the absence of preeclampsia [11]. This data suggests that elevated plasma ADMA may not be limited to preeclampsia and may contribute to the pathophysiology of other pregnancy disorders with reduced placental perfusion [12].

Several studies have been conducted retrospectively in the early detection of preeclampsia, including the use of cell-free total DNA and ADMA separately, but no study yet has compared between them in diagnosis of preeclampsia. Therefore, we aimed in this study to evaluate and compare the predictive values of total cell-free DNA (cfDNA) and ADMA and find out the value of combining the two markers in diagnosis of preeclampsia in Egyptian women.

2. Patients and Methods

This study was conducted on twenty five female patients with multiple pregnancies at 22 week of pregnancy and had preeclampsia (preeclampsia group); attending the out-patient clinics and/or admitted to obstetrics and gynecology Department of Al-Zahra University Hospital and followed by nephrologist. Full medical history and clinical examination and laboratory investigations including blood urea, serum creatinine and uric acid, fasting blood sugar, twenty four hour urinary proteins and urinalysis were done for all included pregnant women. Preeclampsia was determined by the increased blood pressure ≥140 mm Hg systolic and ≥ 90 mm Hg diastolic on two different occasions, more than 4 hours apart in a previously normotensive woman. It's accompanied by proteinuria defined as the urinary excretion of more than ≥ 0.3 g protein in a 24 hour specimen (with no evidence of urinary tract infection), with or without lower limb edema and hyperuricemia, None of our patients had elevated liver enzymes. The control group consisted of twenty healthy pregnant women who had no medical problems, were normotensive throughout pregnancy, and had neither proteinuria nor hyperuricemia, with similar distribution of age, parity, and gestational age (Control group). Subjects with pre-existing hypertension, cardiovascular disorders, renal diseases, diabetes mellitus, autoimmune disease and polyhydramnios were excluded from the study. All women included in the study gave an informed consent.

Venous blood samples were collected aseptically from patients and control subjects in separate test tubes: One sterile serum separator tube was used for biochemical analysis. After coagulation, sample was centrifuged (for 15 minutes at 1000 ×g) and serum was collected, and stored at -20°C until it was assayed for ADMA. Other sterile tube containing EDTA was collected for DNA extraction.

ADMA was assayed by ELISA immunoassay kit according to the manufacturer instructions (Immunodiagnostic AG., Germany), with normal range 0.45±0.19 µmol/L and a lower detection limit of 0.05 µmol/L. This assay is based on the method of competitive enzyme linked immunoassay. The sample preparation includes the addition of a derivatization reagent for ADMA-coupling. Afterwards, the treated samples and the polyclonal ADMA-antiserum were incubated in wells of micro plate coated with ADMA derivative (tracer). During the incubation period, the target ADMA in the sample competed with the tracer immobilized on the wall of the micro titer wells for the biding of the polyclonal antibodies. The ADMA in the sample displaced the antibodies out of the binding to the tracer. Therefore the concentration of the tracer bound antibody was inversely proportional to the ADMA concentration in the sample. During the second incubation step, a peroxidase-conjugated antibody was added to each microtiter well to detect the anti ADMA antibodies. After washing away the unbound components, TMB (tetramethylbenzidine) was added as a substrate for peroxidase, and then the enzymatic reaction was terminated by an acidic stop solution. The absorbance was measured at 450nm.The intensity of the color was inversely proportional to the ADMA concentration in the sample.

Cell-free DNA was extracted from all samples and measured by real-time quantitative polymerase chain

reaction (PCR) amplification. DNA was extracted from peripheral venous blood of each individual using a DNA extraction and purification kit (QIAamp DNA Blood Mini Kit, Qiagen, Germany) fully automated on the (QIAcube - Germany). QIAamp DNA Blood Mini Kits are designed for rapid purification of an average of 6 µg of total DNA from 200 µl of whole blood. -The Kits are based on the selective binding of DNA to silica-based membrane in the presence of chaotropic salts. The cells were digested with Proteinase K at 55°C using an optimized digestion buffer formulation that aids in protein denaturation and enhances Proteinase K activity. Any residual RNA was removed by digestion with RNAase prior to binding samples to the silica membrane. The lysate was mixed with ethanol and Pure Link R genomic binding buffer that allows high DNA binding Pure Link R Spin column. The DNA binds to the silica-based membrane in the column and impurities were removed by thorough washing with Wash Buffers. The genomic DNA was then eluted in low salt Elution Buffer.

*Quantitative estimation of cell free DNA using Real time PCR. Preprepared DNA standard was used to establish a standard curve as a reference for quantifying the results. To determine the amount of cell free DNA we used the Applied Bio systems 7300 instrument. We selected two sets of primers.

Forward: 5'-ACA CAA CTG TGT TCA CTA GC -3'

Reverse: 5'- CAA CTT CAT CCA CGT TCA CC-3'

Master Mix preparation: (ABI master mix)

12.5 ul PCR buffer, 6.0 ul nuclease free water, 2.0 ul primers, 0.5 ul probe, 3.0 ul enhancer, 1 ul Taq enzyme, and 5.0 ul of DNA template.

PCR conditions : 50ºC for 30 min – 1 cycle / 95ºC for 10 min. - 1 cycle / 95ºC for 15 sec. - 60ºC for 30 sec. – 40 cycles.

3. Statistical Analysis

The data were collected and entered to the Statistical Package for Social Science (IBM SPSS) version 20. Qualitative data were presented as number and percentages and compared using Chi-square test while quantitative data with parametric distribution were presented as mean, standard deviations and ranges. The comparison between two independent groups regarding quantitative data with parametric distribution was done using Independent t-test while comparison between two paired groups was done using Paired t-test. The confidence interval was set to 95% and the margin of error accepted was set to 5%. The level of significance was taken at P-value of <0.05" and high significant at P-value of < 0.001.

4. Results

There Was highly significant increase in the level of ADMA, systolic blood pressure, and diastolic blood pressure in patient group compared with control group p value = (0.000) group and There was significant increase in total DNA levels in patient group than control group p value = (0.043). There was significant elevated level of serum creatinine and uric acid and urinary protein in preeclampsia group compared with control group. Also there was significant correlation between ADMA, total DNA levels and serum creatinine and proteinuria P. Value = (0.05). No correlation between levels of total DNA and ADMA, systolic blood pressure, and diastolic blood pressure in patient group P. Value = (0.291).

Receiver operating characteristic (ROC) curves were constructed for value of cell-free total DNA (cfDNA), ADMA with calculation of sensitivity, specificity and accuracy at different cutoff levels.

A ROC curve was drawn to demonstrate the selectivity of ADMA for preeclampsia (fig.3), when the cut of point >0.32, the sensitivity was 100.0, specificity 100.0, and Accuracy was 1.000.

A ROC curve was drawn also to demonstrate the selectivity of cell-free total DNA a for preeclampsia (fig.4), when the cut of point >18, the sensitivity was 100.0, specificity 100.0, and Accuracy was 1.000.

Table (1). Comparison between u.s in patient and control group

U.S.		Groups					
		Patients		Control		Total	
		N	%	N	%	N	%
Negative		20	80.00	20	100.00	40	85.71
IUGR		1	4.00	0	0.00	1	2.86
Oligohydramnos		1	4.00	0	0.00	1	2.86
Oligohydromns		1	4.00	0	0.00	1	2.86
Polyhdromns		2	8.00	0	0.00	2	5.71
Total		25	100.00	20	100.00	45	100.00
Chi-square	X^2	2.333					
	P-value	0.675					

Table (2). Comparison of different parameters in all studied groups

	Patient N = 25	Control N = 20	P	Sig.
	Mean ± SD	Mean ± SD		
Age (year)	28.760 ± 4.176	30.300 ± 4.739	0.349	NS
Systolic Bl. Pressure (mm Hg)	155.652 ± 6.450	112.500 ± 6.346	0.000	HS
Diastolic Bl. Pressure (mm Hg)	93.333 ± 3.807	73.500 ± 4.743	0.000	HS
Serum creatinine (mg/dl)	0.9 ± 0.5	0.5 ± 0.3	0.05	S
Proteinurea (g/d)	0.92 ± 0.40	0.21 ± 0.09	0.05	S
Serum uric acid (mg/dl)	6.8 ± 1.6	3.66 ± 1.28	0.05	S
Total DNA (IU/ml)	21022.2 ± 31290.5	13.300 ± 2.003	0.043	S
ADMA (µmol/L)	1.225 ± 0.716	0.229 ± 0.053	0.000	HS

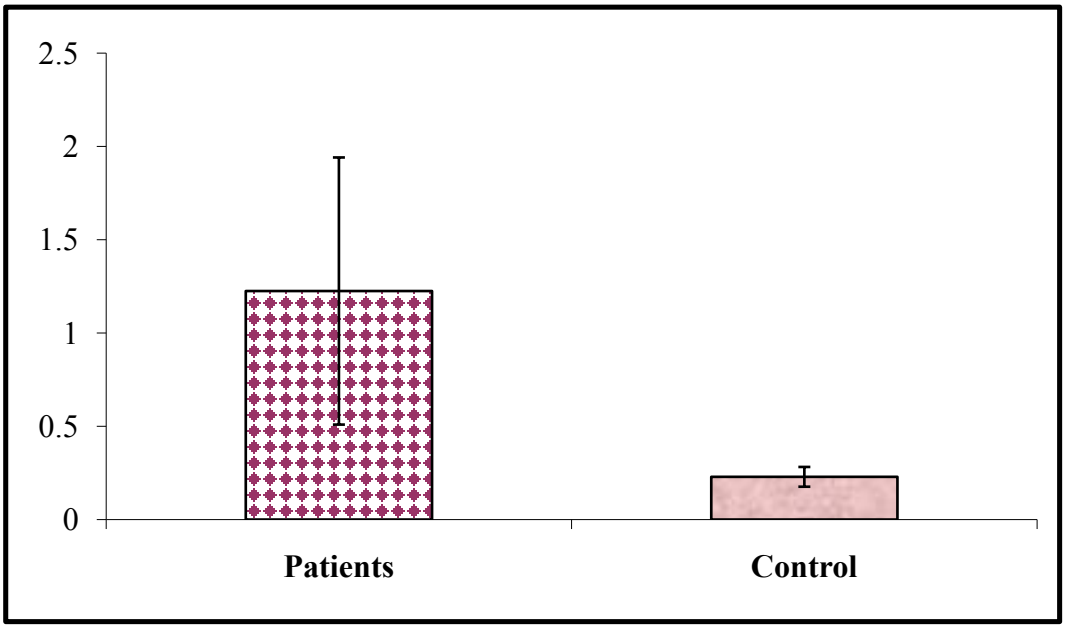

Figure 1. Comparison between ADMA level in patient and control group

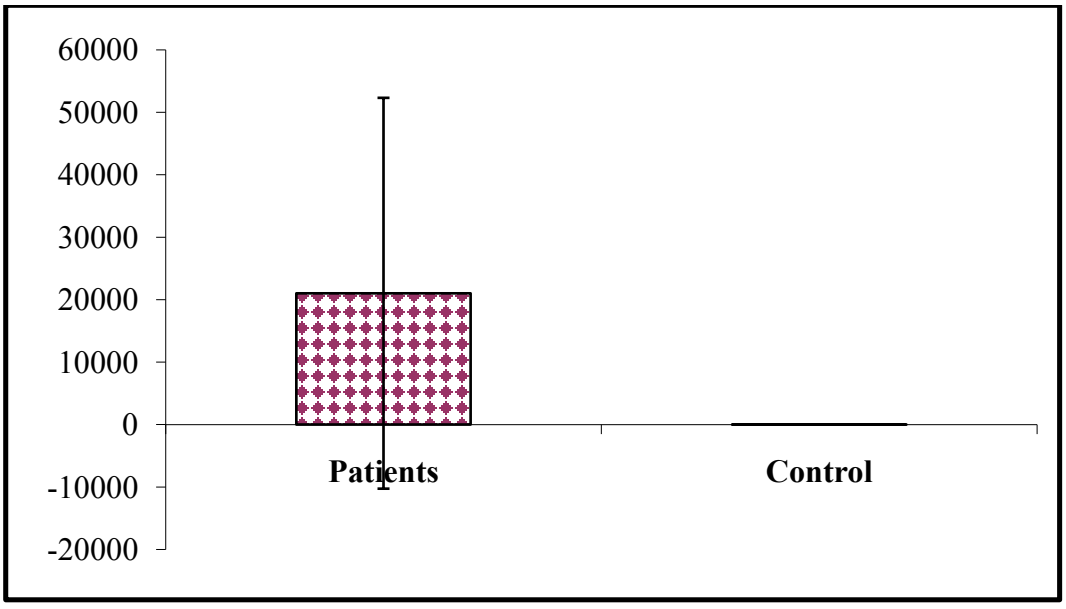

Figure 2. Comparison between Total DNA level in patient and control group

Table (3). Correlation between *Total DNA*, ADMA and lab.parameters

Variable	ADMA R	P. value	Cf-DNA R	P. value
Serum creatinine (mg/dl)	0.490	< 0.05	0.450	< 0.05
Blood urea (mg/dl)	0.281	< 0.05	0.196	< 0.05
Serum uric acid (mg/dl)	0.161	< 0.005	0.290	< 0.05
Proteinurea (g/d)	0.457	< 0.005	0.426	< 0.05
ADMA (µmol/L)			-0.225	< 0.291

Table (4). Correlation between *Total DNA* and, Systolic / Diastolic Bl pressure in patient group

	Total DNA IU\ml	
	R	P-value
Age	-0.003	0.987
Systolic Bl pressure/ mm Hg	0.254	0.243
Diastolic Bl pressure/ mm Hg	0.150	0.485

Table (5). ROC curve of ADMA

ROC curve of ADMA					
Cutoff	Sens.	Spec.	PPV	NPV	Accuracy
> 0.32 *	100.0	100.0	100.0	100.0	1.000

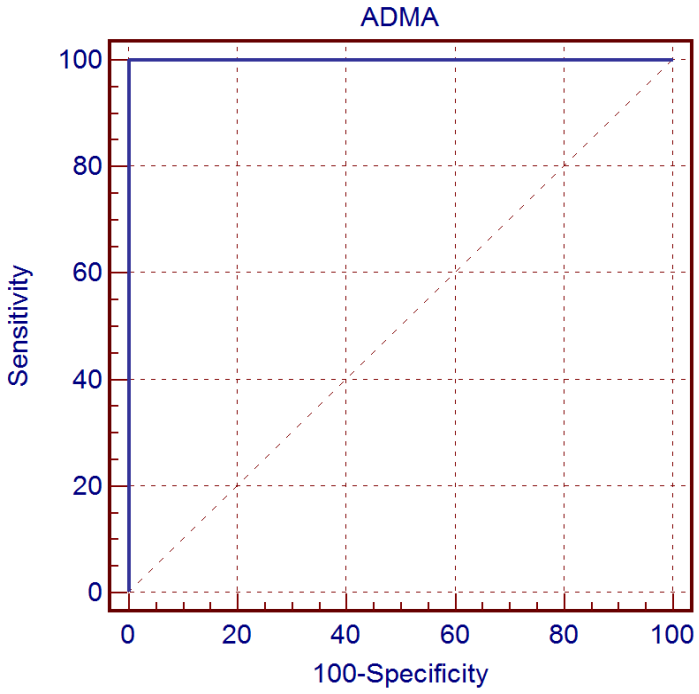

Figure 3. ROC curve of ADMA

Table (6). ROC curve of cell-free total DNA (cfDNA)

ROC curve of cell-free total DNA (cfDNA)					
Cutoff	Sens.	Spec.	PPV	NPV	Accuracy
> 18*	100.0	100.0	100.0	100.0	1.000

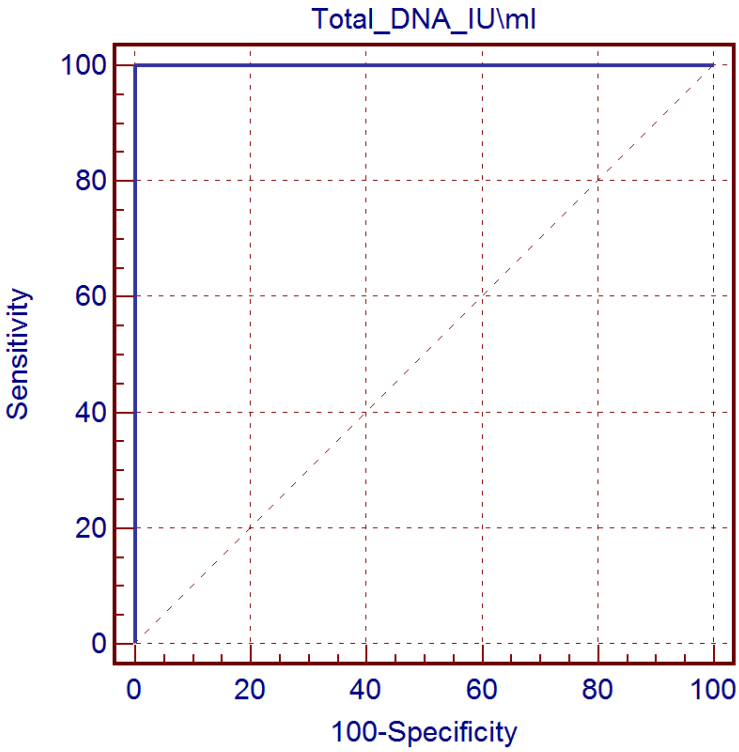

Figure 4. ROC curve of cell-free total DNA (cfDNA)

5. Discussion

Pre-eclampsia is a disorder that occurs in the second half of pregnancy, characterized by high blood pressure, proteinuria and / or edema. It is the leading cause of fetal mortality with a relatively high incidence in the developed countries. It's the result of trophoblast impairment leading to improper placenta. To date, there is no single reliable parameter used to predict the development of preeclampsia [13].

The continuous turnover of villous trophoblast results in extrusion of apoptotic substances into the maternal circulation which includes (cf) DNA. Since the release of cf DNA is closely related to placental morphogenesis, any conditions associated with placental abnormalities, such as preeclampsia, are associated with high levels of cf DNA in the blood of pregnant women [14].

The purpose of this study was to evaluate and compare the predictive values of total cell-free DNA (cfDNA) and ADMA and find out the value of combining the two markers in diagnosis of preeclampsia in Egyptian women.

In present study, the total cf DNA levels were significantly increased in patient group than control group ($p<0.043$), this coincides with most of previous studies [15-19]. ROLNIK and his team found that in late-PE group at 20–24 weeks, there was a significant increase in median total cfDNA and a decrease in median fetal fraction compared with controls [15]. Also, reported that, twofold increase in cf-DNA was detected in the severe PE group over the mild group, and both were significantly higher than the control group [16].

Kim and his colleagues also mentioned that the median levels and multiples of the median (MoM) values of *HYP2*ما ا were significantly higher in the PE and hypertensive disorders of pregnancy (HDP) groups at 6-14 and 15-23 weeks [17]. Additionally, other studies concluded, a gradual and strong relationship between severity of PE and each of total and fetal circulating cfDNA, soluble endoglin, soluble form of vascular endothelial growth factor receptor, and placental growth factor. The highest levels were found in patients with HELLP (Hemolysis, Elevated Liver enzymes, Low Platelet count) syndrome. These findings have been assigned to increased apoptosis of trophoblastic cells resulting from placental ischemia with reduced clearance of the cfDNA from the maternal circulation. Maternal DNA, which constitutes the majority of the total cf-DNA, has been suggested to originate from activated leukocytes that are present in increased numbers in PE, which might reflect a generalized state of inflammation [18, 19].

Contradicting with our study, Silver and his team reported that, non significant difference in levels of cell-free total DNA in the first trimester in women who subsequently develop preeclampsia. They explained that total cell free DNA is elevated once the patient manifests the phenotype of preeclampsia, perhaps due to endothelial cell activation and damage. However, it is not elevated prior to the clinical onset of disease. They suggested that differences among studies are likely due to gestational age at assessment, populations studied and methods used to detect total cell free DNA [2].

We also found significant correlation between serum creatinine and cfDNA in preeclamptic patients. Which was not the same in another study [20].

In this study, ADAMA levels were highly significantly increased in preeclamptic women than healthy controls ($p<0.000$). This is supported by other authors [12, 21, 22].

A study by Speer and his friends found that maternal ADMA concentrations were elevated at mid-pregnancy and remained elevated at delivery in women who later developed preeclampsia compared to women with uncomplicated pregnancies and women with small gestational age (SGA) infants [12].

Moreover, Braekke and his team mentioned that, not only maternal concentrations of ADMA and SDMA but also L-arginine were significantly higher in women with preeclampsia than in controls. On the other hand, only SDMA concentrations were higher in fetal samples of the preeclamptic group [22].

All previous studies discussed ADMA or cf DNA per si, but non of them had addressed the combination of both markers together in early diagnosis of PE. In our study there was no correlation between them. But we assume that combining both will improve the diagnosis of early stages of PE. Further studies are needed to support or deny this presumption.

In our study we found significant elevated level of serum creatinine and uric acid and urinary protein in preeclampsia group compared with control group. Also there was significant correlation between ADMA and serum creatinine and proteinuria. This goes in hand with *Fliser's study* on nondiabetic patients with kidney disease, point to long term elevation of ADMA as risk factor for progression to renal disease, *via* endothelial damage as a consequence of reduced NO availability [23].

These results could be explained by the fact of ADMA is an antiangiogenetic factor that decreases VEGF expression in endothelial cells and prevents the formation of nitric oxide (NO) increase peripheral vascular resistance [24, 25]. Increased ADMA levels in endothelium-dependent vascular dysfunction were also detected in diabetic, hypertensive, and hypercholesterolemic patients. ADMA levels may decrease at the beginning of a normal pregnancy but its concentration increases significantly in PE [26].

In conclusion, this study shows that the combination of the - cell-free total DNA, ADMA is the best predictor for detection of PE.

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