Circulating Maternal Serum Cell Free Fetal DNA Levels for prediction of Preeclampsia

Original Article

Mohammad N. Soroura, Maged M. El Shorbagy, Mohamed E. Shawky, Tamer F. Borg, Karim A. Wahba, Ahmed M. Reyad

Department of Obstetrics and Gynecology, Faculty of Medicine, Ain-Shams University, Cairo, Egypt

ABSTRACT

Background: Pre-eclampsia is a pregnancy-specific disorder that has a worldwide prevalence of 5-15%. It is one of the main causes of maternal and perinatal morbidity and mortality globally and accounts for 50000–60000 deaths annually, with a predominance in the low- and middle-income countries. There is a great need to have a test for predication of women at high risk of developing preeclampsia.

Aim: This work aimed to assess the accuracy of maternal serum Cff-DNA concentration levels in primigravidas 10-20 weeks gestation as a predictive test for the development of preeclampsia.

Materials and Methods: A nested case-control study was conducted on 26 patients with PE and 26 matched controls rimigravidas in 10-20 weeks gestation recruited from Ain-Shams University Maternity Hospital. Laboratory work was done in Ali Khalifa laboratory of the biochemistry department, Faculty of Medicine, Ain-Shams University**.**

Results: The study showed a statistically significant increase median of severe preeclampsia compared to non-severe preeclampsia and control group according to cff-DNA (GE/ml). Also the study results revealed a statistically significant increase median of preeclampsia group compared to control group according to BMI, GA (wks), SBP and DBP,

Conclusion: Cff-DNA quantification can be considered as a promising marker for preeclampsia prediction, especially for the development of early-onset or severe preeclampsia.

Key Words: Cff-DNA, Preeclampsia, primigravida

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Corresponding Author: Mohammad N. Soroura**,** Department of Obstetrics and Gynecology, Faculty of Medicine, Ain-Shams*,* Cairo, Egypt, **Tel.:** 01021544424, **E-mail:** mohammadsoroura@gmail.com

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INTRODUCTION

Preeclampsia (PE) is one of the leading causes of maternal and fetal/neonatal morbidity and mortality worldwide[1]. The disease complicates up to 10 % of pregnancies in the developing world, where emergency care is often inadequate or even lacking[2].

Although the diagnosis of PE is based on the measurement of maternal blood pressure and proteinuria, the sensitivity and specificity of these parameters are low with regard to the prediction of adverse maternal and fetal outcomes. Therefore, there is an urgent demand for widely applicable and affordable tests that can accurately identify women at risk as well as predict which fetus may have complications, in order to avoid morbidity and improve neonatal outcome through providing the best prenatal care for these patients and their children[3].

In Redman's[4] model of a 2-stage disorder of PE, the first stage is reduced placental perfusion and the second stage is the maternal syndrome (inflammatory/oxidative stress). The first stage is where the spiral arteries of the placental bed fail to undergo normal physiological change. Nguyen et al. described the Doppler changes in the uteroplacental flow in the midtrimester of normal nulliparous pregnancy, observing the development of a low-resistance circulation [5].

In the second stage of PE there is excessive embolization of trophoblast that releases cell-free fetal DNA (Cff-DNA) into the maternal circulation. The Cff-DNA is then broken down in the maternal liver where the purines are catabolized to uric acid by xanthine oxidoreductase. It is hypothesized that when the hepatocytes are presented with excessive amounts of purines for catabolism, in patients who subsequently develop PE, there is activation of xanthine oxidase, the more toxic isoenzyme of xanthine oxidoreductase, with the generation of reactive oxygen species (ROS) as byproducts. Excessive ROS production overwhelms the normal antioxidant ability of the tissues to produce oxidative stress. Oxidative stress is most likely the

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horse driving the development of the second stage of PE. Cff-DNA does play an important part in the pathogenesis of PE, being the early substrate for production of ROS but it is the cart not the horse (Mcmaster et al., 2008).

Cell-free fetal DNA (Cff-DNA) is a molecular biomarker that has revolutionized the field of non-invasive prenatal diagnosis or screening. It was first discovered in 1997 by Ke *et al*. who demonstrated the presence of fetal DNA sequences in maternal plasma and serum[7]. Subsequent studies revealed that significantly greater levels of Cff-DNA are present in blood of pregnant women compared to fetal DNA extracted from nucleated fetal cells found in the maternal blood[8]. Based on these findings, further investigations have extended the detection techniques of Cff-DNA by means of quantitative PCR and have focused on the clinical applications of this biomarker with particular emphasis on the prediction of pregnancy-related complications, as well as in the prenatal diagnosis or screening of fetal disorders of genetic origin. The latter approach has received considerable attention, since it has certain advantages over the isolation of initial fetal cells, such as speed, reliability, low cost and less laborious protocols[9].

The exact origin of Cff-DNA remains unknown. It is suggested that it is derived mainly from the placenta, as demonstrated by its very rapid clearance from maternal blood following delivery, in contrast to the majority of fetal cells that can survive several weeks post-partum[10].

Several studies have reported that in women with established PE, the plasma concentrations of both total cell-free DNA (Cf-DNA) and cell-free fetal DNA (Cff-DNA) are higher than in normotensive controls, and that the increase is particularly marked in those with severe PE[11,12]. These findings have been attributed to accelerated apoptosis of trophoblastic cells resulting from placental ischemia and reduced clearance of the Cf-DNA from the maternal circulation in women with PE[13]. The serum level of Cff-DNA in the maternal circulation may provide indirect clues to the underlying physiology and eventual pathology of the fetoplacental unit during all trimesters[14]; however, data are conflicting as to whether these altered levels are related to time of delivery and fetal outcome in women with PE[15].

AIM OF THE STUDY

This study aims to assess the accuracy of maternal Cff-DNA concentration levels in primigravidas in 10-20 weeks gestation as a predictive test for the development of preeclampsia.

PATIENTS AND METHODS

This cohort study was conducted on a total of 26 patients with PE and 26 matched controls primigravidas in 10-20 weeks gestation recruited from Ain-Shams University Maternity Hospital. Laboratory work was done in Ali Khalifa Laboratory of the Biochemistry Department, Faculty of Medicine, Ain-Shams University.

The study included initially healthy primigravida in the reproductive age (20-30 years) attending Ain-Shams university maternity hospital, single viable fetus 10-20 weeks gestational age and no maternal or fetal comorbidities whether medical, surgical or obstetrical e.g. Hypertension, Diabetes mellitus, Congenital fetal malformation, etc.

Patients with any maternal or fetal comorbidities whether medical, surgical or obstetrical e.g.: diabetes mellitus, gestational hypertension, congenital fetal malformation, fetal hydrops, threatened abortion, etc., patients age ≥ 30 years or ≤ 20 years and maternal BMI: less than 18.5kg/m2 or more than 25 kg/m2 were excluded from the study.

Patient group: It targeted reaching a sample of 26 initially healthy primigravidas who subsequently developed preeclampsia in the same pregnancy.

Criteria for the diagnosis of preeclampsia[16]. Systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on two occasions at least four hours apart after 20 weeks of gestation in a previously normotensive patient. If systolic blood pressure is ≥ 160 mmHg or diastolic blood pressure is ≥ 110 mmHg, confirmation within minutes is sufficient and Proteinuria ≥ 0.3 grams in a 24-hour specimen or protein (mg/dL) creatinine (mg/dL) ratio ≥ 0.3. Dipstick ≥ 1 + if a quantitative measurement is unavailable.

In patients with new-onset hypertension without proteinuria, the new onset of any of the following is diagnostic of preeclampsia: platelet count < 100,000/ microliter, serum creatinine > 1.1 mg/dL or doubling of serum creatinine in the absence of other renal disease, liver transaminases at least twice the normal concentrations, pulmonary edema and cerebral or visual symptoms.

The presence of one or more of the following indicates a diagnosis of "preeclampsia with severe features[16], symptoms of central nervous system dysfunction. New onset cerebral or visual disturbance, such as: Photopsia, scotomata, cortical blindness, retinal vasospasm. Severe headache (i.e., incapacitating, the "worst headache I've

ever had") or headache that persists and progresses despite analgesic therapy and altered mental status. Hepatic abormality: Severe persistent right upper quadrant or epigastric pain unresponsible to medication and not accounted for by an alternative diagnosis or serum transaminase concentration ≥ twice normal, or both. Severe blood pressure elevation: Systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg on two occasions at least four hours apart while the patient is on bedrest (unless the patient is on antihypetenive therapy). Thrombocytopenia: < 100,000 platelets/microL. Renal abnormality: Progressive renal insufficiency (serum creatinine> 1.1 mg/dL or doubling of serum creatinine concentration in the absence of other renal disease) and pulmonary edema.

The control group: It targeted reaching a matched sample of 26 healthy primigravidas who continued their pregnancies without any maternal or fetal complication whether medical, surgical or obstetrical.

All cases were subjected to full history taking: Personal history, present history, past history, family history, menstrual history and obstetric history. Clinical examination included: General examination: Body weight measurement, vital signs, heart rate, body temperature, blood pressure and respiratory rate. Inspection and palpation of head and neck, chest, back and lower Limbs. Local (abdominal) examination: Including: inspection, palpation (fundal level, fundal grip, umbilical grip and first pelvic grip) and auscultation of fetal heart rate using the sonicaid. Investigations include: Laboratory investigations: A-Rh B-Coagulation profile: prothrombin time, concentration and INR. C-Complete blood picture: Hb level. Platelet count. Heamatocrit. D-Liver and Kidney functions: Urea Creatinine ALT AST E-Detection of cell free fetal DNA for patients fulfilling study design criteria. Radiological investigations: A-Abdominal U/S (fetal biometry) using SONOACEX6MEDISION U/S machine. B-U/S Doppler (uterine artery, umbilical artery and middle cerebral artery) using the same U.

Detection of plasma cell free fetal DNA level
Blood sampling: 10 ml of venous blood samples in plain vacutainers were taken from all healthy pregnant primigravidas 10-20 weeks gestational age attending Ain-Shams University Maternity Hospital. Samples were left to clot.Cell-free serum samples were obtained by centrifugation of the whole blood at 1600g for 10min.

The supernatant was collected and centrifuged serum, free of blood cells, were removed from the micro-centrifuge tubes and stored at -70°C. DNA was extracted by use of the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's "Blood and Body Fluid Spin Protocol".

Amplification of Cff-DNA, PCR amplification using 7500 fast real time PCR (Applied biosystems, Foster City, USA), Universal fetal DNA marker RASSF1A is quantified in both samples and fully methylated genomic DNA standard (Qiagen, Germany) using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, USA) with the following primers and probe: RSF-b151F5-AGCCTGAGCTCATTGAGC TG-3, RSF-dsgnR5-ACCAGCTGCCGTGTG G-3, RSF-dsgnT5-FAM-CCAACGCGCTGCGCAT (MGB)-3.

Analyzing results using real time 7500 fast SDS software v. 2.05 (Applied biosystems, Foster City, USA).

STATISTICAL ANALYSIS

Recorded data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were expressed as mean± standard deviation (SD). Qualitative data were expressed as frequency and percentage. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the *p-value* was considered significant as the following: *P-value <0.05* was considered significant, *P-value <0.001* was considered as highly significant and *P-value >0.05* was considered insignificant.

RESULTS

Table 1 showed statistically significant increase mean of preeclampsia group compared to control group according to BMI at first visit, GA at dekivery(wks), SBP and DBP at time of delivery.

Table 2 showed statistically significant increase mean of preeclampsia group compared to control group according to ALT, Creatinine and PCR at delivery, also significant decrease mean of preeclampsia compared control according to platelets.

Table 4 showed statistically significant difference between non severe, severe and control according to BMI, GA, SBP, DBP and onset.

Table 5 showed statistically significant difference between non severe, severe and control according to laboratory data.

Table 6 showed statistically significant difference between non severe, severe and control according to neonatal ICU admission, fetal birth weight and UMB-RI at delivery

Table 7 showed statistically significant increase median of severe preeclampsia compared to non severe preeclampsia and control group according to cff-DNA (GE/ml).

Positive correlation and significant between cff-DNA with SBP, DBP, ALT, Creat, PCR and UMB-RI, while negative correlation and significant with fetal birth weight in preeclampsia group (Table 8).

Receiver operating characteristics (ROC) curve was used to define the best cut off value of preeclampsia vs. control: we predict through cut-off 222, that sensitivity 70.2%, specificity 84.%, PPV 82%, NPV 73.4% and accuracy 80.8%.

Preeclampsia non severevs severe: we predict through cut-off 378, that sensitivity 85.7%, specificity 83.3%, PPV 85.7%, NPV 83.3% and accuracy 91.7% (Table 9).

Table 1: Comparison between preeclampsia and control according to maternal baseline characteristics

Maternal baseline characteristics	Patients (n=26)	Control (n=26)	t/χ^2#	*p-value*
Age (years)				
Mean±SD	26.73±2.27	25.85±2.17	2.064	0.157
Range	23-30	21-30		
BMI [wt/(ht)^2]				
Mean±SD	29.63±1.17	27.58±2.03	11.482	<0.001**
Range	26.7-31	23.61-30		
GA (wks) at delivery				
Preterm <37 wks	12 (46.2%)	2 (7.7%)	9.774#	0.002*
Term >37wks	14 (53.8%)	24 (92.3%)		
SBP				
Mean±SD	161.46±14.43	111.73±9.14	220.466	<0.001**
Range	141-189	102-134		
DBP				
Mean±SD	99.73±5.96	72.08±6.69	247.677	<0.001**
Range	91-113	61-86		
Onset				
LOPE ≥34 weeks	12 (46.2%)	--	--	--
EOPE <34 weeks	14 (53.8%)	--		

t-Independent Sample t-test; t/χ^2#: Chi-square test

p-value >0.05 NS; **p-value <0.05 S*; ***p-value <0.001 HS*

Table 2: Comparison between patients "preeclampsia" and control according to laboratory data

Laboratory	Patients (n=26)	Control (n=26)	t-test	*p-value*
ALT				
Mean±SD	70.54±49.59	22.19±6.73	24.261	<0.001**
Range	29-281 U/L	12-37U/L		
Platelets				
Mean±SD	212.08±61.82	260.96±61.83	8.128	0.006*
Range	105-320x109/L	136-363x109/L		
Creat				
Mean±SD	0.84±0.19	0.71±0.12	9.523	0.003*
Range	0.52-1.4mg/dL	0.5-0.92mg/dL		
PCR				
Mean±SD	636.19±238.50	211.77±59.64	77.494	<0.001**
Range	367-1506mg/dl	81-330mg/dl		

t-Independent Sample t-test;

**p-value <0.05 S*; ***p-value <0.001 HS*

Table 3: Comparison between preeclampsia and control according to cff-DNA (GE/ml)

cff-DNA, GE/ml	Patients (n=26)	Control (n=26)	z-test	*p-value*
Median	472	101.5		
25^{th} -75^{th}	88.5-773.75	41.25-204	8.346	0.006*
Range	60-5026	33-419		

*z-Mann-Whitney test; *p-value <0.05 S*

Table 4: Comparison between non severe, severe preeclampsia and control according to maternal baseline characteristics

Maternal baseline characteristics	Non severe preeclampsia (n=12)	Severe preeclampsia (n=14)	Control (n=26)	F/χ^2#	*p-value*
Age (years)					
Mean±SD	26.58±2.23	26.86±2.38	25.85±2.17	1.061	0.354
Range	23-30	23-30	21-30		
BMI [wt/(ht)^2]					
Mean±SD	29.28±1.42	29.93±0.85	27.58±2.03	10.529	<0.001**
Range	26.7-31	28.56-31	23.61-30		
GA (wks) at delivery					
Preterm <37 wks	3 (25.0%)	9 (64.3%)	2 (7.7%)	14.843	<0.001**
Term >37wks	9 (75.0%)	5 (35.7%)	24 (92.3%)		
SBP(mmHg)					
Mean±SD	149.50±5.65	171.71±11.31	111.73±9.14	211.022	<0.001**
Range	141-159	156-189	102-134		
DBP(mmHg)					
Mean±SD	96.17±4.88	102.79±5.15	72.08±6.69	145.315#	<0.001**
Range	91-105	93-113	61-86		
Onset					
LOPE ≥34 weeks	8 (66.7%)	4 (28.6%)	--	3.773#	0.049*
EOPE <34 weeks	4 (33.3%)	10 (71.4%)	--		

F-One way analysis; #x2: Chi-square test

p-value >0.05 NS; **p-value <0.05 S*; ***p-value <0.001 HS*

Table 5: Comparison between non severe, severe preeclampsia and control according to laboratory data

Laboratory	Non severe preeclampsia (n=12)	Severe preeclampsia (n=14)	Control (n=26)	ANOVA	*p-value*
ALT					
Mean±SD	54.33±13.03	84.43±64.31	22.19±6.73	15.639	<0.001**
Range	29-74	45-281	12-37		
Platelets					
Mean±SD	218.75±63.89	206.36±61.79	260.96±61.83	4.132	0.022*
Range	122-320	105-300	136-363		
Creat					
Mean±SD	0.79±0.14	0.89±0.22	0.71±0.12	6.037	0.005*
Range	0.52-0.95	0.54-1.4	0.5-0.92		
PCR					
Mean±SD	550.50±157.22	709.64±275.47	211.77±59.64	45.560	<0.001**
Range	367-872	443-1506	81-330		

F-One way analysis;

**p-value <0.05* S; ***p-value <0.001* HS

Table 6: Comparison between non severe, severe preeclampsia and control according to secondary outcome

Secondary outcome	Non severepreeclampsia (n=12)	Severe preeclampsia (n=14)	Control (n=26)	F/χ^2#	*p-value*
Fetal Gender					
Male	10 (83.3%)	9 (64.3%)	22 (84.6%)	2.443#	0.295
Female	2 (16.7%)	5 (35.7%)	4 (15.4%)		
Fetal death					
Yes	0 (0.0%)	2 (14.3%)	1 (3.8%)	2.779#	0.249
No	12 (100.0%)	12 (85.7%)	25 (96.2%)		
Neonatal ICU admission					
Yes	5 (41.7%)	10 (71.4%)	2 (7.7%)	17.371#	<0.001**
No	7 (58.3%)	4 (28.6%)	24 (92.3%)		
Fetal birth weight					
Mean±SD	2.99±0.46	2.53±0.51	3.07±0.34	7.840	<0.001**
Range	2.23-3.69	1.58-3.39	2.17-3.67		
UMB-RI					
Mean±SD	0.75±0.07	0.81±0.05	0.66±0.06	26.410	<0.001**
Range	0.65-0.87	0.71-0.89	0.59-0.81		

F-One way analysis; #x^2: Chi-square test
p-value >0.05 NS; ***p-value <0.001* HS

Table 7: Comparison between non severe, severe preeclampsia and control according to cff-DNA (GE/ml)

cff-DNA, GE/ml	Non severe preeclampsia (n=12)	Severe preeclampsia (n=14)	Control (n=26)	H	*p-value*
Median	88	744.5	101.5	11.661	<0.001**
25^{th}-75^{th}	65.75-356.75	483-1278.75	41.25-204		
Range	60-723	264-5026	33-419		

H-Kruskal Wallis test; ***p-value <0.001* HS
p-value <0.001 = Highly significant

Table 8: Correlation between cff-DNA (GE/ml) with all parameters, using Pearson Correlation Coefficient in preeclampsia

	cff-DNA, GE/ml	
	r	*p-value*
Age (years)	0.129	0.529
BMI [wt/(ht)^2]	0.115	0.575
SBP	0.544	0.004*
DBP	0.511	0.008*
ALT	0.898	<0.001**
Platelets	-0.140	0.494
Creat	0.762	<0.001**
PCR	0.783	<0.001**
Fetal birth weight	-0.569	0.002*
UMB-RI	0.693	<0.001**

-Pearson Correlation Coefficient
p-value >0.05 NS; **p-value <0.05* S; ***p-value <0.001* HS

Table 9: Diagnostic Performance of preeclampsia and control in discrimination of cff-DNA, GE/ml

Cut-off	Cut-off.	Sen.	Spe.	PPV	NPV	Accuracy
Preeclampsia vs. control	>222	70.2%	84.4%	82.0%	73.4%	80.8%
Preeclampsia non severevs. severe	>378	85.7%	83.3%	85.7%	83.3%	91.7%

DISCUSSION

Pre-eclampsia is one of the leading causes of maternal and perinatal morbidity and mortality in the developed world[17]. Although the pathogenesis of this condition is not fully understood, but it is widely accepted that vascular endothelial cell dysfunction is the final common pathway responsible for the maternal syndrome. The underlying pathological changes that lead to the endothelial cell dysfunction remain incompletely understood, but poor placentation has been proposed as a major contributory factor. As a result of incomplete or failure of trophoblastic invasion of the spiral arteries, placental ischemia results, leading to the release of one or more factors that are responsible for the damage of the maternal vascular endothelium[19].

Doppler ultrasonography has been used as a modality to evaluate placental circulation and fetal well being for about three decades. The normal process of trophoblastic invasion is completed by 20 weeks of gestation. Hence, the initiating placental pathology should exist prior to this stage of pregnancy, long before the onset of the clinical syndrome. Therefore, it might be possible to develop new plasma/serum biochemical markers for identifying subjects at increased risk of developing pre-eclampsia. Women with established pre-eclampsia have a fivefold increase in circulating fetal DNA concentrations in their plasma compared with control pregnant subjects[20].

Quantitative changes of cffDNA in maternal plasma as an indicator for impending pre-eclampsia have been reported in different studies, using real-time quantitative PCR for the male specific SRY or DYS 14 loci. The increased levels of cffDNA before the onset of symptoms may be due to hypoxia within the intervillous space leading to tissue oxidative stress and increased placental apoptosis and necrosis. In addition to the evidence for increased shedding of cffDNA into the maternal circulation, there is also evidence for reduced renal clearance of cffDNA in pre-eclampsia[21].

A new test for the detection of fetal DNA in maternal plasma has been discovered. This test is based on the detection of a hypermethylated placental (fetal) DNA sequence in the maternal circulation. The methylation pattern of the RASSF1A promoter in the placenta and maternal blood cells allows the use of methylation-sensitive restriction enzyme digestion for specifically cutting the maternally derived background RASSF1A sequences while leaving the placentally (fetal) derived RASSF1A sequences intact. It can be used as a marker regardless of the fetal sex. Also, this marker allows the detection of false-negative results caused by low fetal DNA concentrations in maternal plasma, when applied to prenatal RhD genotyping. So, we detected cffDNA by detection of hypermethylated RASSF1A in this study.

Our study showed that cffDNA level was higher in pre-eclamptic women compared to normotensive control women. Its level increased about five folds and this difference was statistically significant (Table 7) .The results of this study were in accordance with[19, 26, 27, 17], but they measured fetal DNA using real-time quantitative PCR for the SRY gene on the Y chromosome[25] who examined 20 pre-eclamptic women and 20 normotensive pregnant women as control subjects. Male fetal DNA in maternal serum was measured using real-time quantitative PCR for the SRY gene on the Y chromosome. They found that the mean circulating fetal DNA was increased fivefold in pre-eclamptic women compared with control pregnant women ($P<0.001$)[19] and [26,27] also proved that there was significant increase in the number of copies of fetal DNA (Y-sequences) in pre-eclamptic women[17] examined 67 pre-eclamptic women and 70 normotensive pregnant women as control subjects. They found that the levels of fetal DNA(Y-sequences) were significantly elevated in pregnancies complicated by pre-eclampsia compared to the normotensive control subjects ($P < 0.001$).

According to our results; the cffDNA level increased about 2 folds in non severe pre-eclampsia and 7 folds in severe pre-eclampsia compared to control group (Table 7). This result was in accordance with[29,28]. They found that there was positive correlation between fetal-derived hypermethylated RASSF1A levels and the severity of pre-eclampsia[29] examined 60 normotensive and 20 pre-eclamptic pregnant women as study groups and 20 non-pregnant women as control group. They found that

there was positive correlation between fetal-derived hypermethylated RASSF1A levels and the severity of pre-eclampsia. Also,[28] examined 60 pre-eclampsia women and 60 women with normal pregnancy were studied as control group. They found that the mean concentration of hypermethylated RASSF1A gene was 3.31-fold higher in samples from pre-eclamptic pregnancies than that in control group. There was significant difference between the non severe and severe pre-eclamptic subjects ($P<0.05$). They proved that hypermethylated

In this study, umbilical artery resistance index increased in pre-eclamptic women compared with normotensive control subjects and these changes tend to be greater in the group of severe pre-eclampsia (Table 6). This difference was statistically significant[24] results were in accordance with our results.

CONCOLUSION

Based on the results obtained by this study, Cff-DNA quantification can be considered a promising marker for preeclampsia prediction, especially for the development of early-onset or severe preeclampsia.

CONFLICT OF INTEREST

There are no conflicts of interests.

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