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Nitric oxide/Asymmetric Dimethylarginine Axis may Interplay With Some Sex Hormones for Pathogenesis and Severity of Pre-eclampsia: Study controlled by Uterine Arterial Doppler

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Keywords

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- Uterine arterial Doppler

Abstract

Objectives: To evaluate relations of asymmetric dimethylarginine (ADMA) and nitric oxide (NO) with estrogen (E_2) and progesterone (Pg) and its role in pathogenesis and severity of pre-eclampsia (PE). **Patients & Methods**: At 12th week gestational age (GA) constitutional data, systolic and diastolic blood pressures (SBP and DBP), uterine artery pulsatility index (UAPI) and serum NO, ADMA, E_2 and Pg levels were determined for primigravida attending the antenatal care unit. During pregnancy, women who developed PE were categorized as early or late and mild or severe according to ACOG Practice Bulletin of PE guidelines. Studied parameters were evaluated statistically as early predictors for development and severity of PE. **Results**: Ninety pregnant women developed PE; 39 early and 51 late. Only 13 women had severe and 77 women had mild PE. At 12th week GA, UAPI and serum ADMA levels were significantly higher with significantly lower E_2 and NO levels in PE women than women free of PE and in early than in late PE. High SBP at time of PE diagnosis showed positive significant correlation with body mass index (BMI) and serum ADMA levels but showed negative significant correlation with serum NO and E_2 levels. Serum ADMA levels showed positive significant correlation with BMI, but negative significant correlation with serum E_2 levels. High UAPI, serum ADMA and high BMI were significant early predictors for PE development and severity. **Conclusion:** Disturbed maternal serum ADMA/NO axis and low E_2 serum levels may underlie PE development. High BMI and low E_2 level may have a role in PE pathogenesis through induction of high serum ADMA levels. High UAPI and serum ADMA at 12th week GA could be used as early predictors of PE especially early-onset and severe PE.

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Introduction

Pregnancy is marked by cardiovascular changes and adaptations involving vasodilation, angiogenic or remodeling. These adaptations are important for the maintenance and growth of the placenta and fetus. Estrogen is partially responsible for facilitating this dramatic increase in uterine blood flow during pregnancy (1).

Uterine arteries from non-pregnant women respond to nitric oxide (NO) liberated from the endothelium and nitrergic nerves with relaxations. Endothelial NO liberation is influenced by the phase of the estrous cycle, with its enhanced release at the follicular phase when the estrogen level is high (2).

Placental vascular tone is critically influenced by NO derived from endothelial NO synthase (eNOS) activity (3). NO bioavailability in the uteroplacental circulatory system is gradually increased during pregnancy (2). Placental vessels from complicated pregnancies present altered NOS-dependent vasodilation (3).

L-Arginine, a semi-essential cationic amino acid involved in multiple areas of human physiology and metabolism, is a precursor of NO and, recently, it has been found to crucially influence endothelial function (4). L-Arginine can be broken down by transformation into guanidinoacetate and creatine, a reaction catalyzed by L-arginine:glycine amidinotransferase and guanidinoacetate N-methyltransferase and take place mainly in kidney and liver (5).

Nitric oxide is synthesized enzymatically from L-Arginine by three NOS isoforms, inducible, neuronal NOS and eNOS. NO synthesis is selectively inhibited by guanidino-substituted analogs of L-Arginine or methyl-arginines such as asymmetric dimethylarginine (ADMA), which results from cellular protein degradation (6). ADMA as a competitive endogenous inhibitor of NOS, thus elevated levels of ADMA may play a key role in the pathophysiology of endothelial dysfunction, in the progression of atherosclerosis and in cardiovascular diseases (7). Although NO synthesis, as well as generation of ADMA, symmetric DMA and the arginine homolog homoarginine, occurs intracellularly, these biomarkers are usually measured in plasma (8).

The study aimed to evaluate the relations of ADMA and NO with estrogen (E2) and progesterone (Pg) and its role played in the pathogenesis and severity of pre-eclampsia (PE).

Patients & Methods

The current prospective comparative study was conducted at Obstetrics & Gynecology and Medical Biochemistry Departments at Benha University, Clinical Pathology Department at Tanta University Hospital in conjunction with Medical Biochemistry, Applied Science College, October 6 University since March 2015. The study protocol was approved by the Local Ethical Committees.

The study was designed to include all primigravida with singleton fetus and attended the antenatal care unit prior to the 12th week GA to allow selecting women who develop PE throughout pregnancy. All study participants signed a fully informed written consent to participate in the study and to attend the clinic 4-

weekly till delivery for follow-up. Throughout their antenatal visits, women who developed PE diagnostic criteria were grouped as PE group. PE was diagnosed by the development of gestational hypertension after the 12th week GA in women who were normotensive at time of 1st antenatal visit with systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg on at least two occasions, 4 hours apart, and proteinuria (one dipstick measurement ≥2+ on a voided random urine sample) (9, 10).

Pre-eclamptic women were categorized according to time of development of PE into Early PE if diagnosed around the 20th week GA and Late PE if diagnosed later to the 20th week GA and within each group, PE was categorized as mild and severe. Severe preeclampsia was diagnosed according to the criteria published in the National High Blood Pressure Education Program Working Group Report on High Blood Pressure in Pregnancy (11) including SBP >160 mmHg, DBP>110 mmHg, and proteinuria >5 g/in a 24-h period. Also, patients were considered to have severe preeclampsia if they had 1 or more of the following clinical manifestations: renal abnormalities (oliguria), hematologic abnormalities (thrombocytopenia and microangiopathic hemolysis), HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count and right-upper quadrant pain), or neurologic symptoms (headache, visual disturbances and seizures). The study also included a cross-matched number of pregnant women who completed their pregnancy free of manifestations of PE as control group.

Exclusion criteria included multiple pregnancy, fetal abnormalities, conception after ovarian hyperstimulation program, pre-conception diabetes, essential hypertension and autoimmune diseases especially those accompanied by vasculitis. Also, patients with renal, hepatic or cardiac diseases were excluded from the study.

At time of study enrolment patients underwent complete clinical and gynecological examination to assure absence of exclusion criteria. Gestational age was calculated since the 1st day of the last menstrual period and confirmed by crown–rump length measurement. In all cases, Doppler examinations for determination of uterine arteries pulsatality index (UAPI) were performed using transabdominal ultrasound as described by **Hollis et al.** (12, 13). High-resistance cases were defined as cases showed bilateral uterine artery notches with mean UAPI >95th centile and normal-resistance cases included those showed no uterine artery notches with mean UAPI <95th centile (12).

Investigations

Sampling: blood samples were obtained from all study participants at time of 1st antenatal visit. Venous blood samples (5 ml) were collected from the antecubital vein under complete aseptic conditions. Collected blood samples were allowed to clot and then were centrifuged at 1500×g for 15 min and the serum samples were stored at –70°C until assayed.

Estimated parameters

1. Serum estradiol (E2) and progesterone (Pg) levels were measured using an ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to manufacturer's instructions (14).

2. Serum NO level was estimated using the two-step Nitrate/Nitrite assay depending on the fact the best index of total NO production is the sum of both nitrite and nitrate. The first step is the conversion of nitrate to nitrite by the use of NADH or NADPH-dependent nitrate reductase and the converted nitrite was quantified by the addition of Griess Reagent, which converts it to a purple azo compound. The absorbance was measured by a microreader at a wavelength of 540 nm and endogenous nitrite concentration of the samples was determined by comparing to the nitrite standard reference curve. In the second step, the total nitrate/nitrite concentration of a sample was measured using the nitrate assay procedure and the calibration curve of nitrate. Endogenous nitrate serum concentration was calculated by subtracting the endogenous nitrite concentration obtained in the 1st step from the total nitrate/nitrite concentration obtained in 2nd step (15).

3. Serum ADMA levels were measured using ELISA kit (Eagle Biosciences, Hamburg, Germany) that relies on competing of ADMA in serum with solid phase-bound ADMA for a fixed number of rabbit anti-ADMA antiserum binding sites. When the system is in equilibrium, after overnight incubation, free antigen and antigen-antiserum complexes were washed and then anti-rabbit/peroxidase was used to detect the antibody bound to the solid phase ADMA, which is inversely proportional to the ADMA concentration of the sample. The substrate/peroxidase reaction was monitored at 450 nm and result was interpreted versus the typical standard curve (16).

Statistical analysis

Sample size was calculated using the standard nomogram proposed by **Kraemer & Thiemann** (17) and a sample size of 30 patients was determined to be sufficient to detect a difference at the 5% significance level and give the trial 60% power (18). Sample size and power were re-calculated and assured using Power and Sample Size Calculation Software program provided by Department of Biostatistics, Vanderbilt University. Obtained data were presented as mean±SD, numbers and ratios. Results were analyzed using One-way ANOVA with post-hoc Tukey HSD Test and Chi-square test (X2 test). Sensitivity & specificity of estimated parameters as predictors were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) compared versus the null hypothesis that AUC=0.05. Regression analysis (Stepwise method) was used for stratification of studied parameters as specific predictors. Statistical analysis was conducted using the IBM SPSS (Version 23, 2015) statistical package. P value <0.05 was considered statistically significant.

Results

The study included 90 primigravida developed PE during their course of pregnancy (PE group). Another 90 primigravida who completed their pregnancy free of PE were collected as control

group. There was non-significant ($p>0.05$) difference between both groups as regards enrolment data determined at 12th week GA as shown in table 1.

Among women who developed PE, 39 women developed early PE; 33 had mild and 6 had severe PE. The other 51 women developed late PE; 44 had mild and 7 had severe PE. Women who developed mild early PE had significantly higher SBP ($p=0.027$), DBP ($p=0.020$) and extent of proteinuria ($p=0.025$) compared to those who developed late mild PE. On contrary, women who developed severe early PE showed non-significantly ($p>0.05$) higher measures compared to those had severe late PE. Moreover, total women who developed early PE showed non-significantly higher measures than those who had late PE. Details of clinical findings at time of development of PE are shown in table 2.

The 12th week GA estimated serum levels of E_2, Pg and NO were significantly ($P_2=0.001$) lower with significantly ($P_2=0.001$) higher serum ADMA levels in PE women than in PE free women. Moreover, estimated UAPI was significantly ($P_2=0.001$) higher in controls than in PE women. Differentially women developed early PE, both mild and severe PE, showed significantly ($P_1=0.001$) higher UAPI compared to women who developed late PE. Furthermore, mean serum levels of E_2 and NO were significantly lower with mild early PE with significantly higher ADMA serum level, but non-significantly ($P_1>0.05$) lower serum Pg levels than with mild late PE. On the other hand, women developed severe early PE showed significantly higher serum ADMA ($P_1=0.006$) and significantly ($P_1=0.015$) lower NO level than women developed severe late PE. Details of laboratory and US findings are shown in table 3.

Severity of PE as judged by SBP at time of development of PE showed positive significant correlation with high maternal age and with higher 12th week GA maternal BMI, UAPI and serum ADMA levels, while showed negative significant correlations with 12th week GA maternal serum levels of NO, E_2 and Pg. At 12th week GA maternal serum ADMA levels showed positive significant correlation with maternal BMI and UAPI, while showed negative significant correlations with maternal serum levels of NO and E_2, but the correlation was negative non-significant with maternal serum Pg. Details of significance of correlations are shown in table 4.

Evaluations of predictors for development of PE depending on data obtained at 12th week GA for all study population, using ROC curve analysis, defined high UAPI, low maternal serum levels of NO, Pg and E_2 and high maternal serum ADMA levels, in decreasing order of significance (Fig. 1). On the other hand, high maternal serum ADMA levels estimated at 12th week GA was the only significant sensitive predictor for development of severe PE among women of PE group. Details of significance of PE predictors are shown in table 5.

Verification of significant early predictors for PE development using Regression analysis defined high UAPI, high maternal serum ADMA levels and maternal BMI as specific independent predictors, in decreasing order of significance. On the other hand, Regression analysis defined high UAPI and high maternal serum ADMA levels as

specific independent predictors for severe PE, in decreasing order of significance. Details of significance of PE predictors are shown in table 6.

Table (1): The 12th week GA data of studied women categorized according to development of PE

Data		Control (n=90)	PE (n=90)	P value
Age (years)		27.8±2.4	28.4±2.7	**0.076**
BMI data	Weight (kg)	80.7±10.7	82.3±11.6	**0.057**
	Height (cm)	169.7±3.4	170.3±3.1	**0.162**
	BMI (kg/m^2)	28±3.7	28.4±	**0.074**
Blood pressure measures (mmHg)	SBP	120.3±6.1	122.4±8.3	**0.056**
	DBP	75.7±6.8	77.3±7.3	**0.127**
Proteinuria‡	No	64 (71.1%)	57 (63.3%)	**0.266**
	Present (+)	**26 (28.9%)**	**33 (36.7%)**	

Data are presented as mean±SD & numbers; percentages are in parenthesis; PE: Pre-eclampsia; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; p>0.05: indicates non-significant difference; ‡: Level of protein in urine as judged by dipstick measurement and expressed as number of + marks

Table (2): Clinical data of PE women determined at time of development of PE

Data		Mild PE	Severe PE
SBP (mmHg)	Early PE	149.4±4.3	**172±3.6**
	Late PE	147±4.8	**170.1±3.4**
	P=	0.027	**0.347**
DBP (mmHg)	Early PE	99.5±4	**118.5±2.7**
	Late PE	97.1±4.8	**116.6±2.1**
	P=	0.020	**0.182**
Proteinuria ‡	Early PE	2.5±0.5	**3.7±0.5**
	Late PE	2.3±0.4	**3.6±0.5**
	P=	**0.025**	**0.762**

Data are presented as mean±SD; PE: Pre-eclampsia; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; P: significance versus women developed late PE; p<0.05: indicates significant difference; p>0.05: indicates non-significant difference; ‡: Level of protein in urine as judged by dipstick measurement and expressed as number of + marks

Table (3): Laboratory and US findings of PE women determined at the 12th week GA compared to women free of PE

Parameter		Mild	Severe	Total	Total PE	Control
E_2 (pg/ml)	Early PE	8284±1009	6961±573	8080±1065	8452±1143	**11393±1036**
	Late PE	8946±1048	7416±609	8736±1128	P_2=0.001	
	P_1=	0.007	0.194	0.006		
Pg (pg/ml)	Early PE	172.6±9.4	164.3±9	170.5±9.6	170.7±9.6	**191.7±11.2**
	Late PE	173±9	159±5.2	170.8±9.7	P_2=0.001	
	P_1=	0.555	0.208	0.426		
ADMA (ng/ml)	Early PE	116±12.6	136.8±8.9	119.2±14.2	108.4±15.8	**95.7±10.3**
	Late PE	98.1±9.5	113±15	100.2±11.5	P_2=0.001	
	P_1=	0.001	0.006	0.001		
NO (µmol/L)	Early PE	29.6±3.1	28.4±3.4	29.5±3.2	32.4±4.1	**42.4±4.4**
	Late PE	34.6±3.1	32.9±2.2	34.6±3.3	P_2=0.001	
	P_1=	0.001	0.015	0.001		
UAPI	Early PE	0.87±0.01	0.91±0.01	0.87±0.02	0.87±0.02	**0.55±0.05**
	Late PE	0.86±0.01	0.88±0.01	0.86±0.01	P_2=0.001	
	Late PE	**0.001**	**0.001**	**0.001**		

Data are presented as mean±SD; PE: Pre-eclampsia; E2: Estradiol; Pg: Progesterone; ADMA: NO: Nitric oxide; UAPI: uterine artery pulsatility index; P1: significance versus women developed late PE; P2: significance versus women free of PE (Control women); p<0.05: indicates significant difference; p>0.05: indicates non-significant difference

Discussion

The current study detected significantly higher maternal uterine artery pulsatility index (UAPI) and serum ADMA levels with significantly lower E2 and NO levels estimated in women who developed PE compared to women free of PE and in early than in late PE. Moreover, disturbed serum levels of studied parameters showed significant correlation with possibility of development and severity of PE.

In line with these findings, Alpoim et al. (19) detected significantly increased serum ADMA level in early versus late PE and versus normotensive pregnant women, so suggested that high ADMA levels in early PE could compromise NO synthesis contributing to endothelial dysfunction (ED), leading to impaired placentation and the onset of PE. Also, Khalil et al. (20) reported that women with PE and low birth weight offspring had ADMA, L-arginine, and homoarginine levels >75th percentile of control levels and concluded that PE was associated with changes in circulating markers that might represent early ED. Moreover, Bian et al. (21) found first-trimester serum ADMA levels of women who developed PE were significantly higher compared to women with normal pregnancies

Recently, Gumus et al. (22) found maternal and umbilical serum ADMA levels were significantly higher in women with PE and PE with intrauterine growth retardation (IUGR) than women free of PE and concluded that both maternal and umbilical serum ADMA levels correlate with severity of PE. Also, Zheng et al. (23) reported that ADMA concentrations in placental tissue and maternal serum were significantly higher with significantly lower serum E2 levels in severe than in mild PE, while maternal serum Pg levels showed significant difference only between women with severe PE and control women.

Statistical analyses defined high UAPI and the 12-w GA maternal serum ADMA level as the significant early predictors for development and severity of PE; similarly, Bian et al. (21) documented that logistic regression and ROC curve identified first-trimester placental growth factor (PGF) and ADMA to be sensitive and selective predictors of PE. Also, López-Alarcón et al. (24) reported gradually increased ADMA levels throughout pregnancy in PE and concluded that increased ADMA levels precede clinical manifestations of PE.

Table (4): Correlation between laboratory and US findings of PE women determined at the 12th week GA

Parameters	SBP (mmHg)		ADMA (ng/ml)	
	r	p	r	**p**
SBP (mmHg)	-	-	0.422	0.0008
Age (years)	0.243	0.022	0.052	0.629
BMI (kg/m^2)	0.291	0.006	0.211	0.047
UAPI	0.690	0.00001	0.327	0.002
NO (µmol/L)	-0.221	0.037	-0.377	0.0009
E_2 (pg/ml)	-0.325	0.002	-0.344	0.001
Pg (pg/ml)	-0.214	0.044	-0.167	0.117
ADMA (ng/ml)	**0.422**	**0.0008**		

SBP: systolic blood pressure; BMI: Body mass index; UAPI: Uterine arteries resistance index; E2: Estradiol; Pg: Progesterone; ADMA: NO: Nitric oxide; Pg: Progesterone; $p<0.05$: indicates significant difference; $p>0.05$: indicates non-significant difference

Table (5): ROC curve analysis of laboratory and US findings at the 12th week GA as predictors for development and severity of PE

Parameter	Early prediction of PE				Prediction of severity of PE			
	AUC	SE	p	CI	AUC	SE	p	**CI**
SBP (mmHg)	0.575	0.043	0.084	0.49-0.66				
Age (years)	0.573	0.044	0.095	0.487-0.658	0.422	0.119	0.649	**0.19-0.655**
BMI (kg/m^2)	0.530	0.044	0.487	0.445-0.616	0.610	0.053	0.517	**0.507-0.714**
UAPI	1.000	000	0.0001	1	0.607	0.085	0.532	**0.44-0.773**
NO (µmol/L)	0.735	0.037	0.0004	0.663-0.808	0.506	0.197	0.973	**0.12-0.892**
E_2 (pg/ml)	0.057	0.016	0.0007	0.026-0.088	0.589	0.117	0.601	**0.359-0.819**
Pg (pg/ml)	0.024	0.008	0.0004	0.007-0.04	0.417	0.130	0.625	**0.161-0.672**
ADMA (ng/ml)	**0.069**	**0.021**	**0.0009**	**0.028-0.11**	**0.151**	**0.075**	**0.041**	**0.004-0.298**

AUC: Area under curve; SE: Standard Error; CI: Confident interval; SBP: systolic blood pressure; BMI: Body mass index; UAPI: Uterine arteries pulsatality index; E2: Estradiol; Pg: Progesterone; ADMA: NO: Nitric oxide; Pg: Progesterone; p<0.05: indicates significant difference; p>0.05: indicates non-significant difference

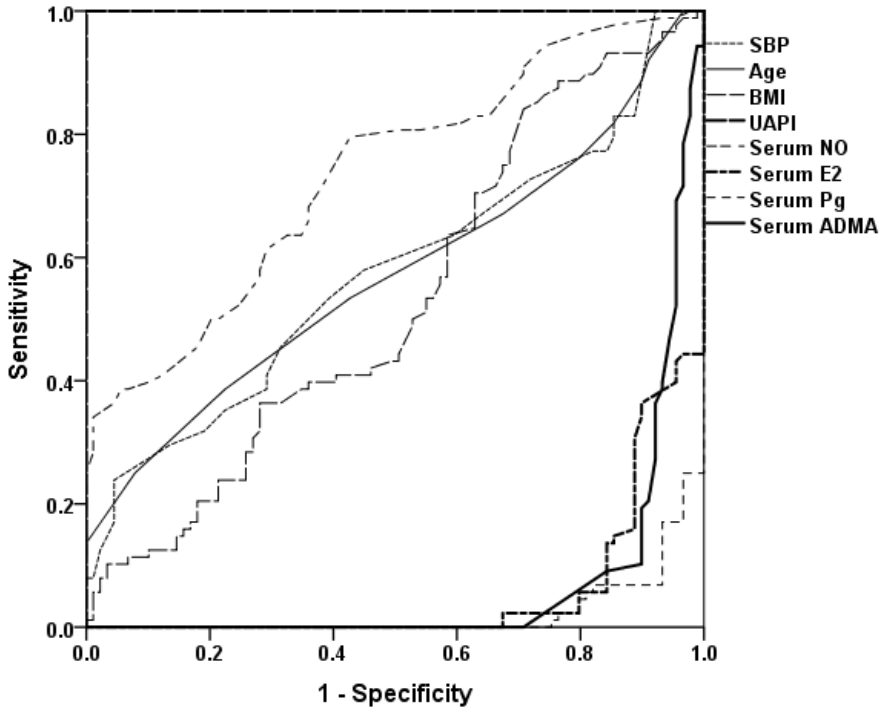

Fig. (1): Laboratory and US findings at the 12th week GA as predictors for development of PE

Table (6): Regression analysis for laboratory and US findings at the 12th week GA as predictors for development and severity of PE

Early prediction of PE				Prediction of severity of PE			
Model	Parameter	β	p	Model	Parameter	β	**p**
No. 1	BMI (kg/m^2)	-0.023	0.004	No. 1	ADMA (ng/ml)	0.219	**0.007**
	ADMA (ng/ml)	-0.026	0.003		UAPI	0.618	**0.0006**
	UAPI	1.009	0.0009	No.2	UAPI	0.690	**0.0004**
No. 2	ADMA (ng/ml)	-0.027	0.003				
	UAPI	1.006	0.0006				
No. 3	**UAPI**	**0.994**	**0.0004**				

β: ; UAPI: Uterine arteries resistance index; ADMA; p<0.05: indicates significant difference

Recently, Chang et al. (25) found the predictive model consisting of mean arterial pressure (MAP), UAPI and serum biomarkers showed predictive value for the early (11-13 weeks GA) diagnosis of early PE with fetal growth retardation of 73.2%. Moreover, Tsiakkas et al. (26) reported that combined screening by maternal factors, MAP, UAPI and biomarkers predicted 98% of preterm-PE and 49% of term-PE, at a false-positive rate of 5%. Also, Li et al. (27) reported that in pregnancies that developed PE, the UAPI was increased and a combination of biomarkers and UAPI gave an AUC of 0.915 with 91% sensitivity at 82% specificity.

The reported positive significant correlation between high SBP, a diagnostic criterion for PE and at 12-w GA high BMI and serum ADMA levels and the negative significant correlation between high SBP and serum NO and E2 levels spot light on a possible relationship between disturbed NO/ADMA axis, obesity, disturbed sex hormonal milieu on one side and the development of PE on the other side. In support of this assumption, the present study detected that serum ADMA levels showed positive significant correlation with BMI, but negative significant correlation with serum E2 levels and both high serum ADMA and BMI were of the persistently significant early predictors for PE development and severity in regression analysis models.

Multiple experimental and clinical studies go in line with these findings and proposed assumption; experimentally, Chakraborti et al. (28) found administration of estrogen synthesis blockers increased ADMA and decreased NO metabolites levels in female rats; El Assar et al. (29) reported that increased ADMA and up-regulated arginase contribute to ED related to insulin resistance (IR) in obese rats and morbidly obese humans and Tain et al. (30) found animal-induced hypertension was associated with increased plasma ADMA, decreased plasma L-arginine/ADMA ratio, and decreased renal dimethyl-arginine dimethyl-aminohydrolase (ADMA-metabolizing enzyme) activity.

Clinically, Patle et al. (31) reported that bariatric surgery for morbid obesity induced significant weight loss, significantly decreased MAP and plasma ADMA concentration with significantly increased plasma total nitrite concentration. Also, Maeda et al. (32) found the 12-week lifestyle modification program significantly decreased BMI, brachial-ankle pulse wave velocity, an index of arterial stiffness and plasma ADMA concentrations in overweight and obese individuals. Moreover, Karakurt et al. (33) reported significantly decreased ADMA levels and IR after 6 months treatment of women had polycystic ovaries with ethinyl estradiol and cyproterone acetate. Laskowska et al. (34) found impaired NO bioavailability in pregnancies complicated by severe PE result from increased ADMA levels not from a reduced level or activity of eNOS or from its disturbed intracellular transport. Recently, de Giorgis et al. (35) reported that increased BP already occur moving from the pre-pubertal to the pubertal period in obese children, and modifications in IR and ADMA seem to be implicated in this early progression in BP.

Conclusion:

Disturbed maternal serum ADMA/NO axis and low E_2 serum levels may underlie development of PE. High BMI and low maternal serum E_2 level may have a role in PE pathogenesis through induction of

high serum ADMA levels. High UAPI and serum ADMA at the 12th week GA could be used for early prediction of PE especially early-onset and severe PE

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