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Endothelial and Platelet-Derived Microparticles in Preeclamptic and Normal Pregnant Women

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ABSTRACT

Background: Preeclampsia (PE) is a vascular disorder with uncertain etiology. Increased levels of circulating microparticles (MPs) have been suggested as a possible cause of vascular damage.

Aim: To explore the MPs involved in the control of hemostatic equilibrium: platelets and endothelial MPs as well as total MPs, defined as annexin V positive MPs, in pregnant women.

Patients and Methods: The study was conducted on 25 women with PE and 25 normal pregnant women (NP); another 25 normal non-pregnant women served as a normal control. Endothelial microparticles (EMPs) and platelets microparticles (PMPs) were measured by flowcytometry using monoclonal antibodies (anti-PECAM-1, FITC-CD31 for EMPs; anti-GPIIbIIIa, PE-CD41 for PMPs; and FITC-labeled annexin V for total MPs).

Results: The mean level of EMPs ($CD31^+/CD41^-$) was significantly elevated in PE group compared to NP group ($p<0.001$). EMPs level in PE positively correlated with systolic blood pressure ($p=0.006$; $r=0.36$) and diastolic blood pressure ($p=0.001$; $r=0.42$). The mean level of PMPs ($CD31^+/CD41^+$) as well as mean level of the total MPs were not different among the three groups. No significant correlation was found between PMPs or total MPs and mean arterial pressure in cases or control subjects.

Conclusions: The significant elevation in EMPs supports the theory of endothelial injury in the pathogenesis of PE. Further studies are needed to evaluate diagnostic and prognostic value of EMPs in PE at earlier gestational ages.

Key Words: *Preeclampsia – Circulating microparticles – Platelets – Endothelial microparticles.*

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INTRODUCTION

Normal pregnancy is a hypercoagulable state, which is associated with increased levels of coagulant factors and decreased levels of naturally occurring anticoagulants and fibrinolysis **[1]**. Preeclampsia (PE) is a multiorgan vascular disorder that complicates 5 to 7% of pregnancies and presents with hypertension, proteinuria, and fetal growth delay **[2]**. Approximately 10% of pregnancies are associated with hypertension, 75% of them are related to PE **[3]**.

Despite extensive research, the mechanisms involved in the vascular dysfunction in PE are not well understood. An endothelial dysfunction because of placental ischemia was proposed **[4]**. More recently, elevated plasma concentration of shed membrane microparticles (MPs) during PE has been reported **[5]**.

MPs are sub-cellular fragments (0.1-1.0 mm in diameter) that are released from the plasma membrane of stimulated or apoptotic cells into body fluids **[6-9]**. Elevated levels of MPs may reflect either increased cell activation or impairment of clearance by the reticuloendothelial system **[10]**. In addition, MPs exert prothrombotic activity by exposing negatively charged phospholipids and tissue factor. Thus, MPs are increased in conditions involving hypercoagulation, or systemic inflammation such as idiopathic thrombocytopenia, sepsis, and metabolic syndrome **[11-14]**. Normal pregnancy as well as PE, enhances MPs release from intravascular cells **[15,16]**.

Endothelial MPs (EMPs) provide markers of activated endothelium and increased levels

were seen in diseases associated with endothelial injury such as atherosclerosis, acute coronary syndromes, hypertension, and PE **[12,17-19]**. In addition, a proportion of women with pregnancy loss had elevated EMPs suggesting that endothelial damage or activation might be involved in the pathogenesis of pregnancy loss **[20]**.

Circulating platelet MPs (PMPs) could be markers of platelet activation **[17]**. In addition, increased levels of PMPs were demonstrated in patients with thrombotic disorders such as transient ischaemic attacks and myocardial infarction **[11]**. However, the physiological significance of PMPs is still unclear.

PE is a serious pregnancy complication in less developed countries due to the poor antenatal care and low socioeconomic state. In Egypt, PE was reported as an important cause of maternal and fetal mortality as well as premature deliveries **[21]**. Additionally, more than 83% of Egyptian women with toxemia of pregnancy reported severe form of PE **[22]**. The present study aimed to explore the MPs involved in the control of hemostatic equilibrium, i.e. platelets-derived, endothelial-derived, as well as total MPs (annexin V positive) micropaticles, in both PE and normal pregnant women.

PATIENTS AND METHODS

This is a case control study that was carried out on women attending the outpatient clinic as well as the internal department of Obstetrics and Gynecology at Suez Canal University Hospital in the period from October 2007 to April 2008.

The institutional review board (of Suez Canal University) had approved the study. All patients have given an informed consent. The study included 3 groups as follows: (Group 1) pregnant women diagnosed with PE (PE, n=25), (Group 2) normal [normotensive] pregnant women (NP, n=25) and, (Group 3) non-pregnant women as a control group (C, n=25). The following matches were considered: a) Age (±5 years) and parity (for women in the three groups), b) gestational age (±2 weeks) for PE and NP groups.

Definition of PE:

A- de novo appearance of hypertension (diastolic blood pressure ≥110mmHg on any occasion, or ≥90mmHg on two separate occasions (at least four hours apart).

B- New onset of proteinuria (at least 0.3g protein/24 hours or ≥2+ on dipstick/24 hours) detected for the first time after 20 weeks of gestation.

C- Symptoms of PE developed after 20 weeks gestational age in a previously normotensive woman (according to standard criteria, Brown et al., 2001).

Patients with preexistent hypertension, gestational diabetes mellitus, coagulation disorders, previous renal or hepatic disease, intra-uterine growth retardation, patients in labor, and patients on regular drug treatment other than antihypertensive medications (i.e. oral contraceptive pills and aspirin) were excluded from the study. The control group consisted of healthy women not using any medications including oral contraceptives.

Methods:

Venous blood was drawn with minimal stasis into citrated vacutainer tubes (BD Biosciences; Oxford, UK) containing 0.5mL of 3.8% 0.129 mol/L trisodium citrate, 9:1 v/v.

Platelet-poor plasma (PPP) was obtained by centrifugation at 1500g for 15 min within 15min. of venipuncture. Plasma samples were divided in 250µl aliquots, snap frozen in liquid nitrogen to preserve MPs structure, and stored at –80°C until further analysis.

Flowcytometer analysis of MPs:

A- *Isolation of MPs:* MPs were isolated from plasma samples as described previously **[23]**. A sample of 250µl frozen plasma was centrifuged for 30 minutes at 19000g at 20°C to pellet the MPs. After centrifugation, 225µl of the supernatant was removed. The MPs pellet and remaining supernatant were re-suspended in 225µl phosphate-buffered saline with citrate (154mmol/L NaCl, 1.4mmol/L phosphates, 10.9mmol/L trisodium citrate, pH 7.4). After centrifugation for 15 minutes at 13000g at 20°C, 225µl of the supernatant was removed again. The MPS pellet was then re-suspended in 75µl

citrated PBS, of which five µl was used per incubation.

B- *Labeling of MPs:* Five µl of MPs suspension was diluted in 35µl of PBS containing calcium chloride (2.5mol/L), and separated equally into two tubes (A and B): In tube (A), 5µl FITC-labelled annexin V (BD Biosciences pharmingen, USA) was added to measure total MPs. In tube (B), 5µl of the monoclonal antibody against endothelial and platelet antigens were used (anti-PECAM-1, FITC-CD31, and anti-GPIIbIIIa, PE-CD41, Diaclone, France) to measure EMPs and PMPs respectively. Samples were incubated in the dark for 15 minutes at room temperature. After incubation, 200µl citrate-containing PBS was added to tube (A) while, 200µl of calcium-containing PBS was added to tube (B).

C- *Analysis:* Samples were analysed in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software (Becton Dickinson, San Jose, CA). Both forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. To identify marker positive events, thresholds were set based on microparticle samples incubated with same concentrations of isotype matched control antibodies (fluorescein isothiocyanate (FITC)-labelled IgG1 and phycoerythrin (PE)-labelled IgG1, Becton Dickinson, San Jose, CA) and an internal standard was added immediately prior to flow cytometry (Enumeration beads 1.01mm in diameter), (Sigma, USA). Calculation of the number of microparticles per ul plasma is based upon the particle count per unit time, the flow rate of the flowcytometer, and the net dilution of the microparticle suspension. MPs were identified on basis of their size (< mean diameter of the latex beads), density and capacity to bind to a cell type-specific antibody. The inter-assay and intra-assay coefficients of variation were <8% and <5%, respectively.

D- *Interpretation of the results:* Events with 0.1- to 1-µm size on a FS-SS graph were gated as MPs. MPs were estimated as the difference in labeling between specific antibody and their isotype. Mps expressing only CD31 ($CD31^+$/$CD41^-$) were defined as endothelial-derived MPs, Particles co-expressing both antigens ($CD31^+$/$CD41^+$) were defined as platelet-derived MPs while, (annexin V^+) MP were defined as prothrombotic MPs **[23]**.

Statistical analysis:

Statistical analyses were performed with the use of the software SSPS for windows 11.5. Values were expressed as median, range, and percentages as appropriate. Mann-Whitney U Test was used to compare MPs between two groups. While, Kruskall Wallis test was used to compare MPs among the three groups. Correlation between EMPs and blood pressure was performed by linear regression analysis. *p*-values of 0.05 or less were regarded as significant.

RESULTS

Baseline characteristics of study participants are shown in (Table 1). Blood pressure was significantly higher in PE group compared to NP group ($p<0.001$).

Hematologic parameters were compared between PE group and NP group. Platelet count was significantly lower in PE group compared to NP group ($p=0.02$), however, it was within the normal range for NP and C groups. Comparing NP group to C group revealed a significant decrease in all hematologic parameters in NP group compared to C group ($p=0.002$, 0.002, 0.004 and 0.03 for hemoglobin levels, red blood cells, white blood cells and platelet counts respectively) (Table 2). The total number of prothrombotic circulating MPs were analyzed based on whether they were annexin V positive or not. The mean level of MPs showed no significant difference among the three groups (Table 3, Fig. 1). The mean level of Platelets microparticles (PMPs) ($CD31^+$/$CD41^+$ PMPs, counts $x10^3$/ml) were not significantly different among the three groups ($p=0.20$) (Table 3, Fig. 2). In addition, no correlation was found between PMPs levels and either systolic or diastolic blood pressure ($p=0.47$, $p=0.67$ respectively). The mean level of endothelial cell microparticles (EMPs) ($CD31^+$/$CD41^-$ EMPs, count $x10^3$/ml) revealed a statistically significant difference among the three groups ($p=0.04$). EMPs was significantly higher in PE group compared to NP group ($p=0.001$). While, no difference between NP group and C group was noted ($p=0.62$). EMPs levels in PE group positively correlated with both systolic and diastolic blood pressure ($r=0.36$; $p=0.006$) and ($r=0.42$; $p=0.001$) respectively (Table 3, Figs. 2-4).

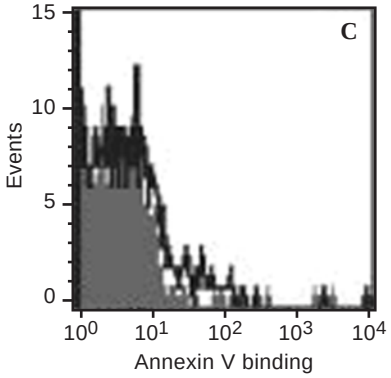

Fig. (1): Representative histogram of positive FITC annexin-V microparticles in PE group (preeclampsia).

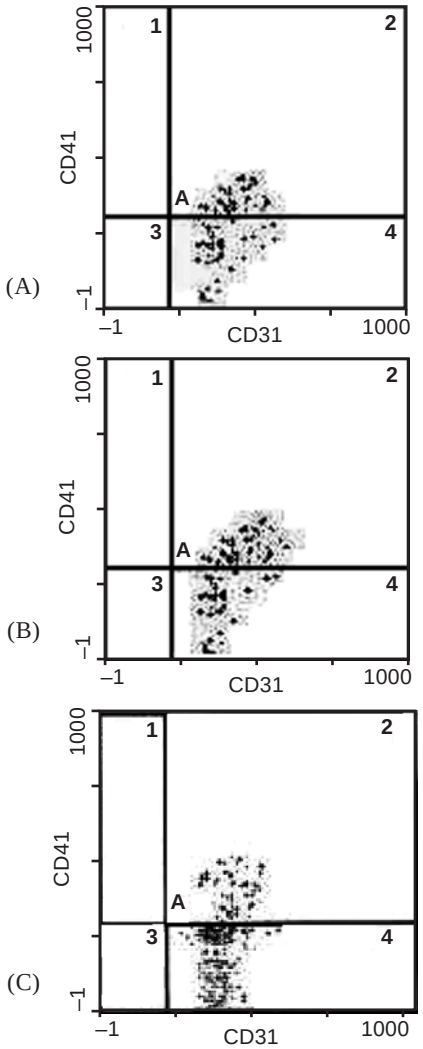

Fig. (2): Flow cytometric determination of EMP ($CD31^+/CD41^-$) and PMP ($CD31^+/CD41^+$): (A) C group (non-pregnant); (B) NP group (normal pregnant); (C) PE group (preeclampsia). Region 4 (lower right) is $CD31^+/CD41^-$, which represents EMP. Region 2 (top right) is $CD31^+/CD41^+$, which represents PMP.

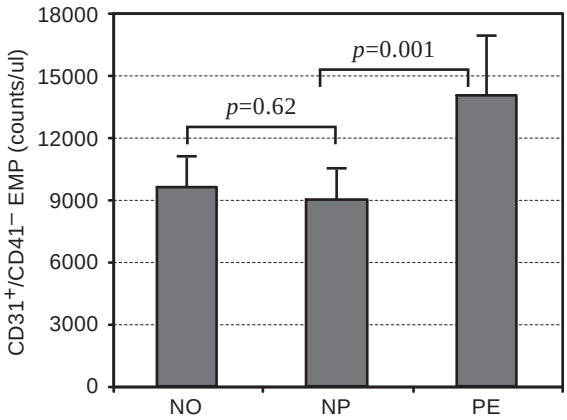

Fig. (3): Endothelial microparticles in: Non pregnant group (C), normal pregnant group (NP) and preeclampsia group (PE).

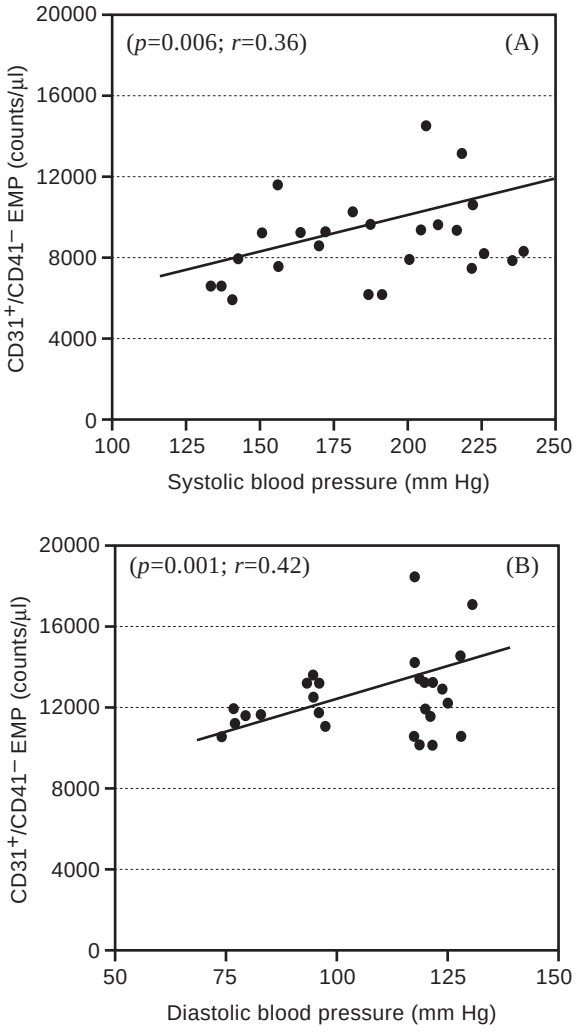

Fig. (4): Correlation between endothelial microparticles and systolic blood pressure (A), diastolic blood pressure (B) in Preeclamisa.

Table (1): Study population characteristics.

Characteristic	Preeclamptic (PE) (n = 25)	Normal pregnant (NP) (n = 25)	Control (C) (n = 25)
Demographic data:			
Patient age (yrs)	29 (27.7-30.4)	29 (28.0-30.6)	30 (23.5-34)
Gestational age (weeks)	35 (33.9-36.3)	36 (33.7-36.3)	–
Blood pressure (mmHg):			
Systolic	160 (148-200)	110 (109-118)	108 (95-133)
Diastolic	100 (97-103)	70 (68-74)	69 (65-85)
Proteinuria (g/L)	3.2 (0.4-5.2)	–	–
Parity:			
Primiparous	15 (60%)	12 (48%)	116 (64%)
Multiparous	10 (40%)	13 (52%)	99 (36%)

Values are given as medians, ranges and percentages.

Table (2): Hematological data of the study groups.

Hematologic data	Preeclamptic (PE) (n=25)	Normal pregnant (NP) (n=25)	P^a	Control (C) (n=25)	P^b
Hemoglobin (g/dl)	11.9 (10.9-12.6)	11.9 (11-12.3)	0.80	13.1 (12.6-13.8)	0.002
Red cell count ($x10^6$/L)	3.8 (3.6-3.9)	3.9 (3.7-4.2)	0.36	4.6 (4.2-4.8)	0.002
White cell count ($x10^3$/L)	8.7 (7.6-10.4)	8.4 (7.7-10.0)	0.65	6.5 (4.9-8.2)	0.004
Platelet count ($x10^3$/L)	154 (93-229)	203 (186-340)	0.02	288 (223-390)	0.03

Values are given as medians and ranges.
Statistical significant difference at $p<0.05$.
P^a = Preeclamptic group (PE) compared to normal pregnancy group (NP): Mann-Whitney U test.
P^b = normal control group (C) compared to normal pregnancy group (NP): Mann-Whitney U test.

Table (3): Microparticles in the study groups.

	Preeclamptic (PE) (n = 25)	Normal pregnant (NP) (n = 25)	Non pregnant (C) (n = 25)	P	P^b	P^c
Total MPs [a] (counts $x10^3$/µl)	23.4±9.1	19.8±7.6	22.7±8.8	0.62	–	–
PMPs [a] (counts $x10^3$/µl)	10.6±6.5	8.8±4.4	9.9±5.4	0.20	–	–
EMPs [a] (counts $x10^3$/µl)	13.9±7.9	8.5±3.0	9.8±2.6	0.04	0.001	0.62

MPs = Microparticles; PMPs = Platelet-derived microparticles; EMPs = Endothelial microparticles.
[a] =Values are given as mean ± SDÆ
P : Statistical difference between all groups (Kruskal-Wallis test).
P^b : Statistical difference between PE and NP groups (Mann-Whitney test).
P^c :Statistical difference between NP and C groups (Mann-Whitney test).

DISCUSSION

Preeclampsia (PE) is a major obstetrical health problem that threatens the survival of both mother and baby and, it becomes even more serious in less developed countries **[5]**. According to a WHO study on 7993 pregnancies in developing countries, hypertensive disorders of pregnancy was one of the most common obstetric events leading to perinatal deaths **[24]**.

PE is relatively a disorder of unknown etiology; however, many markers of endothelial dysfunction have been reported in women who develop PE, suggesting that PE is an endothelial cell disorder **[25,26]**. In theory, circulating MPs as pro-coagulant factors could cause the exaggerated hemostatic response 'hypercoagulability' seen in PE **[15]**.

Data about MPs in normal pregnancy and preeclampsia are controversial. Up to our knowledge, this is the first report about the pattern of MPs in Egyptian pregnant women with or without PE. In the present study, the total numbers of prothrombotic circulating MPs showed no

difference among the three studied groups, with no correlation with arterial blood pressure. This result came in conformity with the results of VanWijk et al. **[15,27]** who investigated the cellular origin and numbers of circulating MPs in normal pregnancy and reported no correlation between the total number of circulating MPs and blood pressure. In contrast, Bretelle et al., **[28]** was the first to demonstrate that normal pregnancy was associated with increased numbers and procoagulant activity of cell-derived MP. Unexpectedly according to the same study, pathological pregnancies did not show higher levels of MPs, instead they were associated with lower number of total (annexin V+) MPs. However, Desprez et al., **[29]** reported a progressive increase in procoagulant MPs level during normal pregnancy but these high levels did not exceed MPs level obtained in non-pregnant women. Recently, Redman and Sergent, **[30]** had found that MPs were increased during normal pregnancy, and they increased further with PE. A recent study reported a decrease in MPs at 12 weeks in normal pregnancy, and then returned to normal values postpartum. While a significant decrease in MPs in PE was observed at 28 and 36 weeks **[31]**. In our study, the mean gestational age was 35 weeks for normal pregnancy and 36 weeks for PE, which means that we might have missed the period of possible changes in MPs according to the previous study. The differences between the results of the previous studies may be related to the differences in number of study population, methods used to detect MPs, and gestational age during the study.

Previous studies showed that the levels of PMPs were reduced **[28]**, increased **[5]**, or unchanged **[18]** between PE and normal pregnancy. In this study, numbers of platelets-derived MPs were neither different among the three study groups nor related to arterial blood pressure. Same results were reported previously **[18,32]**. However, others reported significant fewer absolute numbers of PMPs in PE women compared to normal pregnant women **[28,31,33]**. Surprisingly, PMPs were not significantly lower only in PE, but also in non-pregnant women compared to normal pregnancy **[27]**.

Recently, Lok et al. **[16]** suggested that the decrease in PMPs in PE women is possibly due to the concurrent decrease in platelet numbers. They reported a ratio of 0.02 between PMPs count and platelets number that was consistent among all the studied groups suggesting a direct association between number of circulating platelets and number of PMPs. Another possible explanation was that PMPs from PE patients might attach to leucocytes via p-selectin glycoprotein ligand-1 (PSGL-1) and being removed from circulation. In our study, platelet counts were significantly lower in PE compared to normal pregnancy and control group, yet it was within the normal range of platelets count in NP and C groups. Thus, the absence of a difference in PMPs in our patients could be explained partially by the absence of a true decrease in platelet count. Also, there is a possibility that PE may be associated with increased procoagulant potential of MPs without a change in their total number, as reported previously **[15,27,28]**. In favor of the latter explanation is the lack of a correlation between numbers of MPs and severity of PE suggesting that MPs numbers alone do not explain the reported vascular effects of MP **[31]**.

The measurement of plasma EMPs is emerging as a useful marker of endothelial injury. Normally, EMPs represent 10-15% of the total microparticle population, and exist within a concentration range of 1-70 $x10^3$ (EMPs/ml) **[28,34,35]**. In the current study, EMPs showed a significant increase in PE group compared to normal pregnant group with significant correlation to both systolic and diastolic blood pressure. The same results were recorded by González-Quintero et al. **[18]**. These results are in favor of the theory that suggested EMPs as an important pathogenetic factor in the development of endothelial cell disorder (ECD). Previously, circulating EMPs were reported to directly affect the endothelium via impairment of ACh-induced vasorelaxation and nitric oxide production and thus not only act as a marker for ECD but also aggravate preexisting ECD **[36]**.

Accordingly, a prospective study on patients with PE, and those with gestational hypertension reported a significant elevation in $CD31^+/CD42^-$ EMPs in PE compared to both gestational hypertension and normal pregnancy. In addition, PE plasma elicited a significantly greater level of $CD31^+$ EMP release from the cultured renal microvascular endothelial cells compared to gestational hypertension or control plasma

[37]. However, Bretelle et al. **[28]** enumerated EMPs using specific monoclonal antibody directed against αvβ3 (CD51). They reported a significant increase in plasma EMPs in normal pregnant women compared to non-pregnant controls; in the mean time, no difference was observed in PE group compared to normal pregnant women.

In this work systolic and diastolic blood pressure significantly correlated with the levels of $CD31^{+}/CD41^{-}$ EMP, indicating that the severity of hypertension is likely indicative of progressive endothelial damage. This supports previous findings in which non-pregnant patients with severe hypertension exhibited elevated EMP values when compared with mild hypertension and control groups **[19]**. This increase in blood pressure goes hand in hand with the elevation of EMPs since both reflect the possible endothelial damage in PE patients.

Some limitations to our study included the cross sectional type (tests were done in a single time point), the relatively small population number, and the late gestational age (35-36 weeks). Further prospective studies using larger population of pregnant women at different maternal and gestational ages are needed to enhance our results and, evaluate MPs diagnostic and prognostic value in PE. Still, this is the first study investigating MPs levels in Egyptian pregnant and preeclamptic women. In conclusion, the significant elevation in EMPs in this study supports the theory of endothelial injury in the pathogenesis of PE.

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