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Circulating microparticles in severe preeclampsia

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ABSTRACT

The present study aimed to evaluate microparticles (MPs) from different sources in women with severe preeclampsia (PE) compared with normotensive pregnant women and non-pregnant women. This case–control study evaluated 28 pregnant women with severe PE, 30 normotensive pregnant women, and 29 non-pregnant women. MPs from neutrophils, endothelial cells, monocytes, platelets, leukocytes, erythrocytes, and syncytiotrophoblast were evaluated using flow cytometry. A higher total number of MPs were observed in women with severe PE compared with normotensive pregnant women and non-pregnant women ($P=0.004$ and $P=0.001$, respectively). MPs derived from erythrocytes were increased in women with severe PE compared with normotensive pregnant women ($P=0.002$). A trend towards association was observed between platelet count and the number of MPs derived from platelets ($P=0.09$) in severe PE group. A positive correlation was also found between the number of endothelial cell-derived MPs and the number of platelet-derived MPs, leukocyte-derived MPs, neutrophil-derived MPs, and lymphocyte-derived MPs ($P<0.05$) in severe PE pregnant women. MP counts can be increased in severe PE, and erythrocyte and endothelial cell-derived MPs seem to be associated to severe PE.

1. Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome characterized clinically by hypertension and proteinuria after 20 weeks gestation [1,2]. The etiology of PE remains unknown, but it is a multifactorial disorder. The clinical spectrum ranges from mild to severe [3,4]. In its severe form, PE is an important cause of maternal and fetal morbidity and mortality worldwide [3,5]. The origin of PE remains enigmatic despite considerable research, but the placenta undoubtedly plays a role in its pathogenesis because delivery inevitably leads to recovery [6,7].

Pregnancy is a controlled inflammatory state. It is believed that an excessive systemic inflammatory response is the basis of clinical manifestations of PE, but the causes of this inflammatory response in normal pregnancy and PE are not known [8,9]. Some studies have shown that all network components of intravascular inflammation (leukocytes, endothelial cells, and the coagulation cascade) contribute to the exacerbation of the inflammatory response in PE [10]. In addition to placental cytokines and angiogenic factors, apoptotic fragments released into the maternal blood are candidates that trigger this systemic inflammatory process [9,11–13].

Microparticles (MPs) are vesicles (0.1–1 μm) that are shed from the plasma membranes of several cell types in response to activation or apoptosis. The initial step in their formation is membrane remodeling with the formation of blebs. This step requires increased intracellular calcium levels resulting in the rearrangement and loss of phospholipidic membrane asymmetry with externalization of phosphatidylserine. Concomitant to the loss of membrane asymmetry, calcium-sensitive enzymes are activated and promote cleavage of the cytoskeletal filaments, leading to bleb formation on the membrane and MP release.

MPs are considered potent vectors of biological information and protagonists of cellular communication networks, such as the induction of endothelial modifications, inflammation, differentiation, and angiogenesis, because they mediate cell–cell communication by transferring mRNAs and microRNA from the cell of origin to the target cells [14–16].

MPs of various cellular origins are found in the plasma of healthy subjects, and their amounts increase under pathological conditions [15]. Several groups have reported elevated circulating levels of MPs during pregnancy, but this increase is especially important in preeclampsia, suggesting their involvement in the hypertension associated with this disease [17,18]. Measurement of MP phospholipid content (mainly phosphatidylserine) has allowed their quantification and characterization [15].

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Few studies have evaluated the MPs of different cells in severe PE. Because severe PE is associated with procoagulant and pro-inflammatory states, studies involving MP pathways should be conducted to clarify a possible role of MPs in PE.

2. Material and methods

2.1. Study design

This study included 87 women: 28 pregnant women with severe PE, 30 normotensive pregnant women, and 29 non-pregnant women. Women with severe preeclampsia were enrolled from Maternidade Odete Valadares, Santa Casa de Misericórdia de Belo Horizonte, and Hospital Municipal Odilon Behrens — Belo Horizonte/Brazil and included into the study. Normotensive pregnant women and non-pregnant women were enrolled from Centro de Saúde Guanabara, Betim/Brazil. Clinical data were obtained from the patients' medical records.

2.1.1. Inclusion criteria

Severe PE was defined as systolic blood pressure ≥160 mm Hg or diastolic blood pressure ≥110 mm Hg on at least 2 consecutive occasions, 4 h apart; and proteinuria ≥2 g/l or at least 3+ protein by dipstick. The normotensive pregnant women had systolic/diastolic blood pressure <120/80 mm Hg and no history of hypertension or proteinuria. The non-pregnant women had neither clinical alterations nor a history of PE or hypertension [3].

2.1.2. Exclusion criteria

Exclusion criteria common to the 3 groups were chronic hypertension, hemostatic abnormalities, cancer, diabetes, obesity, and cardiovascular, autoimmune, renal, and hepatic diseases.

2.2. Ethical aspects

This study was approved by the Ethics Committee of Universidade Federal de Minas Gerais (COEP), no. ETIC 0343.0.203.000-10, and informed consent was obtained from all participants.

2.3. Blood samples

Blood samples were drawn in sodium citrate (0.129 mol/l) in a 9:1 volume ratio. The samples were centrifuged at 2500×*g* for 15 min to obtain plasma. Samples were aliquoted and stored at −70 °C until analysis.

2.4. Flow cytometry assay

MPs were prepared as described elsewhere [19]. Briefly, samples were centrifuged at 13,000×*g* for 3 min to obtain platelet-free plasma, which was then diluted 1:3 in citrated phosphate buffered saline (PBS) containing heparin and centrifuged at 14,000×*g* for 90 min at 15 °C. The subsequent MP pellet was resuspended in 1× annexin V binding buffer (Sigma-Aldrich, MO).

MPs isolated from plasma were gated on the basis of their forward (FSC) and side (SSC) scatter distribution of synthetic 0.7–0.9 μm SPHEROTM Amino Fluorescent Particles (Spherotech Inc., Libertyville, IL, USA) (Fig. 1). Taking into account the presence of phosphatidylserine residues on the MP surfaces, events present in the gate were assessed for their positive staining for annexin V (Sigma-Aldrich) — a classical marker for microparticles — using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against annexin V. Labeling with cell-specific monoclonal antibodies was corrected for isotype-matched control antibodies.

FITC-labeled immunoglobulin G1 (IgG1) and PE-labeled IgG1 isotype controls, monoclonal antibodies directed against neutrophils (CD66-PE), endothelial cells (CD51-PE), monocytes (CD14–PERCP), platelets (CD41–PERCP), leukocytes (CD45–APC), and erythrocytes (CD235a–PECy5), were purchased from BD Biosciences® (CA, USA). Monoclonal antibody directed against T lymphocytes (CD3-PE) was purchased from Beckman Coulter Immunotech (Marseille, France). We used the following combination: tube 1 — annexin FITC, CD66-PE, CD45–APC, CD14–PERCP; tube 2 — annexin FITC, CD41–PERCP, CD3-PE and tube 3 — annexin FITC, CD235a–PECy5, CD51-PE.

A placental MP assessment was performed using an indirect staining procedure. NDOG2 (specific primary antibody; BD Biosciences®, San Jose, CA) and a goat anti-mouse IgM secondary antibody PE-conjugate (Thermo Scientific®, Fairlawn, NJ) were used. MPs were incubated with unlabeled NDOG2, washed with PBS, and incubated with secondary antibody PE.

The samples were analyzed for 60 s in a Flow Cytometry FACSCalibur (Becton-Dickinson). The following final dilutions of antibodies were used: anti-CD235a–PECy5 (1:400), NDOG2 (1:20), and anti-mouse IgM secondary antibody (1:25). The other antibodies were used in concentrations according to each manufacturer's instructions.

2.5. Determination of MP plasma levels

To investigate the absolute MP plasma levels and to determinate the number of plasma MPs per microliter (MPs/μl), the cytometer was set to operate at a high flow rate setting for 60 s for each sample.

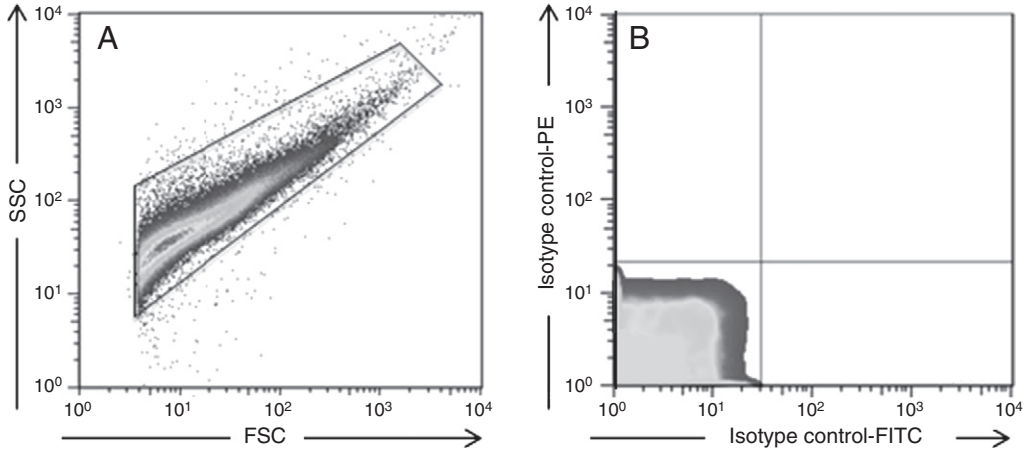

Fig. 1. Identification of plasma microparticles. (A) Microparticles isolated from the plasma were gated on the basis of their forward (FSC) and side (SSC) scatter distribution. (B) Mouse IgG FITC and PE-conjugated isotype control monoclonal antibodies were used to accurately place the gates.

Table 1
Characteristics of the women studied.

Characteristic	Severe PE (n = 28)	Normotensive pregnant (n = 30)	Nonpregnant women (n = 29)	P value
Age (y)	29 (26–34)	24 (20–28)	22 (18–30)	0.008[a]
BMI	25.2 ± 5.4	24.6 ± 4.1	22.6 ± 3.5	0.009[b]
GA (weeks)	33.5 ± 3.7	33.9 ± 3.9	—	NS[c]
SBP (mm Hg)	170 (160–180)	100 (100–110)	120 (110–120)	<0.001[a]
DBP (mm Hg)	110 (100–120)	70 (70–75)	80 (70–80)	<0.001[a]
Parity				
Nulliparous	11 (39%)	10 (33%)	12 (41%)	NS
Multiparous	17 (61%)	20 (67%)	17 (59%)	

Age, SBP: systolic blood pressure and DBP: diastolic blood pressure. Values are presented as median (25th–75th percentiles). GA: gestational age. Values are presented as mean ± standard deviation. (−): does not apply. Significant difference when P<0.05. NS: non significant.
[a] Kruskal–Wallis test.
[b] Anova.
[c] T test.

The MPs/μl of plasma was calculated as described elsewhere [20]: MPs/μl = (N × 400)/(60 × 100), in which N = number of events, 400 = total volume of sample in the tube before analysis, 60 = sample volume analyzed, and 100 = original volume of MP suspension.

2.6. Statistical analysis

Statistical analyses were performed using SPSS software version 13.0 (SPSS Inc., Chicago, IL). Shapiro–Wilk tests were used to verify if the variables were normally distributed. Data not normally distributed were compared using the Kruskal–Wallis test. Comparison between 2 groups was done using the Mann–Whitney *U* test with Bonferroni's correction (non-normal data) or *t*-test (normal data). Normal data are presented as mean and standard deviation, while non-normal variables are presented as median and interquartile range (25th–75th percentiles). Correlations were analyzed using the Pearson or Spearman 2-sided test and Pearson χ^2 test to frequency differences. Differences were considered significant when P<0.05.

3. Results

The characteristics of the women enrolled in this study are summarized in Table 1. The mean proteinuria value (g/l/24 h) for pregnant women with PE was 4.16 ± 2.1, confirming the presence of severe PE. All women with severe PE had significantly increased systolic (P<0.001) and diastolic blood pressure (P<0.001) compared with the 2 other groups.

No differences in gestational age were noted between the women with severe PE and the normotensive pregnant women. Body mass index before pregnancy did not differ among the 3 groups (P = 0.009). Differences were found in age among the 3 groups (P = 0.008).

Most participants in the 3 groups were multiparous. Eleven (39%) of the 28 women with severe PE were nulliparous. Five of the multiparous women had PE in their previous pregnancy. Eleven women with PE had abnormal liver function markers or decreased platelets counts but did not fulfill the criteria for HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count). The most common symptom among women with PE was headache (21 women), scotomata (9), epigastric pain (3), and patellar reflex alteration (1).

Table 2 summarizes the cellular origin and number of circulating MPs studied. A higher total number of MPs were observed in women with severe PE compared with normotensive pregnant women and non-pregnant women (P = 0.004 and P = 0.001, respectively). However, the 2 last groups did not display different numbers of MPs (P = 0.154).

Normotensive pregnant and non-pregnant women showed higher levels of circulating platelet-derived MPs. Unlike these 2 groups, most circulating MPs in women with severe PE originated from the endothelial cells. The number of erythrocyte-derived MPs was increased in women with severe PE compared with normotensive pregnant

Table 2
Cellular origin and number of circulating microparticles.

MPs	Severe PE (I)	Normotensive pregnant (II)	Non-pregnant women (III)	P value*
Total	8.43 (1.60–30.48)	4.87 (1.23–19.20)	3.53 (0.80–14.73)	**0.004**[a]
				0.001[b]
				NS[c]
Platelet	30.93 (11.08–86.92)	38.27 (11.43–132.93)	60.13 (11.87–129.80)	NS[a]
				NS[b]
				NS[c]
Endothelial	36.77 (5.48–73.03)	28.67(3.55–95.48)	7.93 (2.77–38.90)	NS[a]
				NS[b]
				NS[c]
Leukocyte	19.76 (5.20–63.77)	16.57 (2.70–58.07)	16.67 (4.43–79.70)	NS[a]
				NS[b]
				NS[c]
Erythrocyte	12.77 (1.87–37.40)	5.27 (1.22–10.08)	2.73 (1.23–14.20)	**0.002**[a]
				NS[b]
				0.005[c]
Neutrophil	9.13 (1.37–17.78)	3.00 (1.42–9.25)	3.47 (0.63–8.60)	NS[a]
				NS[b]
				NS[c]
Placenta	6.37 (1.62–12.45)	5.00 (1.00–13.08)	2.00 (0.17–3.03)	NS[a]
				0.002[b]
				0.002[c]
Monocyte	1.93 (0.55–5.40)	1.53 (0.48–2.70)	1.00 (0.23–3.60)	NS[a]
				NS[b]
				NS[c]
Lymphocyte	0.90 (0.15–3.37)	1.20 (0.22–4.20)	0.73 (0.13–2.53)	NS[a]
				NS[b]
				NS[c]

Data are presented as median (25th–75th centiles), MPs/μl. *Differences between 2 groups (Mann–Whitney *U* test and Bonferroni correction). * Significant: P<0.05. NS: non significant.
a = group I × group II.
b = group I × group III.
c = group II × group III.

women (P = 0.002) and was higher in normotensive pregnant women compared with non-pregnant women (P = 0.005) (Fig. 2A).

Placenta-derived MPs (NDOG2-positive) were detected in the circulation of women with severe PE and in normotensive pregnant women. Curiously, some placenta-derived MPs were detected in non-pregnant women. However, those levels were lower than what was seen in the women with severe PE or normotensive pregnant women (P = 0.002 in both cases) (Fig. 2B).

No significant differences were observed among the 3 groups regarding the number of platelet-, endothelium-, leukocyte-, neutrophil-, monocyte-, and T lymphocyte-derived MPs. Nevertheless, there was a clear reduction in platelet-derived MP levels in women with severe PE and normotensive pregnant women but an increase in neutrophil- and endothelial cell-derived MPs in women with severe PE.

Correlation analysis showed no correlation between MP levels and gestational age or systolic/diastolic blood pressure considering all types of MPs in women with PE (P > 0.05). Similarly, no correlation was found among trophoblast-, endothelial cell-, and platelet-derived MPs (P > 0.05). No correlation was found between platelet-, erythrocyte-, and leukocyte-derived MPs and their respective cell numbers in the circulation of women with PE. However, we observed a trend towards association between platelet count (categorized according to cutoff = 150,000/mm^3) and the number of MPs derived from platelets (categorized considering the median of the control group) (P = 0.09).

Positive correlations were found between the number of endothelial cell-derived MPs and platelet-derived MPs (r = 0.483; P = 0.009), leukocyte-derived MPs (r = 0.519; P = 0.005), neutrophil-derived MPs (r = 0.394; P = 0.038), and T lymphocyte-derived MPs (r = 0.616; P < 0.001) in PE group.

4. Discussion

This study showed that MPs were significantly increased in women with severe PE compared with normotensive pregnant women and non-pregnant women. Similarly, Lok et al. [21] and Orozco et al. [22] demonstrated higher number of MPs in women with PE compared with normotensive pregnant women.

The majority of circulating MPs detected in severe PE were derived from endothelial cells, while most MPs in normotensive pregnant women and non-pregnant women were derived from platelets. Although we observed a reduced number of platelet MPs in women with severe PE compared with non-pregnant women, this difference was not significant, probably due to the high dispersion of the data in this variable. Similarly, Alijotas-Reig et al. [23] found no difference in platelet-derived MPs in women with severe PE vs. non-pregnant women. However, there was a positive correlation between platelet-derived MPs and platelet cell count when these variables were categorized. Lok et al. [24] also noted a reduction in platelet-derived MPs in women with severe PE and a correlation with platelet count.

The number of platelet-derived MPs may reflect the turnover of platelets in the plasma. Although platelet activation has been observed in PE, we were not able to identify increased number of platelet-derived MPs. A possible explanation for this finding could

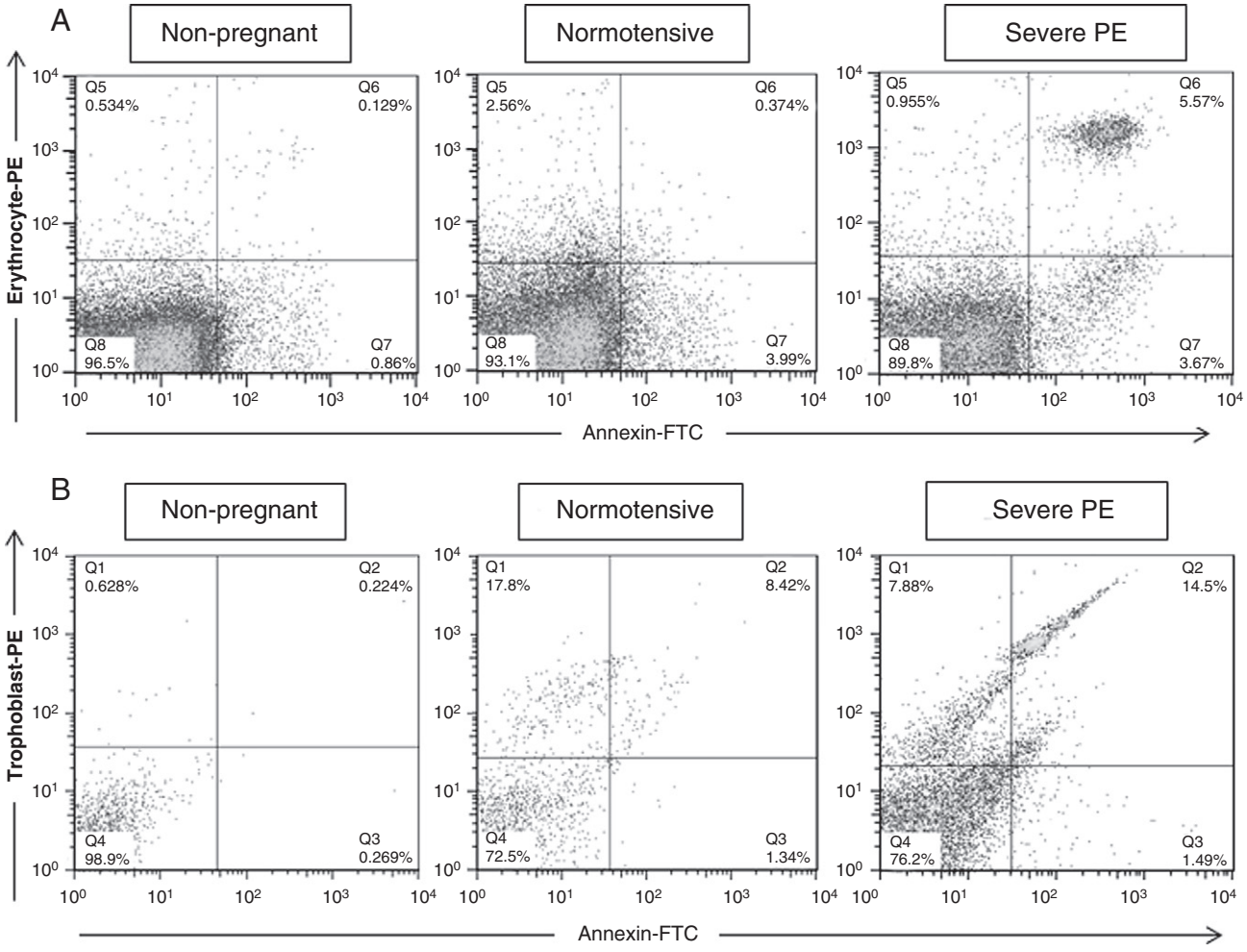

Fig. 2. Flow cytometry plots of microparticles derived from erythrocytes (A) and trophoblasts (B) in non-pregnant woman, normotensive pregnant women, and women with severe preeclampsia.

be that platelet MPs would remain trapped in the fibrin clots that are frequently evidenced in the placental microvasculature of women with severe PE [23]. Therefore, a lower platelet count in severe PE is associated with exacerbated platelet activation and high consumption [23,25]. Thus, the decreased platelet counts in PE may explain the decreased number of this MP type [24].

PE is believed to be a disorder of the maternal endothelium [6]. Although there was a tendency for a higher number of endothelial cell-derived microparticles in women with severe PE compared with normotensive pregnant women and non-pregnant women, the difference was not significant. Contrarily, González-Quintero et al. [26] documented higher number of endothelial cell-derived MPs in women with PE compared with women with gestational hypertension and non-pregnant women. Endothelial cell activation may contribute to both inflammatory response and vasoconstriction. In the kidney, the endothelial defect can cause proteinuria and endothelium-dependent dilatation failure, which can contribute to hypertension and intense vasoconstriction in different organs [6]. Therefore, endothelium activation should be detectable by an increased number of endothelial cell-derived MPs in the circulation using more highly specific MPs [27]. Although we were not able to show a significant increase in the number of endothelial cell-derived MPs in women with severe PE compared with the other groups, the number of endothelial cell-derived MPs was associated with higher levels of lymphocyte-, leukocyte-, and platelet-derived MPs, which suggests a correlation between endothelium activation and these cell types [15].

Our data showed increased number of erythrocyte-derived MPs in women with severe PE. This finding could be explained by hemolysis, which is commonly observed in this disease [28]. Because fibrin clots have been observed in the microvasculature of women with PE, one hypothesis is that erythrocytes are lysed by colliding with such clots and result in MP release [29]. However, no correlation between erythrocyte-derived MPs and erythrocyte number in the circulation was found.

Our data do not reveal significant differences in leukocyte-, monocyte-, lymphocyte-, and neutrophil-derived MPs, although there was a tendency toward increased number of neutrophil-derived MPs in women with severe PE. In contrast, monocyte-, lymphocyte-, and neutrophil-derived MPs were previously determined to be associated with PE [24,30,31]. Elevated number of leukocyte-derived MPs may reflect activation of these cells because this disease is associated with the local inflammatory response that results in an enhanced leukocyte endothelial interaction [32].

Stallmach et al. [33] observed higher number of activated lymphocytes in the placentas of women with severe PE, which could generate increased number of MPs released into the maternal circulation. Leukocyte-derived MPs induce endothelial cell and cytokine gene activation. This may be a mechanism for amplification of the local concentration of inflammatory and chemotactic cytokines and induction of adhesion molecule-facilitated intercellular communication and cross-signaling between leukocytes and endothelial cells [34]. Leukocyte-derived MPs cause endothelial damage, which could explain the correlation between neutrophil-, leukocyte-, and lymphocyte-derived MPs and endothelial-derived MPs observed in this study [30].

The placenta has been shown to play an important role in the pathogenesis of PE. Trophoblast invasion is impaired, which results in placental factor release in the maternal circulation that causes generalized vascular dysfunction [35]. Syncytiotrophoblast (STBM)-derived MPs have been considered a candidate for this factor, mainly because increased trophoblast apoptosis was observed in PE [36,37]. Our data showed that a number of placenta-derived MPs were not significantly elevated in women with severe PE compared with normotensive pregnant women. However, there were an elevated number of placenta-derived MPs in women with severe PE and normotensive pregnant women compared with non-pregnant women. This finding is in contrast to the findings of other authors, who reported an elevated number of placenta-derived MPs in women with PE compared with normotensive pregnant women [17,38].

NDOG-2 is a specific antibody that recognizes placental alkaline phosphatase [36]. Similar to the study of Vanwijk et al. [30], we used the NDOG2 antibody to detect and quantify STBM. These antibodies bound to placenta-derived MPs in both women with severe PE and normotensive pregnant women. However, some NDOG-2 was detected in non-pregnant women, even in women who had never been pregnant. This finding suggests that NDOG2 is not STBM-specific and could explain these opposite results comparing previous studies [30]. Despite this low specificity, the high capacity of these MPs to damage the vascular endothelium or to activate neutrophils should be considered [39]. Moreover, trophoblast-derived MPs bind to monocytes and B cells, stimulate the production of inflammatory cytokines, and may be related to placental ischemia and oxidative stress [38,40,41].

In conclusion, a higher number of endothelial cell-derived MPs in women with severe PE suggest endothelium activation. Therefore, the level of erythrocyte-derived MPs is increased in preeclampsia. In the future, new therapeutic targeting erythrocyte-derived MPs could be proposed. However, considering the limited sample of the current study, other studies are needed to elucidate the mechanisms involved in their effects to contribute to additional intervention strategies for the management of severe PE.

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