



Elevated plasma endothelial microparticles: Preeclampsia versus gestational hypertension

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KEY WORDS

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Objective: Elevated plasma endothelial microparticle levels have been found to be elevated in women with preeclampsia. However, their role in distinguishing preeclampsia from gestational hypertension remains to be elucidated. The objectives of this study were to compare endothelial microparticle levels among patients with preeclampsia, gestational hypertension, and healthy pregnant control subjects and to evaluate the effect of plasma from women with preeclampsia and gestational hypertension on the release of endothelial microparticles by renal microvascular endothelial cells.

Study design: A prospective study was conducted on 52 women with preeclampsia, 20 women with gestational hypertension, and 38 healthy pregnant control subjects. Endothelial microparticles were measured by flow cytometry with fluorescent monoclonal mouse anti-human antibodies against CD31, CD42b, and CD62E.

Results: CD31⁺/42b⁻ endothelial microparticle levels were 10497 ± 5145 counts/ μ L in women with preeclampsia versus 6768 ± 1810 counts/ μ L in women with gestational hypertension ($P < .01$). In control subjects, CD31⁺/42b⁻ endothelial microparticle levels were 6119 ± 3592 counts/ μ L. CD62E⁺ endothelial microparticle levels were 1930 ± 966 counts/ μ L in women with preeclampsia versus 822 ± 150 counts/ μ L in women with gestational hypertension ($P < .01$). In control subjects, CD62E⁺ endothelial microparticle levels were 712 ± 160 counts/ μ L. Incubation of renal microvascular endothelial cells with plasma from women with preeclampsia resulted in a rise in CD31⁺ and CD62E⁺ endothelial microparticle levels as compared with women with gestational hypertension and control subjects.

Conclusion: Endothelial microparticle levels are higher in women with preeclampsia than in women with gestational hypertension and control subjects. The measurement of endothelial microparticles may be useful as a diagnostic tool for preeclampsia in pregnant women.

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Hypertension is the most common medical disorder during pregnancy.¹ The spectrum of this pregnancy-specific condition ranges from uncomplicated hypertension, known as *gestational hypertension*, to a syndrome of blood pressure elevation with associated proteinuria,

known as *preeclampsia*. Preeclampsia can range from mild to severe, with subsequent manifestations of critical end-organ damage (such as cerebral hemorrhage, pulmonary edema, coagulopathy, and renal or hepatic failure). Gestational hypertension is the most frequent cause of hypertension during pregnancy. Some women with gestational hypertension will progress subsequently to preeclampsia, depending on gestational age at time of diagnosis.^{2,3}

The actual cause of preeclampsia is unknown, although many different theories have been described. The theory of endothelial dysfunction may account for many of the pathologic changes, which include maternal vasospasm, edema, proteinuria, coagulopathy, and end-organ damage, that are associated with preeclampsia.^{4,5} It is perhaps the damage to the normally nonthrombogenic, nonadhesive endothelial lining of the vascular tree that causes deregulation in vascular tone and permeability of the blood vessels.⁶⁻⁹

On endothelial disturbance, it has been shown that the endothelial cell releases vesicles of its plasma membrane into the circulation. These endothelial microparticles (EMPs), which measure $<1\ \mu\text{m}$ in size, carry both cytoplasmic components and cell-surface antigens that are expressed on the endothelia (such as CD31 and CD62E).¹⁰⁻¹³ Our laboratory has developed flow cytometric methods for the detection and quantification of these EMP as indicators of endothelial injury. These methods have been used to identify elevated levels of EMP in other disease states of endothelial dysfunction (such as thrombotic thrombocytopenic purpura,¹⁰ multiple sclerosis,¹¹ lupus,¹² malignant hypertension,¹³ and coronary heart disease¹⁴). Recently, we have reported an elevation of EMP levels in women with preeclampsia.¹⁵ The objectives of this study were to compare EMP levels among patients with preeclampsia, gestational hypertension, and healthy pregnant control subjects and to evaluate the effect of plasma from women with preeclampsia and gestational hypertension on the release of EMPs by renal microvascular endothelial cells (RMVEC).

Methods

Patient and control population

This prospective, case-control study was conducted with 52 women with preeclampsia, 20 women with gestational hypertension, and 38 healthy pregnant women who were recruited from our university hospital from July 2002 to February 2003. Approval from the University of Miami Institutional Review Board was obtained, and each woman provided informed consent before enrollment. The Working Group Report on High Blood Pressure in Pregnancy defined the criteria that

were used for the selection of study patients.¹ *Severe preeclampsia* was defined as women with blood pressure of $>160\ \text{mm Hg}$ systolic or $>110\ \text{mm Hg}$ diastolic and/or proteinuria of $\geq 2\ \text{g}$, platelet count of $<100,000\ \text{cells}/\text{mm}^3$ or HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome. Control subjects were healthy pregnant women at >34 weeks of gestation. Exclusion criteria for all subjects included chronic hypertension, pregestational diabetes mellitus, antiphospholipid syndrome, and renal/hepatic disease or women in labor. Mean arterial pressure (MAP) was calculated in all patients before phlebotomy. Results of 24-hour urine protein collections for all patients with preeclampsia and gestational hypertension were also recorded.

In vitro study

RMVEC culture

RMVECs (catalog no. ACBRI 376; Cell Systems Inc, Kirkland, Wash) were cultured to confluence in $25\ \text{cm}^2$ tissue culture flasks in CS-C complete medium (10% serum, CS-C growth factor, heparin; Cell Systems Certified) at 37°C in 5% carbon dioxide, 100% humidity. All cells were used from passages 2 through 6. On confluence, cells were detached with 0.05% trypsin/0.53 mmol/L ethylenediaminetetraacetic acid (Gibco BRL; Life Technologies, Grand Island, NY) for 3 to 5 minutes, washed with CS-C medium, and centrifuged at 100g. The cells were resuspended in CS-C medium and replanted in 12-well tissue culture multiwell clusters (Corning Costa, Cambridge, Mass) that were precoated with attachment factor (Cell Systems Inc) at a density of $1 \times 10^5/\text{well}$ 48 hours before the experiments.

Antibodies and other reagents

The following fluorescent-tagged anti-human monoclonal antibodies were obtained from BD PharMingen, San Diego, Calif: anti-human CD31 (catalog no. 555446; phycoerythrin), anti-human CD62E (catalog no. 551145; phycoerythrin), anti-human CD42 (catalog no. 555472; fluorescein isothiocyanate), and anti-human CD45 (catalog no. 555482; fluorescein isothiocyanate).

Sample preparation for the study of the effect of preeclamptic plasma on RMVEC culture

All plasma was filtered through a $0.1\text{-}\mu\text{m}$ filter (Whatman, Clifton, NJ) just before use to eliminate approximately 95% of detectable preexisting EMPs.¹⁰ Then, 200 μL of preeclampsia, gestational hypertension, or control plasma was added to each well of 1.0 mL media and maintained for 18 hours. The culture dishes contained 1 mL of supernatant fluid; 50 μL of the plasma was used per test. To the 50- μL sample was added (in a $12 \times 75\ \text{mm}$ polypropylene tube) 4 μL of either anti-CD31 or anti-CD62E. Because there are no platelets or leukocytes present in culture, it was not necessary to use anti-CD45 or anti-CD42b antibodies for in vitro experiments.¹⁰ The

Table Baseline characteristics of study participants

Variable	Women with preeclampsia (n = 52)	Women with gestational hypertension (n = 20)	Control subjects (n = 38)	P value
Age (y)*	28.8 ± 6.9	31.0 ± 9.9	27.8 ± 5.6	.236
Gravity (n)†	2 (1-5)	2 (1-8)	2 (1-7)	.259
Estimated gestational age at enrollment (wk)*	35.1 ± 3.8	35.1 ± 4.4	37.5 ± 2.1	< .001
MAP (mm Hg)*	110.6 ± 11.7	100.1 ± 9.9	82.5 ± 9.2	< .01
24-h urine protein level (mg/24 h)†	1800 (308-17138)	181 (95-290)	—	< .01

* Values are given as mean ± SD.

† Values are given as mean (range).

sample was then incubated at room temperature for 15 minutes with gentle shaking (orbital shaker; 120 revolutions/min). After incubation, 0.5 mL of phosphate-buffered saline solution was added, and the sample was ready for flow cytometry.

In vivo study

Sample preparation for clinical studies

Blood samples were collected in sterile 5-mL sodium citrate tubes and assayed within 4 hours of venipuncture to avoid contamination with microparticles that are released ex vivo. The samples were centrifuged 10 minutes at 160g to prepare platelet-rich plasma. The platelet-rich plasma was further centrifuged 6 minutes at 1500g to obtain platelet-poor plasma. Then 30- μ L aliquots of platelet-poor plasma were transferred into 12 \times 75-mm polypropylene tubes and were used to measure EMP in the following manner: (1) to measure CD62E⁺ EMP, the platelet-poor plasma was incubated with 5 μ L of phenylethylamine-labeled anti-CD62E. (2) To measure CD31⁺/CD42b⁻ EMP, the platelet-poor plasma was incubated with 5 μ L each of phenylethylamine-labeled anti-CD31 and fluorescein isothiocyanate-labeled anti-CD42b. The purpose of double labeling with anti-CD42b was to exclude CD31⁺ microparticles of platelet origin. CD31⁺/CD45⁺ particles account for a negligible percentage of all CD31⁺ microparticles, which would represent a subpopulation of leukocyte microparticles and would not significantly alter EMP counts.^{10,15} Samples were incubated and diluted as described earlier for in vitro study and were then ready for flow cytometry.

Flow cytometry

EMP are defined as particles bearing antigens CD31⁺ or CD31⁺/42b⁻ or CD62E⁺ particles that were found in vitro or in vivo. Briefly, as previously described,^{10,15} EMPs were analyzed on a Coulter EPICSXL (Beckman Coulter, Miami, Fla) at medium flow rate. The detection of particles was set to trigger by a fluorescence signal

greater than a predetermined value. Light scatter and fluorescence channels were set at logarithmic gain. The fluorescence-positive particles were further separated on another histogram on the basis of size (forward light scatter); only particles <1.5 μ m were assayed. To convert flow cytometer counts to an estimate of the number of EMPs per milliliter of the original supernatant or blood, the count was determined by the standard beads that 19 μ L of sample was actually aspirated and counted in a 30-second run at medium setting. Therefore, because 50 μ L of RMVEC supernatant or platelet-poor plasma was used per test, the conversion factor was ascertained with the following formula: $F = (1.06 \text{ mL}/0.018 \text{ mL})(1.0 \text{ mL}/0.05 \text{ mL}) = 1178$. Daily calibration with fluorescent beads ensured that fluctuations were <2%. Isotype-matched control values for each fluorophore were subtracted from EMP counts.¹⁰

Analysis

Analysis of variance with post hoc test multiple comparisons (Scheffe's test) was used to compare continuous parametric variables (such as maternal age, gravidity, estimated gestational age, and MAP). The 2-tailed sample *t* test was used to compare levels of proteinuria between women with preeclampsia and women with gestational hypertension. In cases in which data failed, the Kolmogorov-Smirnov normality test and the Mann-Whitney rank sum test were used. Spearman correlations were performed for *R* value determinations in linear regressions with logarithmic values used for nonparametric data. Statistical significance was defined as a probability value of < .05.

Results

No significant difference was noted between study and control women with respect to age or gravity. However, there were statistical differences in the gestational age at study enrollment between the patients with preeclampsia and gestational hypertension relative to the control

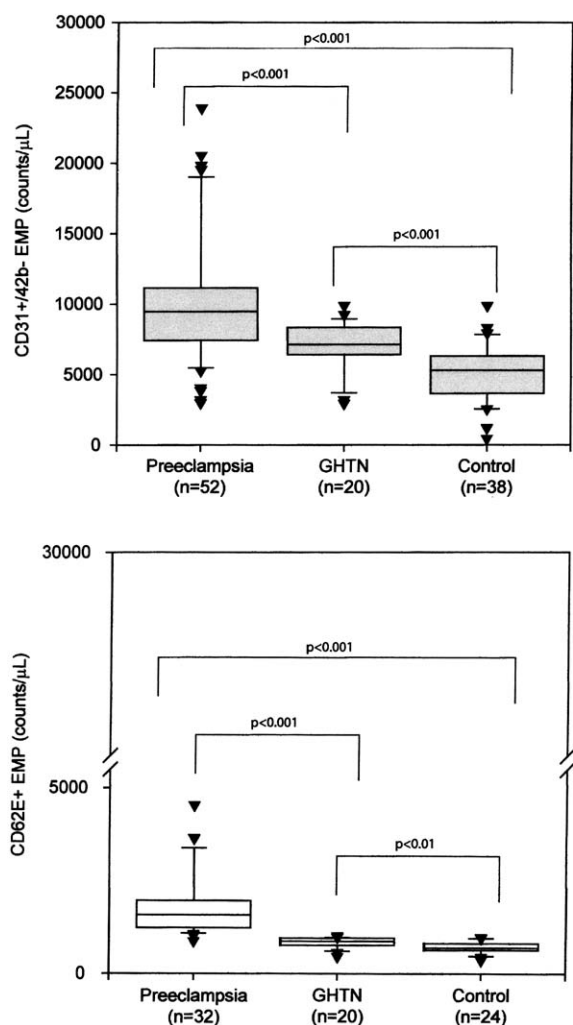


Figure 1 In vivo quantification of plasma CD 31⁺/CD42b EMP (A) and CD62E⁺ EMP (B) among study groups. Values are given as means; error bars, SD. Statistical significance was determined by the Mann-Whitney rank sum test.

subjects. There were 9 cases of severe preeclampsia that met inclusion criteria. As anticipated by the diagnostic criteria that were used for recruitment of study participants, the MAP pressures were elevated significantly in the women with preeclampsia versus the women with gestational hypertension versus control subjects. Also as expected with diagnostic criteria, 24-hour proteinuria levels were significantly different between the 2 hypertensive groups (Table).

In vivo

The levels of CD31⁺/CD41⁻ EMP were shown to be significantly elevated in patients with preeclampsia over the levels that were found in women with gestational hypertension (10497 ± 5145 counts/μL vs 6768 ± 1810 counts/μL; P < .01; Figure 1, A). Both these groups

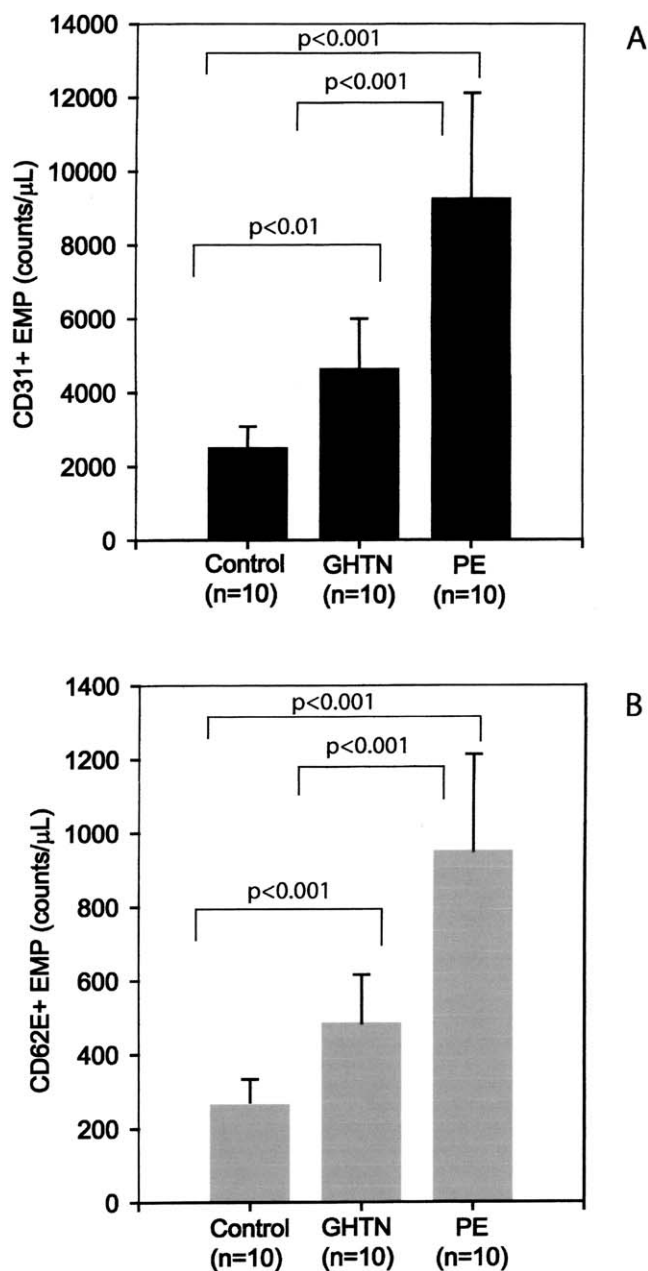


Figure 2 In vitro quantification of CD31⁺ EMP (A) and CD62E⁺ EMP (B) release from cultured RMVEC after incubation with study plasma. Values are given as means; error bars, SD. Statistical significance was determined by the Mann-Whitney rank sum test.

also exhibited significantly elevated CD31⁺/CD41⁻ EMP levels over control subjects (6119 ± 3592 counts/μL; P < .01). The plasma EMP assay also revealed significant elevations in CD62E⁺ counts among the women with preeclampsia, the women with gestational hypertension, and control subjects (1930 ± 966 counts/μL vs 822 ± 150 counts/μL vs 712 ± 160 counts/μL, respectively; P < .01; Figure 1, B). The data were also analyzed, with the exclusion of the 9 cases of

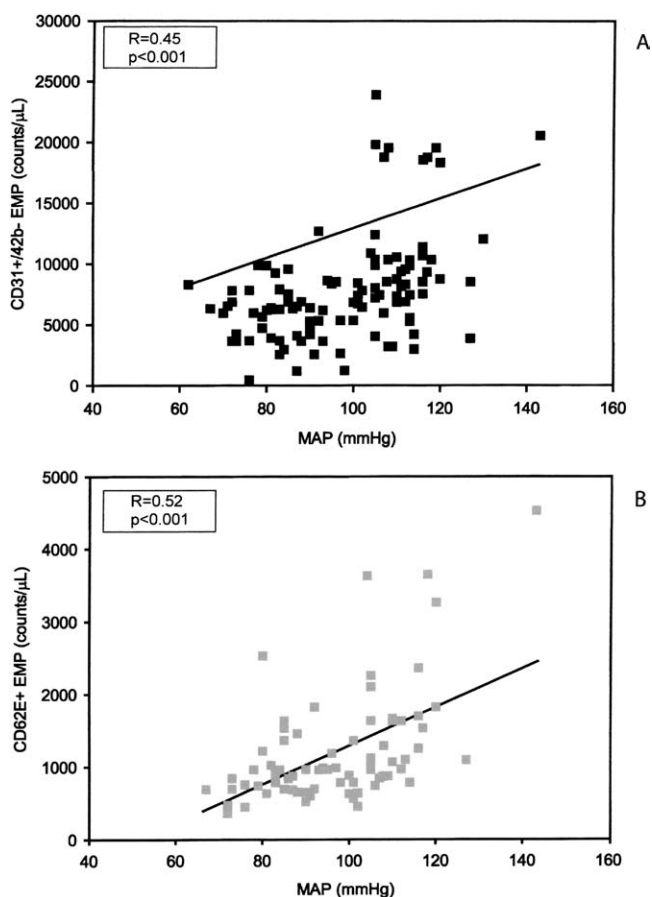


Figure 3 Correlation of (A) CD31⁺/CD42⁻ EMP versus MAP and (B) CD62E⁺ EMP versus MAP among all study groups. Statistical significance was determined by linear regression; *R* values were determined by Spearman correlation.

severe preeclampsia. The levels of CD31⁺/CD41⁻ EMP and CD62E⁺ counts were also shown to be elevated significantly, even after the exclusion of the women with severe preeclampsia.

In vitro

Preeclamptic plasma elicited a significantly greater level of CD31⁺ EMP release from the cultured RMVEC cells, as compared with gestational hypertension and control plasma (9254 ± 2865 counts/ μ L vs 4635 ± 1364 counts/ μ L vs 2500 ± 325 counts/ μ L, respectively; $P < .01$; Figure 2, A). Additionally, CD62E⁺ EMP levels were significantly different among the 3 groups of study (preeclampsia [950 ± 263 counts/ μ L] vs gestational hypertension [485 ± 133 counts/ μ L] vs control [263 ± 45 counts/ μ L]; $P < .01$; Figure 2, B).

In a comparison of EMP with clinical parameters, there was a significant positive correlation between CD31⁺/CD41⁻ EMP and MAP ($R = 0.45$; $P < .01$; Figure 3, A). A similar positive correlation was observed between CD62E⁺ EMP and MAP ($R = 0.52$; $P < .01$; Figure 3, B). Additionally, proteinuria that was de-

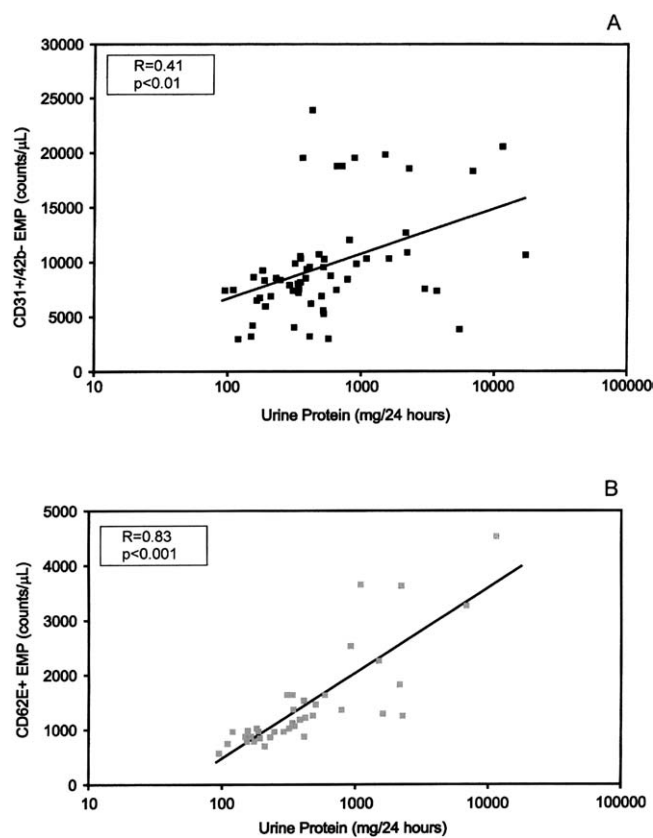


Figure 4 Correlation of (A) CD31⁺/CD42⁻ EMP versus 24-hour urine protein and (B) CD62E⁺ EMP versus 24-hour urine protein among preeclampsia and gestational hypertension study groups. Statistical significance was determined by linear regression; *R* values were determined by Spearman correlation.

termined by the 24-hour urine collection was correlated positively with both CD31⁺/CD41⁻ and CD62E EMP levels ($R = 0.41$ and $R = 0.83$, respectively; $P < .01$; Figure 4).

Comment

The true cause of preeclampsia continues to be debated. In recent years, the theory of endothelial dysfunction has been gaining momentum, with the identification of circulating soluble endothelial adhesion molecules (such as VCAM-1, ICAM-1, CD31, E-selectin, and fibronectin) in the plasma of women with preeclampsia.¹⁶⁻²¹ These adhesion molecules are expressed constitutively on the endothelial cell surface and are cytokine-inducible, which is known to regulate the trafficking of circulating inflammatory cells to sites of cell damage. Yet, despite their presence predominantly on the endothelium, these circulating surface markers are also identified on leukocytes and platelets. Thus, measurement of these soluble markers may not be a direct marker of endothelial damage.

In contrast, EMPs are vesicles that are derived from the endothelial cell surface itself. When budding occurs as the result of endothelial insult, the surface markers CD31 and CD62E are expressed on the surface of the EMP. This provides a direct measure of endothelial cell damage, as previously described for thrombotic thrombocytopenia purpura, multiple sclerosis, severe hypertension,¹⁰⁻¹³ and preeclampsia.¹⁵ This investigation was designed to determine whether identification of these same microparticles could provide direct evidence of endothelial damage in preeclampsia and help us distinguish preeclampsia from gestational hypertension.

The *in vitro* results that were obtained in this study strongly support the theory of endothelial dysfunction in preeclampsia. Stimulation of RMVECs with filtered preeclamptic plasma elicited the release of significantly elevated levels of CD31⁺ and CD62E⁺ EMP from the exposed endothelial cells. The exact component of the preeclamptic plasma that elicits this EMP release is unknown¹⁹⁻²⁴ but may be proinflammatory cytokines, which are known to be endothelial activators (such as tumor necrosis factor- α and interleukin-1), which have also been shown to be elevated in preeclampsia.²⁰⁻²⁴ *In vivo*, levels of both CD31⁺/42b⁻ and CD62E⁺ EMP were elevated significantly in patients with preeclampsia and patients with gestational hypertension when they were compared with control subjects. As exhibited *in vitro*, these findings show an incremental, severity-specific increase in these markers of endothelial injury. The EMP counts that are seen in the preeclampsia group also exhibited higher levels, in relation to the severity of the disease. But even after the exclusion of women with severe preeclampsia, EMP counts were elevated significantly. Thus, EMP might be used as a direct measure of the extent of endothelial damage, which indicates worsening endothelial damage with severity of the syndrome.

This study also provided correlation between clinical findings and elevations in EMP levels. MAP was significantly correlated with levels of both CD31⁺/CD42b⁻ and CD62E⁺ EMP, which indicates that the severity of hypertension is likely indicative of progressive endothelial damage. This supports recent findings in which nonpregnant patients with severe hypertension exhibited elevated EMP values when compared with the mild hypertension and control groups.¹⁴ The positive correlation between CD31⁺/CD42b⁻ and CD62E⁺ EMP and 24-hour urine protein levels is extraordinarily relevant in regards to the diagnosis of preeclampsia. Our investigation identified that increasing levels of proteinuria corresponded to increasing levels of EMP, with the strongest correlation observed with CD62E⁺, which demonstrates an association between the severity of preeclampsia as determined by degree of proteinuria and the degree of EMP elevation.

The association between CD31⁺/CD42b⁻ and CD62E⁺ EMP and 24-hour urine protein levels may

be indicative of not only the endothelial condition as a whole but also of the renovascular endothelium itself. Glomerular endotheliosis, a lesion that is identified in preeclampsia by renal biopsy, is described with glomeruli as the primary target; the severity of renal morphologic alterations parallels the severity of clinical disease.²⁵ These biopsy findings provide direct, yet excessively invasive, evidence that there is dysfunction in this population of endothelial cells. In this regard, our study provides confirmation of global endothelial damage in preeclampsia.

In conclusion, the measurement of plasma EMP shows great promise in the field of preeclampsia. EMP levels can be assayed readily in small amounts of peripheral blood samples, which provides the most direct measure to date of endothelial damage. This study, however, was limited by sample size and therefore did not allow for the determination of distinct cutoff values as used in diagnostic criteria; yet, the trends exhibited in EMP values among the 3 study groups implies that such diagnostic values could be determined in a larger scale investigation. The determination of the correlation between EMP levels and birth weight could provide a method to predict neonatal outcome. Additional plans for study include the collection of serial plasma samples of women, beginning in early gestation, to monitor for trends in CD31⁺/CD42b⁻ and CD62E⁺ EMP as predictors of eminent preeclampsia. Additionally, it would be advantageous to determine whether trends in EMP levels after diagnosis of preeclampsia could be used to determine whether progressively increasing EMP levels indicate worsening disease before the onset of clinical complications, thus influencing conservative treatment of severe preeclampsia remote from term.

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