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Background: Microparticles (MP) are pro-coagulant vesicles derived from various cells. Evidence is accumulating that MP are of pathophysiological relevance in autoimmune, cardiovascular, and thromboembolic diseases and inflammatory disorders. Therefore, their role in the development of preeclampsia was investigated and MP from preeclamptic patients influenced endothelial-dependent vasodilatation. Knowledge about changes in circulating MP numbers during pregnancy and preeclampsia is lacking. We determined this longitudinally and investigated whether these numbers related to the severity of preeclampsia.   *Methods*: Samples were obtained from pregnant women and preeclamptic patients during pregnancy and postpartum. MP were isolated and studied by flow cytometry.   *Results*: During pregnancy, MP were decreased at 12 weeks gestation and then returned to postpartum values. In patients with preeclampsia, MP numbers were reduced at 28 and 36 weeks (both $p = 0.04$). Monocyte-derived MP were elevated in preeclampsia at 28 ($p = 0.007$), 32 ($p = 0.02$), and 36 weeks ($p = 0.01$), as were erythrocyte-derived MP at 28 weeks ($p = 0.04$). Placenta-derived MP increased in pregnancy and preeclampsia. During pregnancy, a correlation was present between placenta-derived MP and systolic blood pressure ($r = 0.33$, $p = 0.015$). No other correlations were found.   *Conclusions*: During pregnancy, numbers of MP initially decrease and subsequently normalize. Placenta-derived MP increase, possibly because of placental growth. In preeclampsia, reduced numbers of PMP are due to decreased platelet counts. Increased numbers of monocyte-derived MP reflect monocyte activation, which

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may be an expression of the systemic inflammation in preeclampsia. Lack of correlation between numbers of MP and severity of preeclampsia suggests that MP numbers alone do not explain the reported vascular effects of MP.

Keywords Microparticles, Longitudinal study, Pregnancy, Preeclampsia.

BACKGROUND

Microparticles (MP) are small, procoagulant membrane vesicles, which are budded from the cell surface and released into the circulation during apoptosis or activation of blood cells or endothelial cells. MP play a definite role in both inflammation and coagulation (1, 2). Since normotensive pregnancy and, even more, preeclampsia are both characterized by activation of coagulation pathways and an altered inflammatory state (3), changes in circulating MP numbers or their cellular origin and function may underlie the activation of these processes.

Previously, we showed that the majority of MP originates from platelets (4). This subpopulation is often decreased in preeclampsia, depending on the patient selection (5). Despite their decrease in preeclampsia, a subpopulation of PMP exposing P-selectin, i.e., MP originating from activated platelets, was increased compared with pregnant and non-pregnant controls (6). Furthermore, numbers of T cell- and granulocyte-derived MP were significantly elevated in patients with preeclampsia compared with controls (4). These elevations could be a part of the exaggerated inflammatory response of preeclampsia. The contribution of MP to the development of preeclampsia, however, is still not completely understood. Whereas MP from women with preeclampsia impaired endothelium-dependent vasodilatation in isolated myometrial arteries (7), the RNA expression of inflammation-related genes in endothelial cells was unaffected (8). Impairment of endothelial dilatation was also found when small arteries were incubated with placenta-derived MP (6). Although placenta-derived MP constitute only a small fraction of the total number of circulating MP in the maternal blood, elevated numbers of placenta-derived MP have been reported in preeclamptic women compared with normotensive pregnant controls (9).

Previously, in non-pregnant hypertensive patients significant correlations were reported between blood pressure and both endothelium-derived MP and PMP (10). Currently, data on changes in MP numbers or subpopulations during the time course of uncomplicated pregnancies are not available. Moreover, it is unclear how changes in circulating MP are associated with the severity of preeclampsia.

At present, MP numbers and cellular origin in preeclampsia have always been investigated using *cross-sectional* studies in the second half of pregnancy. In the present study, we analyzed MP using a *longitudinal* design. Therefore, we

investigated circulating MP numbers throughout normotensive pregnancy, and evaluated whether MP or subsets thereof are related to disease severity in preeclampsia and/or to numbers of circulating blood cells.

MATERIALS AND METHODS

Patients

The study was approved by the medical ethical committee of the Academic Medical Center, and written consent was obtained from all study patients prior to blood collection. Blood samples were collected from randomly chosen healthy normotensive pregnant patients not using any medication (n = 8) between 12–14, 19–21, 23–26, 27–29, 30–34, and 34–38 weeks gestation and at 6 weeks postpartum. Preeclamptic patients (n = 11) were included upon admission to the hospital, and multiple blood samples were obtained until delivery and 6 weeks postpartum. Preeclampsia was defined as: diastolic blood pressure of 110 mm Hg or more on any occasion or 90 mm Hg or more measured on two separate occasions at least 4 hours apart; proteinuria of at least 0.3 g/24 h; and development of these symptoms after 20 weeks gestational age. Data concerning age, parity, medical history, blood pressure at 12 weeks gestational age, Body Mass Index (BMI) and laboratory results (level of hemoglobin, liver enzymes and platelets) were collected from the patient files. The delivery method, birth weight and gestational age at delivery were recorded postpartum.

Collection of Blood Samples

Two blood samples were taken from the antecubital vein without tourniquet through a 20-gauge needle with a vacutainer system. The first sample was collected into a 4.5 mL tube containing 0.105 M buffered sodium citrate (Becton Dickinson, San Jose, CA). Within 30 minutes after collection, cells were removed by centrifugation for 20 minutes at 1560 g and 20 °C. Plasma samples were then divided in 250 μL aliquots, immediately snap frozen in liquid nitrogen to preserve MP structure and then stored at −80 °C until further analysis. The second sample was collected in a 4.5 mL tube containing ethylenediaminetetraacetic acid (EDTA; Becton Dickinson, San Jose, CA) and used to determine numbers of erythrocytes, platelets and leukocytes.

Reagents and Assays

Fluorescein isothiocyanate (FITC)-labeled IgG_1 and phycoerythrin (PE)-labeled IgG_1 and monoclonal antibodies directed against $T_{suppressor}$-cells (anti-CD8-PE), monocytes (anti-CD14-PE), and B-cells (anti-CD20-FITC) were

obtained from Becton Dickinson (San Jose, CA). To detect T_{helper}-cells and granulocytes, anti-CD4-FITC and anti-CD66e-FITC were purchased from the Central Laboratory Bloodtransfusion (Amsterdam, The Netherlands). Anti-CD61-FITC (anti-GP-IIIa) was obtained from Pharmingen (San Jose, CA) and CD62e-PE (anti-E-selectin) from Ancell (Bayport, MN). Antibodies directed against P-selectin (anti-CD62p-PE) and anti-CD63-PE were purchased from Immuno Quality Products (Groningen, The Netherlands) and anti-glycophorin A-FITC was obtained from DakoCytomation (Carpionteria, CA). Allophycocyanin (APC)-conjugated annexin V was purchased from Caltag (Burlingame, CA). ED822, an antibody to an unknown antigen expressed on the apical surface of the syncytiotrophoblast used to detect trophoblastic cells (12), was received from Dr. S. F. Sooranna from the Department of Maternal Fetal Medicine, Imperial College School of Medicine (London, UK). This antibody was shown to be more specific than other suggested antibodies used to detect trophoblast cells with flow cytometry (10). The alternative antibody, NDOG2, seemed to be less specific in an earlier study by our group (4). The second antibody for the indirect labelling with ED822, GAM(Fab)2-FITC, was obtained from Dako (Glostrup; Denmark). The following final dilutions of antibodies were used: IgG_1-FITC (1:10), IgG_1-PE (1:10), anti-CD4-FITC (1:2.5), anti-CD8-PE (1:5), anti-CD14-PE (1:20), anti-CD20-FITC (1:10), anti-CD61-FITC (1:30), anti-CD62p-PE (1:15), CD62e-PE (1:30), anti-CD63 (1:5), anti-CD66e-FITC (1:10), anti-glycophorin A-FITC (1:5), annexin V-APC (1:40), ED822 (1:2.5), and GAM(Fab)2-FITC (1:20).

Whole blood erythrocyte, platelet and leukocyte counts were determined with a Cell-Dyn 4000 (Abbott Diagnostics Division; Abbott Laboratories; Hoofddorp, The Netherlands) at the department of Clinical Chemistry (Academic Medical Center; Amsterdam, The Netherlands).

Isolation of Microparticles

A sample of 250 µL frozen plasma was thawed on ice and centrifuged for 30 minutes at 18890 *g* and 20 °C to pellet the MP. With this centrifugation condition it is possible to pellet the MP without simultaneous pelleting exosomes (13). After centrifugation, 225 µL of the supernatant was removed. The MP pellet and remaining supernatant were resuspended in 225 µL phosphate-buffered saline (PBS) with citrate (154 mmol/L NaCl, 1.4 mmol/L phosphate, 10.9 mmol/L trisodium citrate, pH 7.4). After centrifugation for 30 minutes at 18890 *g* and 20 °C, 225 µL of the supernatant was removed again. The MP pellet was then resuspended in 75 µL PBS-citrate.

Flow cytometry

Five µL of the MP suspension was diluted in 35 µL $CaCl_2$ (2.5 mmol/L)-containing PBS. Then 5 µL APC-labeled annexin V was added to all tubes plus

5 µL of the cell-specific monoclonal antibody or isotype-matched control antibodies. The samples were then incubated in the dark for 15 minutes at room temperature. After incubation, 900 µL of calcium-containing PBS was added to all tubes (except to the annexin V control, to which 900 µL PBS-citrate was added). Samples were analyzed for one minute 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 a logarithmic gain. MP were identified on basis of their size and density (FSC and SSC respectively) and on their capacity to bind annexin V. Annexin V measurements were corrected for autofluorescence. Labeling with cell-specific monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies. The number of MP per liter plasma was calculated using the following formula: Number/L = N × (100/5) × (955/flow) × (10^6/250), in which *N* is the number of events that stained positive for both annexin V and a cell-specific antibody, *100* (µL) is the (total) volume of the MP suspension after isolation, from which *5* (µL) is used for labeling, *955* (µL) is the total volume of the labeled MP suspension after dilution, *flow* is the volume (µL) analyzed per minute by the flow cytometer, multiplied by 10^6 (from µL to L) and finally, *250* (µL) was the original volume of the plasma aliquot from which MP were isolated.

Flow cytometric analysis of placenta-derived MP was performed using an indirect staining procedure. MP (5 µL aliquots) were incubated for 15 minutes at room temperature in a final volume of 55 µL of PBS containing 2.5 mmol/L $CaCl_2$ (PBS/Ca, pH 7.4) and unlabeled ED822. After incubation, MP were washed with 200 µL of PBS/Ca. Subsequently, GAM(Fab)2-FITC (5 µL) was added for 15 minutes at room temperature, and labeling was terminated by addition of 300 µL buffer. Numbers were estimated using a method comparable to that mentioned earlier.

Statistical Analysis

Data were analyzed with Statistical Package of the Social Science software for Windows, release 11.5 (SPSS Benelux BV, Gorinchem, The Netherlands). Differences between demographic data were analyzed with Mann-Whitney *U* tests. Dichotomous variables were compared with a chi-square test. For groups < 5, a Fisher's exact test was used. A mixed model analysis of variance (ANOVA) for repeated measurements with corrections for missing values) was used to analyze MP numbers as a function of gestational age (repeated measurements). Differences between the normotensive pregnant patients and the preeclamptic patients for the different sampling periods were analyzed with Mann-Whitney *U* tests (cross-sectional measurements). If more than one sample was available of one preeclamptic patient for a certain period, the mean was calculated and used for the analysis. Correlations were analyzed with a Pearsons bivariate two-sided test. Differences were considered significant if *p* < 0.05.

RESULTS

Patient Characteristics

Patient characteristics are summarized in Table 1. The median age in the normotensive pregnant women was 30.8 years compared with 26.5 years in the preeclamptic patients. As expected, both systolic and diastolic blood pressures were elevated in patients with preeclampsia compared with those experiencing normotensive pregnancies. Although, this difference in blood pressure was already present at a gestational age of 12 weeks, none of these patients were hypertensive before 20 weeks gestation. Most preeclamptic patients were nulliparous. Four women in the normotensive group and two preeclamptic patients had a first trimester pregnancy loss before the present pregnancy. One woman in the normotensive group was pregnant after assisted reproductive treatment. Two normotensive pregnant women and one preeclamptic patient smoked during the present pregnancy. Most study subjects were Caucasian (10 preeclamptic patients and 4 controls). Six preeclamptic patients had abnormal liver function tests or decreased platelet counts, but they did not fulfill the criteria for HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome. Although none of the preeclamptic patients had an

Table 1: Patient characteristics.

Characteristic	Normotensive pregnancy (n = 8)	Preeclampsia (n = 11)	*p* Value
Age (years)	30.8 (25.5–34.5)	26.5 (20.8–33.0)	0.03
Blood pressure 12 wks*			
Systolic (mm Hg)	95 (70–110)	120 (110–155)	0.0001
Diastolic (mm Hg)	58 (50–70)	75 (65–90)	0.001
Highest blood pressure			
Systolic (mm Hg)	108 (100–120)	160 (140–210)	0.0001
Diastolic (mm Hg)	68 (55–75)	115 (90–130)	0.0001
Parity			
Nulliparous	2	10	0.006
Multiparous	6	1	
Proteinuria (g/24 h)	–	9.3 (0.7–16.3)	–
Platelets (10^9/L)	252 (107–408)	206 (42–568)	0.03
BMI (kg/m2)	23.8 (18.2–31.9)	21.6 (20.0–40.8)	NS
Delivery			
GA (weeks)	39.3 (37.4–42.0)	34.3 (26.1–36.6)	0.0001
Cesarean section	1	8	0.03
Vaginal birth	7	3	
Birth weight (g)	3450 (2940–3870)	1355 (470–2310)	0.0001

Data are presented as median (range). *p* = probability value for differences between the preeclamptic group and the normotensive pregnant group. NS = non-significant. BMI = body mass index. GA = gestational age.
*In the preeclamptic patients the blood pressure at 12 weeks was retrospectively found in the files of the prenatal care.

eclamptic episode, five patients needed preventive treatment with magnesium sulphate. None of the women suffered from thromboembolic complications. Blood pressure and laboratory values returned to normal after delivery. As mentioned in the Methods section, data were extracted from the medical files concerning BMI, birth weight, gestational age at delivery, and the method of delivery. These data showed that the BMI was comparable between groups and birth weight and gestational age at delivery were significantly lower in the preeclamptic women compared with the normotensive pregnant women. Furthermore, most preeclamptic patients delivered by Cesarean section.

Normotensive Pregnancy

Total numbers of circulating MP were longitudinally determined throughout pregnancy in the plasma from normotensive pregnant women. Figure 1 shows that the number of MP was decreased at 12 weeks gestation (median number of MP 2.9×10^9/L, $p = 0.04$) compared with postpartum. Then the MP number gradually normalized to the postpartum values during pregnancy ($p = 0.02$) with a
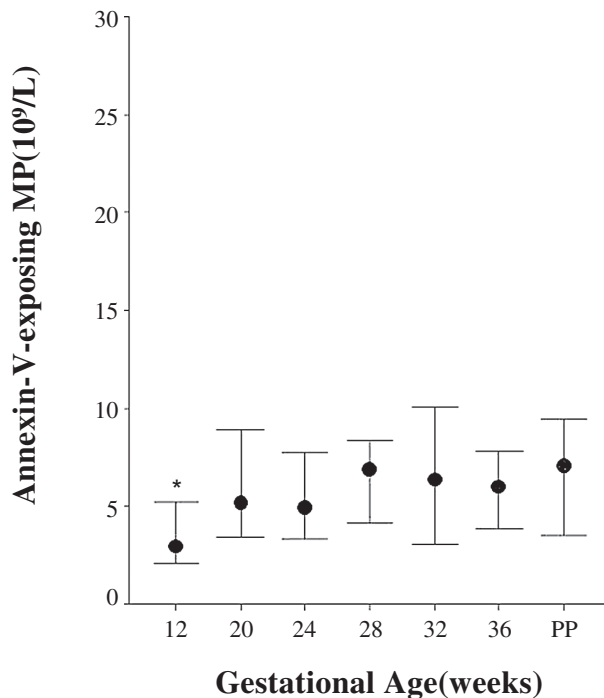

Figure 1: The number of MP during normotensive pregnancy. The number of annexin V-positive MP in normotensive pregnancies is plotted against the gestational age. Data are presented as median with the interquartile range (25% to 75%). In normotensive women, blood collection started at 12 weeks. *: $p = 0.04$ compared with postpartum values.

Table 2: Cellular origin of circulating MP in normotensive pregnant women.

Circulating MP	Gestational Age						
	12	20	24	28	32	36	pp
Total MP (10^9/L)	2.9 (1.8–7.2)	5.8 (3.4–10.0)	5.1 (2.1–13.3)	7.3 (2.2–10.5)	5.7 (2.7–11.7)	5.5 (2.0–17.9)	7.3 (1.5–9.9)
% Platelet-derived MP	97.0 (95.2–98.1)	97.9 (94.6–99.5)	99.0 (97.1–99.6)	97.9 (96.2–98.8)	97.2 (95.1–99.5)	98.1 (88.3–99.8)	98.1 (83.6–98.9)
% Erythrocyte-derived MP	2.7 (0.7–4.0)	1.0 (0.4–4.6)	0.6 (0.3–1.9)	1.6 (0.2–2.5)	1.3 (0.4–3.4)	1.4 (0–6.4)	1.9 (1.2–15.3)
% T$_{helper}$-cell-derived MP	0.2 (0–1.8)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)
% Monocyte-derived MP	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)
% B-cell-derived MP	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)
% Endothelial-cell-derived MP	1.0 (0–1.6)	0.8 (0–1.7)	0.4 (0–1.8)	0.9 (0–2.4)	0.8 (0–3.0)	0.9 (0–5.3)	0.7 (0–2.7)
% Placental-derived MP	2.3 (0.6–5.6)	1.6 (0.8–3.4)	1.9 (0.7–5.4)	1.5 (0.6–5.7)	1.8 (1.0–4.0)	3.0 (1.9–7.0)	1.1 (0.6–1.7)

The total number of annexin V-positive MP is presented as median (range; min-max). The subpopulations are expressed as median percentage of the total number of MP. Percentages were calculated for every patient independently. Therefore, the sum of the median percentages of all subgroups approaches 100% and is not exactly 100%; pp: postpartum, ND: not detectable. The percentage of T$_{suppressor}$-cell-derived MP and Granulocyte-derived MP are < 0.1% for all gestational ages.

maximum number at a gestational age of 28 weeks (7.3×10^9/L). The majority of MP (97% to 99%) originated from platelets (PMP, Table 2). Numbers of PMP correlated with platelet counts ($r = 0.32$, $p = 0.02$) and the PMP/platelet ratio remained constant (0.02 to 0.03) during the course of pregnancy. A minor fraction (0.6% to 2.7%) of the MP originated from erythrocytes. No correlation was present between the numbers of erythrocyte-derived MP and erythrocytes. Numbers of circulating leukocyte-derived MP, staining for CD4, CD8, CD14, CD20, or CD66e, were negligible and did not correlate with the number of leukocytes. Finally, 0.4% to 1.0% of MP stained for E-selectin, which implies an endothelial cell source.

During normotensive pregnancy, the percentage of placenta-derived MP ranged between 1.5 and 3%. A significant increase of the absolute number of placenta-derived MP was found during pregnancy ($p = 0.005$), with the highest level at 36 weeks gestational age (Figure 2). Postpartum the percentage decreased to 1.1%. A modest correlation between the total number of placenta-derived MP and systolic blood pressure was present ($r = 0.33$, $p = 0.015$). In five male volunteers and five healthy non-pregnant women (mean age
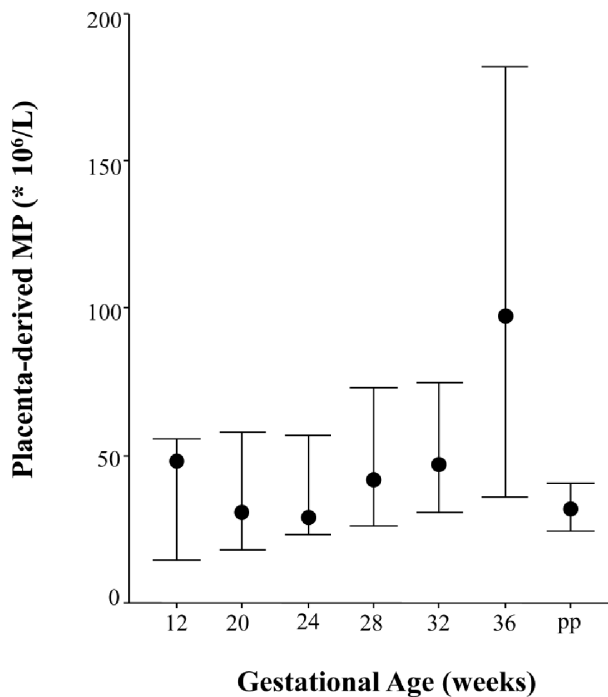

Figure 2: Placenta-derived MP during normotensive pregnancy. The number of MP originating from the placenta in normotensive pregnancies is plotted against the gestational age. The X-axis represents the gestational age in weeks and the Y-axis the number of placenta-derived MP. Data are presented as median with the interquartile range (25% to 75%). A significant increase of the absolute number of placenta-derived MP was found during pregnancy ($p = 0.005$), with the highest level at 36 weeks gestational age.

36 years and not using any medication including oral contraceptives), no relevant staining for ED822 to detect placenta-derived MP was found.

Preeclampsia

The number of MP was determined at weekly intervals in plasma from preeclamptic patients after admission to the hospital. The total number of MP did not change during preeclampsia in the period between 28 and 36 weeks gestation (Figure 3), but total numbers of MP were reduced in preeclamptic patients compared with normotensive controls of the same gestational age. At 28 and 36 weeks gestational age, these differences were statistically significant (both $p = 0.04$). Six weeks postpartum, MP numbers had increased again to normal levels ($p = 0.001$ compared with numbers during preeclampsia, but these (postpartum) numbers were similar to those of normotensive pregnant women.
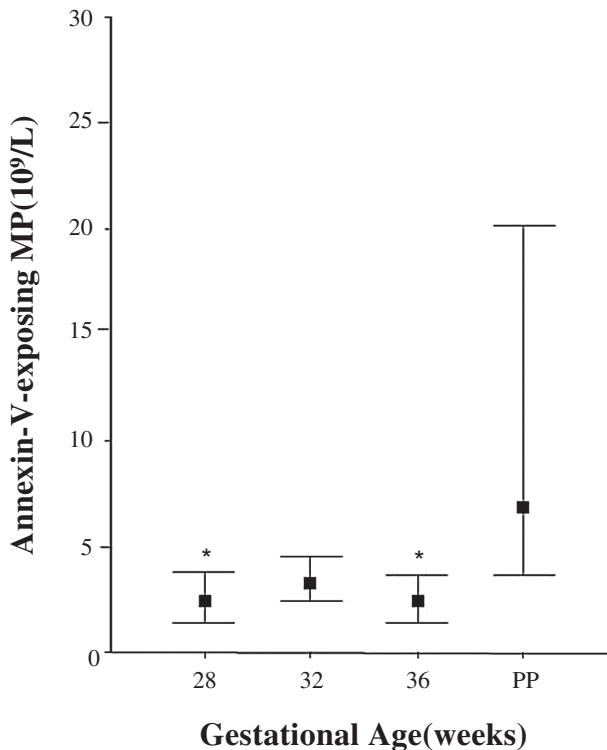

Figure 3: The number of MP in preeclampsia. The number of annexin V-positive MP in preeclampsia is plotted against the gestational age. Blood collection started at admission to the hospital mostly around 28 weeks gestation. Not all preeclamptic patients are represented in all gestational age groups. The median for each gestational age is presented with the interquartile range (25%–75%). Compared with normotensive controls of the same gestational age, there were significant differences at both 28 and 36 weeks gestational age ($p = 0.04$ for both).

Similar to normotensive pregnancy, the majority of MP originated from platelets (93% to 98%, Table 3). Again, numbers of PMP correlated with platelet counts ($r = 0.73$ $p = 0.0001$). The ratio between PMP and platelets was constant throughout pregnancy and comparable to normotensive pregnancy. Compared with normotensive pregnant women, absolute PMP numbers were significantly decreased at 28 weeks in preeclamptic patients ($p = 0.03$). The fraction of erythrocyte-derived MP varied between 1.1% and 5.3%. The absolute number of erythrocyte-derived MP was increased at 28 weeks ($p = 0.04$), 32 weeks ($p = 0.05$), and 36 weeks ($p = 0.06$) compared with normotensive pregnancy, but the fraction of such MP (Table 3) was only significantly elevated at 28 weeks ($p = 0.04$).

Leukocyte-derived MP, staining for CD4, CD8, CD14, and CD20 had negligible (< 0.1%) numbers in normotensive pregnancy. In contrast, T_{helper}-cell-derived MP were detectable (but not significantly elevated) in preeclamptic patients at 28 and 32 weeks gestational age (Table 3). $T_{suppressor}$-cell-derived MP were present only at 28 weeks ($p = 0.02$). Monocyte-derived MP were significantly elevated in preeclampsia at 28 weeks ($p = 0.007$), 32 weeks ($p = 0.04$) and 36 weeks ($p = 0.03$) but not postpartum. In preeclampsia, significantly more B-cell-derived MP (exposing CD20) were present at 36 weeks ($p = 0.008$) compared with normotensive pregnancies. Finally, granulocyte-derived MP were not detectable.

The fraction of endothelium-derived MP ranged between 0.5% and 1.2% of the total MP number. Numbers of endothelium-derived MP were not elevated in preeclampsia.

During normotensive pregnancy, the percentage of placenta-derived MP was higher (2.2% to 5.6%) than postpartum (0.9%). The total number of placenta-derived MP did not change significantly during preeclampsia if measured as function of gestational age (Figure 4). The highest median value was measured at 36 weeks gestational age. Although the percentages of placenta-derived MP were higher than in normal pregnancy at 28, 32, and 36 weeks gestation, the differences did not reach statistical significance ($p = 0.864$, $p = 0.056$, $p = 0.196$, respectively). However, it should be noted that some preeclamptic patients showed a high number of this subset of MP. These patients did not distinguish themselves clinically from the other patients. The hypothesis that placenta-derived MP are not continuously shed into the circulation but episodically, possibly after placental incidents like ischemia, has been proposed before (Knight et al. 1998). If this is true, we possibly sampled some patients during such moments. To illustrate the presence or absence of placenta-derived MP, examples of representative dot plots are shown in Figure 5. Samples of a healthy non-pregnant woman and a male volunteer are shown for comparison with the samples of normotensive pregnant women and preeclamptic patients (Figure 5A and B). During normotensive pregnancy, some placenta-derived MP are detectable (5C), and these MP disappear postpartum (5D). Some preeclamptic patients showed a

Table 3: Cellular origin of circulating MP in preeclamptic patients.

Circulating MP	Gestational Age						
	12	20	24	28	32	36	pp
Total MP (10^9/L)	–	–	–	2.3* (↓) (1.1–4.5)	3.3 (1.9–6.6)	2.3* (↓) (1.2–5.6)	7.2 (2.6–24.8)
% Platelet-derived MP	–	–	–	95.2 (↓) (90.0–98.8)	95.0* (↓) (92.4–98.4)	93.2 (82.4–98.9)	98.0 (87.1–99.2)
% Erythrocyte-derived MP	–	–	–	5.3* (↑) (1.5–12.3)	2.9 (↑) (0.6–5.8)	4.8 (↑) (0.7–12.6)	1.1 (0.3–12.6)
% T_{helper}-cell-derived MP	–	–	–	0.6 (0–5.3)	0.4 (0–4.3)	<0.1 (ND)	<0.1 (ND)
% Monocyte-derived MP	–	–	–	2.1* (↑) (0.9–3.5)	1.3* (↑) (0–3.7)	1.5* (↑) (0.2–3.6)	<0.1 (ND)
% B-cell-derived MP	–	–	–	<0.1 (ND)	<0.1 (ND)	0.3* (↑) (0–0.8)	<0.1 (ND)
% Endothelial-cell-derived MP	–	–	–	0.6 (0–0.8)	0.8 (0–3.0)	1.2 (0.4–4.9)	0.5 (0–1.1)
% Placental-derived MP	–	–	–	2.2 (1.3–3.3)	3.9 (1.3–10.0)	5.6 (1.8–10.3)	0.9 (0.1–6.2)

MP numbers are presented as median (range (min-max)). The subpopulations are expressed as median percentage of the total number of MP. The subpopulations are expressed as median percentage of the total number of MP. Percentages were calculated for every patient independently. Therefore, the sum of the median percentages of all subgroups approaches 100% and is not exactly 100%. pp = postpartum, ND = not detectable. Differences in the fraction of MP between preeclamptic patients and the normotensive women with a probability value of <0.05 were considered statistically significant (*). ↑: elevated or ↓: decreased (total number or fraction of MP) compared with normotensive pregnant women. Granulocyte-derived MP were <0.1% for all gestational ages. The percentage of $T_{suppressor}$-cell-derived MP was only > 0.1% at 28 weeks (0.3%, range 0% to 0.8%) but this was not significantly different compared with normotensive pregnant women.
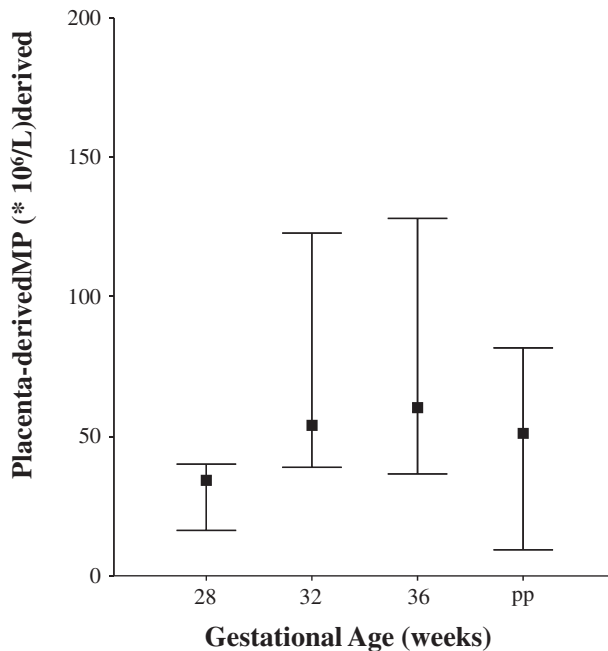
Figure 4: Placenta-derived MP in preeclampsia. The number of MP originating from the placenta in pregnancies complicated by preeclampsia is plotted against the gestational age. The X-axis represents the gestational age in weeks and the Y-axis the number of placenta-derived MP. Not all preeclamptic patients are represented in all gestational age groups. The median for each gestational age is presented with the interquartile range (25%–75%).

high count of this subset of MP (5E), and after delivery the placenta-derived MP were not detectable in the maternal circulation anymore (5F).

No correlations were found between MP (or subgroups) with systolic or diastolic blood pressure, birth weight and proteinuria as markers of the severity of the preeclampsia (data not shown).

DISCUSSION

The present study shows that the number of circulating MP in the maternal venous circulation in normotensive pregnancy is decreased at 12 weeks gestational age compared with postpartum, and gradually increases towards the postpartum values during pregnancy. Compared with normotensive pregnancy, decreased numbers of circulating MP in pregnancies complicated by preeclampsia were found, which is consistent with earlier reports (14,15).

The majority of MP (> 95%) originated from platelets and a positive correlation between PMP numbers and platelet counts was present. This correlation, however, could only partially explain the variation in numbers of PMP. Another possible explanation may be that at least some PMP originate directly from
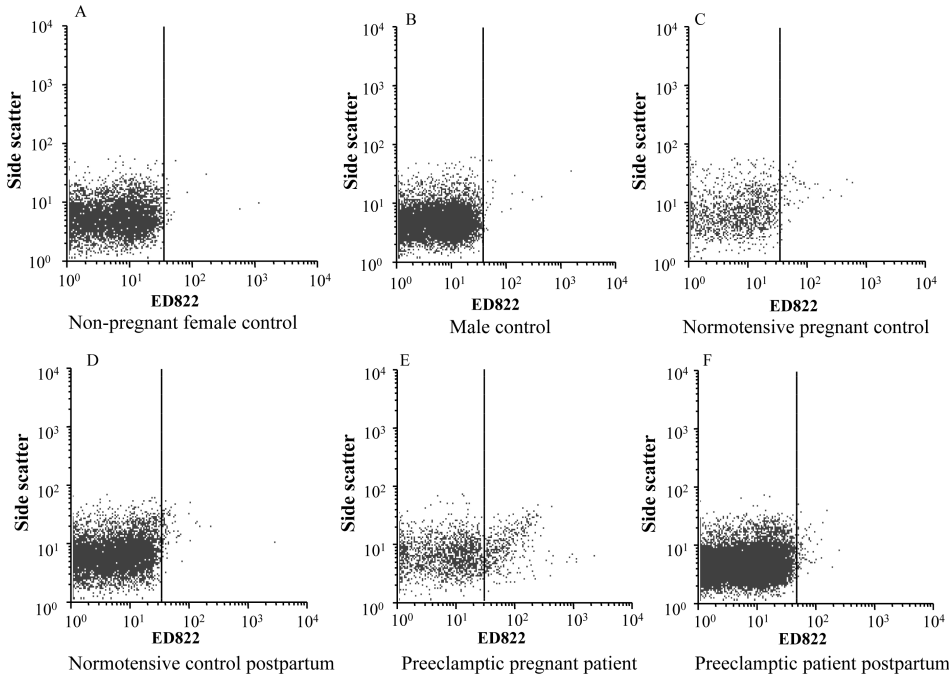
Figure 5: Placenta-derived MP in pregnancy and preeclampsia. Representative dot plots of a non-pregnant woman (*A*), a male control (*B*), a normotensive pregnant woman during pregnancy (*C*) and postpartum (*D*), and a preeclamptic patient during pregnancy (*E*) and postpartum (*F*). All dots are annexin V-binding MP, the MP binding ED822, i.e., MP of placental origin, occur on the right site of the fluorescent threshold (*vertical line*).

megakaryocytes (16). The PMP/platelet ratio was comparable at all time points between normotensive pregnancy and preeclampsia. Thus, PMP may reflect the turnover of platelets in plasma. As a consequence, the decreased platelet counts in patients with preeclampsia may explain the decreased number of circulating (P)MP. If true, than the increase of MP after delivery reflects normalization and sometimes elevation of the platelet count in the maternal blood of preeclamptic patients.

Blood cells, endothelial- and trophoblast cells are presumed to release MP upon activation and apoptosis. However, cell lysis and fragmentation can also be involved. The latter explains the elevated percentages of erythrocyte-derived MP in preeclampsia, because hemolysis frequently accompanies the syndrome.

Elevated numbers of leukocyte-derived MP may reflect activation of leukocytes, which is one of the features of preeclampsia. Especially monocytes and neutrophils become activated, possibly during placental passage (17). Circulating monocytes of preeclamptic patients express significantly higher levels of CD11b and CD14 (18) and produce elevated levels of inflammatory cytokines (IL-1β, IL-6, and IL-8) (19). Although the total number of MP was

decreased in patients with preeclampsia, absolute numbers as well as percentages of monocyte-derived MP were elevated compared to normotensive pregnant women, which probably reflects activation of monocytes in preeclampsia as a consequence of the systemic inflammatory response (3).

A change in numbers of leukocyte-derived MP may be involved in the development of endothelial dysfunction in preeclampsia, since leukocyte-derived MP produced *in vitro* from a T-cell line, impair acetylcholine-induced relaxation of mouse aortic rings (20). A similar decrease in relaxation was seen in rat aortic rings incubated with the total population of circulating MP from patients with myocardial infarction (21). Furthermore, impairment of bradykinin-mediated relaxation in isolated myometrial arteries was demonstrated for the total population of circulating MP from preeclamptic patients (7). Thus, one of the leukocyte-derived MP subpopulations may be responsible for these described effects. Although the fraction of leucocytes-derived MP is small, we cannot exclude that the total number of such subpopulations are higher in vivo, because such MP are likely to adhere to the endothelium of maternal blood vessels (22).

Preeclampsia is thought to be a disorder of the maternal endothelium. Endothelial cell activation may contribute both to the inflammatory response and to vasoconstriction. Theoretically, endothelial cell activation should be detectable by elevated levels of endothelial-derived MP. These circulating endothelium-derived MP affect the endothelium and possibly aggravate pre-existing endothelial dysfunction (23). However, we did not find a difference in the number of endothelial-derived MP between normotensive women and preeclamptic patients, which is in line with our previous work (4) but contradictory to other authors (24).

Placenta-derived MP increased significantly during normotensive pregnancy. This is not surprising, given the increase in placental volume. This rise in placenta-derived MP was also found when measured by enzyme linked immunosorbent assay (ELISA) with NDOG2 as capture antibody (25). In our study, the number of placenta-derived MP was not significantly elevated in preeclampsia compared with normotensive pregnancy. This is in contrast to the findings of other authors (9, 25), who reported elevated numbers of placenta-derived MP in preeclamptic women compared with normal pregnant women. Placenta-derived MP were not found to be increased in late onset preeclampsia or IUGR (26). The difference in results between investigators may be due to variation in patient characteristics, study design and antibodies used to detect placenta-derived MP. Previously, we tested ED822 for the detection of placenta-derived MP. ED822 bound to artificially prepared placenta-derived MP, but failed to detect these MP in diluted samples and was therefore unlikely to detect placenta-derived MP in maternal peripheral blood (4). In that study, our isolation protocol contained a washing step that was omitted from the current (isolation) protocol. Using this new procedure, the yield of MP (sub) populations

improved significantly, which is likely to explain the improved and specific detection of placenta-derived MP in our present study. ED822 has been described in detail and compared to other antibodies used to detect placenta-derived MP or STBM by Knight and colleagues (10).

In conclusion, MP numbers change during normotensive pregnancy and are decreased in preeclampsia. In preeclampsia, circulating MP may reflect activation, apoptosis or hemolysis from parental cells, whereas decreased numbers of circulating PMP and increased numbers of erythrocyte MP and monocyte-derived MP may reflect decreased platelet counts, hemolysis, and activation of monocytes, respectively.

REFERENCES

1. Berckmans RJ, Nieuwland R, Kraan MC, et al. Synovial microparticles from arthritic patients modulate chemokine and cytokine release by synoviocytes. Arthritis Res Ther 2005; 7:R536–44.

2. Nieuwland R, Sturk, A. Platelet-derived microparticles. In: Michelson AD. Platelets. London. Academic Press, 2002: 255–262.

3. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science 2005; 308:1592–1594.

4. VanWijk MJ, Nieuwland R, Boer K, van der Post JA, van Bavel E, Sturk A. Microparticle subpopulations are increased in preeclampsia: possible involvement in vascular dysfunction? Am J Obstet Gynecol 2002; 187:450–456.

5. Toth B, Lok CA, Böing A, Diamant M, van der Post JA, Friese K, Nieuwland R. Microparticles and exosomes: impact on normal and complicated pregnancy. Am J Reprod Immunol 2007; 58:389–402.

6. Lok CA, Nieuwland R, Sturk A, Hau CM, Boer K, Vanbavel E, Vanderpost JA. Microparticle-associated P-selectin reflects platelet activation in preeclampsia. Platelets 2007; 18:68–72.

7. VanWijk MJ, Svedas E, Boer K, Nieuwland R, VanBavel E, Kublickiene KR. Isolated microparticles, but not whole plasma, from women with preeclampsia impair endothelium-dependent relaxation in isolated myometrial arteries from healthy pregnant women. Am J Obstet Gynecol 2002; 187:1686–1693.

8. Lok CA, Boing AN, Reitsma PH, van der Post JA, van BE, Boer K, Sturk A, Nieuwland R: Expression of inflammation-related genes in endothelial cells is not directly affected by microparticles from preeclamptic patients. J Lab Clin Med 2006; 147:310–320.

9. Cockell AP, Learmont JG, Smárason AK, Redman CW, Sargent IL, Poston L. Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. Br J Obstet Gynaecol 1997; 104(2):235–240.

10. Knight M, Redman CW, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. Br J Obstet Gynaecol 1998; 105:632–640.

11. Preston RA, Jy W, Jimenez JJ, Mauro LM, Horstman LL, Valle M, Aime G, Ahn YS. Effects of severe hypertension on endothelial and platelet microparticles. Hypertension 2003; 41:211–217.

12. Contractor SF, SR Sooranna. Monoclonal antibodies to cytotrophoblast and syncytiotrophoblast of human placenta. J Dev Physiol 1986; 8:277–282.

13. Biro E, Nieuwland R, Sturk A. Measuring circulating cell-derived microparticles. J Thromb Haemost 2004; 2:1843–1844.

14. Holthe MR, Lyberg T, Staff AC, Berge LN. Leukocyte-platelet interaction in pregnancies complicated with preeclampsia. Platelets 2005; 16:91–97.

15. Bretelle F, Sabatier F, Desprez D et al. Circulating microparticles: a marker of procoagulant state in normal pregnancy and pregnancy complicated by preeclampsia or intrauterine growth restriction. Thromb Haemost 2003; 89:486–492.

16. Flaumenhaft R. Formation and fate of platelet microparticles. Blood Cells Mol Dis 2006; 36(2):182–187.

17. Mellembakken JR, Aukrust P, Olafsen MK, Ueland T, Hestdal K, Videm V. Activation of leukocytes during the uteroplacental passage in preeclampsia. Hypertension 2002; 39:155–160.

18. Holthe MR, Staff AC, Berge LN, Lyberg T. Leukocyte adhesion molecules and reactive oxygen species in preeclampsia. Obstet Gynecol 2004; 103:913–922.

19. Luppi P, Deloia JA. Monocytes of preeclamptic women spontaneously synthesize pro-inflammatory cytokines. Clin Immunol 2006; 118:268–275.

20. Martin S, Tesse A, Hugel B et al. Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression. Circulation 2004; 109:1653–1659.

21. Boulanger CM, Scoazec A, Ebrahimian T et al. Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. Circulation 2001; 104:2649–2652.

22. Furie B, Furie BC. Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. Trends Mol Med 2004; 10:171–179.

23. Brodsky SV, Zhang F, Nasjletti A, Goligorsky MS. Endothelium-derived microparticles impair endothelial function in vitro. Am J Physiol Heart Circ Physiol 2004; 286:H1910–915.

24. Gonzalez-Quintero VH, Smarkusky LP, Jimenez JJ et al. Elevated plasma endothelial microparticles: preeclampsia versus gestational hypertension. Am J Obstet Gynecol 2004; 191:1418–1424.

25. Germain SJ, Sacks GP, Sooranna SR, Sargent IL, Redman CW. Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. J Immunol 2007; 178:5949–5956.

26. Goswami D, Tannetta DS, Magee LA, Fuchisawa A, Redman CW, Sargent IL, von Dadelszen P. Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction. Placenta 2006; 27:56–61.