Vascular dysfunction in preeclampsia
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Citation for published version (APA):
Chapter 5

Microparticle subpopulations are increased in preeclampsia: possible involvement in vascular dysfunction?


Abstract

Objective: The purpose of this study was to investigate the cellular origin and numbers of circulating microparticles in normal pregnancy and preeclampsia.

Methods: Plasma samples from 10 women with preeclampsia, from 10 normal pregnant women, and from 10 nonpregnant women, matched for age and gestation, were analyzed by flow cytometry.

Results: The total number of circulating microparticles was unaltered in pregnancy and preeclampsia. The largest portion of microparticles was derived from platelets in all groups. T-suppressor cell microparticle numbers were decreased in normal pregnancy (P = .04). In preeclampsia T-suppressor, T-helper cell, and granulocyte microparticle numbers were increased (P = .008, P = .008 and P = .03). Elastase concentration were increased in preeclampsia (P = .02) and correlated with granulocyte microparticle numbers (P = .006). Elastase concentrations correlated with systolic and diastolic blood pressure (P = .001 and .003), and granulocyte microparticle numbers correlated with systolic blood pressure (P = .05).

Conclusions: Numbers of T-cell and granulocyte microparticles are increased in preeclampsia. Whether these altered microparticle numbers cause vascular dysfunction in preeclampsia or are a consequence of the disease remains to be established.
**Introduction**

Microparticles are small membrane fragments that are released from cells during cell activation and apoptosis. Many different cell types (such as endothelial cells, thrombocytes, leukocytes, erythrocytes and vascular smooth muscle cells) release microparticles *in vitro*, the presence of these microparticles *in vivo* has been demonstrated recently in various studies. Microparticle formation is altered in several disease states. A reduced ability to generate microparticles occurs in Scott syndrome and Castaman’s defect, both of which are bleeding disorders [253]. Increased numbers of platelet microparticles have been found in patients who were at increased risk for thromboembolic complications, such as patients with increased coagulation activation, idiopathic thrombocytopenia, ischemic brain diseases, acute coronary syndromes, multiple sclerosis [253], and sepsis [210,211].

The characteristics of microparticles seem to depend on the activation status of the cells from which they originate and on the mechanism of stimulation [154]. Consequently, they vary in size, structure, antigen- and adhesion molecule-expression. The functional importance of microparticles *in vivo*, however, remains to be defined. Microparticles from different cell types have different effects on vascular cells and on blood cells *in vitro*. Platelet microparticles induce COX-2 expression, PGI$_2$ [157] and cytokine [199] production in endothelial cells, up-regulate adhesion molecules on the endothelial surface [199,196], which results in monocyte adherence, cause platelet activation [157], and increase vascular smooth muscle mitogenesis and proliferation [254]. Leukocyte microparticles also induce up-regulation of adhesion molecules on endothelial cells and initiate the production of the cytokines IL-6 and IL-8 [198]. Endothelial cell microparticles can activate neutrophils, which results in increased neutrophil adhesion to the endothelium [239] and can stimulate coagulation [167]. Furthermore, microparticles that have been isolated from patients with acute myocardial infarction can diminish endothelium-dependent relaxation in isolated arteries of rats [235]. Thus, microparticles act as potent pro-inflammatory inducers, which initiate an array of signal transduction pathways and gene expression profiles in endothelial cells, thereby affecting endothelial function. Finally, the surface of microparticles forms a strong procoagulant surface [184] because of the redistribution of membrane components during the formation of microparticles, in particular PS, which flips from the cytosolic leaflet of the membrane to the extracellular leaflet. Therefore, microparticles may, *in vivo*, be involved in regulation of both coagulation and vascular function.

Normal pregnancy is associated with extensive anatomic and functional adaptations of the cardiovascular system to accommodate the demands of pregnancy. In preeclampsia this adaptation is inadequate [255]. Endothelial cells, leukocytes, and the coagulation system are all activated in normal pregnancy, while in preeclampsia this activation seems exaggerated (for review see [256]). In theory, microparticles could cause the alterations that occur in pregnancy and preeclampsia. We therefore determined the numbers and cellular origin of circulating microparticles in pregnancy and preeclampsia. Thus far, the only microparticles that have been studied in pregnancy and preeclampsia are STBM, which were reported to be present in the
maternal circulation in pregnancy and to be increased in number in preeclampsia [64]. *In vitro*, STBM damaged endothelial cell culture structure [65] and function [66] and activated neutrophils [76]. However, at present no information is available on the presence of microparticles from other cells.

**Methods**

**Reagents and assays**

Normal mouse serum, phycoerythrin (PEt)-labeled anti-CD4 (anti-CD4-PEt) and anti-CD66e-PEt were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Anti-glycophorin A-PEt was obtained from Dakopatts (Glostrup, Denmark). Anti-CD8-PEt, anti-CD14-PEt and anti-CD20-PEt, immunoglobulin (Ig) G2a-PEt, IgG1-PEt and fluorescein isothiocyanate (FITC)-labeled IgG1 were obtained from Beckton Dickinson (San Jose, USA). Anti-CD61-PEt was obtained from Pharmingen (San Jose, USA). Anti-CD62e-FITC was obtained from Kordia (Leiden, The Netherlands). Anti-CD144-PEt was obtained from Bender (Vienna, Austria). Allophycocyanin (APC)-conjugated annexin V was obtained from Caltag (Burlingame, USA). Biotinylated NDOG2 and ED822 were donated by C. Redman and I. Sargent (Nuffield Department of Obstetrics and Gynaecology, John Radcliff Hospital, Oxford, UK) and were conjugated with FITC. Plasma concentrations of elastase were determined by enzyme-linked immunosorbent assay (ELISA, Enzygnost), as described by the manufacturer (Boehringer Diagnostics; Marburg, Germany).

**Patients**

The study was approved by the medical ethical committee of the Academic Medical Center. After written informed consent, plasma samples were obtained from 10 women with preeclampsia, from 10 healthy normal pregnant women, and from 10 nonpregnant women. Normal pregnant control subjects were matched with the women with preeclampsia for age (± 5 yr) and gestational age (± 2 wk). Nonpregnant women were matched with the women with preeclampsia for age (± 5 yr).

**Collection of blood samples**

Blood samples were taken from the antecubital vein without tourniquet through a butterfly needle with a Vacutainer blood-collecting system (Beckton Dickinson) into a 4.5-mL tube that contained 1 part of 0.105 mol/L citrate to 9 parts of blood (Beckton Dickinson). Cells were removed by centrifugation for 20 minutes at 1550 g at room temperature. Plasma samples were divided in 250 μL aliquots, snap frozen in liquid nitrogen to preserve microparticle structure, and stored at −80°C until further analysis.
Circulating microparticles

Isolation of microparticles

A 250-µL plasma sample was thawed on ice and then centrifuged for 30 minutes at 17570 \( g \) and 20°C to pellet the microparticles. The plasma from which the microparticles were isolated was not defibrinated to avoid a potential loss of platelet-derived microparticles. After centrifugation, 225 µL of the supernatant was removed and the microparticle-pellet and remaining 25 µL of plasma were resuspended with 225 µL citrate-substituted PBS. After another centrifugation period of 30 minutes at 17570 \( g \) and 20°C, again 225 µL of the supernatant was removed. The microparticle pellet was then resuspended in 75 µL citrated PBS, of which 5 µL was used per incubation.

Flow cytometric analysis

The 5 µL of microparticle suspension was diluted in 35 µL of calcium chloride (2.5 mmol/L)-containing PBS and 5 µL of 5000-fold prediluted microparticle-free normal mouse serum. After an incubation period of 15 minutes at room temperature, 5 µL APC-labeled annexin V was added to all tubes plus 5 µL of one of the labeled cell-specific monoclonal antibodies or isotype-matched control antibody (IgG1-PEt, except for anti-CD14-PEt, which has IgG2b-PEt as a control and anti-CD62e-FITC and NDOG2-FITC, which have IgG1-FITC as a control). The samples were then incubated in the dark for 15 minutes at room temperature.

A panel of cell-specific monoclonal antibodies was used, directed against platelets (CD61), erythrocytes (glycophorin A), endothelial cells (CD62e and CD144), T-helper (T\(_{\text{h}}\)) cells (CD4), T-suppressor (T\(_{\text{s}}\)) cells (CD8), monocytes (CD14), B cells (CD20), granulocytes (CD66e) and syncytiotrophoblast (NDOG2). Anti-CD62e and anti-CD144 were used to detect endothelial cell microparticles on the basis of previous observations [210]. NDOG2 and ED822, both antibodies against placental alkaline phosphatase, were tested for their ability to identify syncytiotrophoblast microparticles. Although ED822 bound to artificially prepared STBM, this antibody could not detect STBM when the samples were diluted more than 50-fold. NDOG2 detected artificially prepared STBM well (figure 1) also at low STBM concentrations and in plasma samples from patients. The following final concentrations were used: anti-CD8-PEt (250 ng/mL), anti-CD14-PEt (250 ng/mL), anti-CD20-PEt (500 ng/mL), anti-CD62e-FITC (1.67 µg/mL), antiglycophorin A-PEt (200 ng/mL), anti-CD144-PEt (500 ng/mL), IgG1-Pet (500 ng/mL), IgG2b-PEt (500 ng/mL), IgG1-FITC (500 ng/mL) and annexin V-APC (3.33 µmol/ml). For some antibodies concentrations were not supplied by the supplier. For these antibodies the final dilutions used were anti-CD4-PEt (1:50), anti-CD61-PE (1:100), anti-CD66e-PEt (1:100) and 1:100 for the NDOG2-FITC antibody. All antibodies were previously validated in our laboratory on various templates (patients with sepsis for anti-CD14, synovial fluid from patients with arthritis for anti-CD66e and anti-CD20, buffy coat for anti-CD4 and anti-CD8, artificially prepared syncytiotrophoblast microparticles for NDOG2, and endothelial cell microparticles from IL-1\( \alpha \)-stimulated human umbilical vein endothelial cells for anti-CD62e and anti-CD144). The choice of antibody concentrations used in this study was based on previous experience in our laboratory [211,186,210].
Figure 1. Histogram presenting the labeling of artificially prepared STBM with NDOG2-FITC (grey area) and with control IgG-FITC (black area). All events on the right side of the vertical line are considered NDOG2-positive microparticles.

After the incubation period, 200 µL of calcium-containing PBS was added to all tubes and the microparticles were pelleted again by centrifugation for 30 minutes at 17570 g and 20°C. Finally, 200 µL of supernatant was removed and the microparticle pellets were resuspended in 300 µL of calcium-containing PBS. Samples were analyzed for one minute in a fluorescence automated cell sorter flow cytometer with CellQuest software (Beckton Dickinson). Both forward scatter and sideward scatter were set at a logarithmic gain. Microparticles were identified on the basis of their size and density, as previously described [211], and on their capacity to bind annexin V and a cell-type-specific monoclonal antibody (the latter was used to establish the cellular origin of the microparticles). Annexin V measurements were corrected for autofluorescence, and binding of cell-specific monoclonal antibodies was corrected with identical concentrations of isotype-matched control antibodies. Microparticles numbers, which were corrected for isotype control and autofluorescence, were calculated as the number per liter plasma with the following formula: \( N \times (100/5) \times (355/60) \times (10^6/250) \), in which \( N \) is the number of events that stained for both annexin V and a cell-specific antibody.

Statistical analysis

Data were analyzed in SPSS for Windows, release 10.0.7 (SPSS Inc; Chicago, USA). The distribution of data was tested for normality using a Kolmogorov Smirnov 2 test. The results of the demographic characteristics of patients were normally distributed and therefore analyzed with a ANOVA test with a Bonferroni post hoc test for differences between groups. Because of the
borderline normal distribution and the small number of patients the Kruskal-Wallis test was used to test for differences in microparticle numbers among groups. When statistically significant differences between groups were found, the Mann-Whitney $U$ test with a Bonferroni correction was used to test for differences between women with preeclampsia and normal pregnant women and differences between normal pregnant and nonpregnant women. Spearman’s $\rho$ correlation test was used to investigate correlations between microparticle numbers and patient characteristics. Differences were considered statistically significant at $P < .05$. All data are given as median (range), except for demographic data, which are presented as mean ± SD.

Results

Patient characteristics

The patient characteristics are presented in table I. As by definition, all women with preeclampsia had significantly increased systolic and diastolic blood pressures and had significant proteinuria. Of the women with preeclampsia one woman also met the criteria for the HELLP syndrome. Women with preeclampsia were delivered at significantly lower gestational ages than normal pregnant women, and the birthweights of their children were also significantly lower. In one woman with preeclampsia an intrauterine fetal death occurred. One normal pregnant woman was excluded from the study because microparticle numbers differed >3 SD from the measurements in all other women. She had an otherwise normal pregnancy outcome. One woman with preeclampsia was excluded from evaluation of elastase values, since her elastase concentration differed >7 SD from the other women in the preeclampsia group.

<table>
<thead>
<tr>
<th>Table I. Patient characteristics.</th>
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<tr>
<td><strong>Sampling</strong></td>
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<tr>
<td>Age (yr)</td>
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<tr>
<td>Gestational age (wk)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
</tr>
<tr>
<td><strong>Delivery</strong></td>
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<tr>
<td>Gestational age (wk)</td>
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<tr>
<td>Birthweight (g)</td>
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</table>

Demographic data of the included subjects at the time of sampling and data at delivery.

* ANOVA $P < .001$
Number and cellular origin of circulating microparticles

Figure 2 shows representative fluorescence automated cell sorter histogram of granulocyte microparticles that were double stained with annexin V-APC and anti-CD66e-PEt of women from the 3 groups. The individual data points for granulocyte-, T-cell-, and endothelial cell-derived microparticles are presented in figure 3. Table II summarizes the cellular origin of the circulating microparticles and their numbers.

![Histograms](image)

**Figure 2.** Example of representative histograms of annexin V-positive microparticles binding to anti-CD66e-PEt (gray area) and control IgG,PEt (black area) in a woman with preeclampsia (A), a normal pregnant woman (B), and a nonpregnant woman (C). All events on the right side of the vertical line are considered anti-CD66e-positive microparticles.

The total numbers of circulating microparticles were similar in all three groups. From all circulating microparticles, the cellular origin could be established because the number of microparticles derived from the investigated cell types added up to the total number of microparticles, as defined by the annexin V-positive events in the microparticle region of the flow cytometric analysis. By far, the largest portion of circulating microparticles originated from platelets in all groups (median percentage: 82.3% in women with preeclampsia, 91.7% in normal pregnant women, and 87.1% in nonpregnant women). Microparticles that were derived from T_s-cells were decreased in normal pregnancy (P =.04). In preeclampsia T-cell and granulocyte microparticles were significantly increased (P =.008 for both T_H and T_s-cell microparticles and P =.03 for granulocyte microparticles). As an extra measure of granulocyte activation plasma concentrations of elastase were determined, which were significantly increased in preeclampsia compared with normal pregnancy (median: 54.3 ng/mL [range 38.5–104.5 ng/mL] vs median 37.7 ng/mL [range 27.9–47.8 ng/mL]; P =.02). The number of granulocyte microparticles correlated significantly with the elastase concentration (r = 0.52, P =.006). NDOG2-positive
Circulating microparticles were not only present in the circulation of normal pregnant and preeclamptic women but also in the circulation of 8 of the 10 nonpregnant women, of whom five women had never been pregnant.

There was no correlation between the total number of circulating microparticles and either age, systolic or diastolic blood pressure, or proteinuria. The number of granulocyte microparticles correlated with systolic blood pressure \( r = 0.39, P = 0.05 \) and elastase concentrations \( r = 0.52, P = .006 \), which also correlated with both systolic and diastolic blood pressure \( r = 0.64, P = .001 \) and \( r =0.58, P = .003 \), respectively.

### Table II. Cellular origin and numbers of circulating microparticles.

<table>
<thead>
<tr>
<th>Microparticle origin</th>
<th>Preeclampsia</th>
<th>Normal pregnant</th>
<th>Nonpregnant</th>
<th>P*</th>
<th>P‡</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>2256 (994-3839)</td>
<td>1960 (410-3714)</td>
<td>2357 (1092-3468)</td>
<td>.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet (CD61)</td>
<td>1818 (638-3561)</td>
<td>1618 (117-3450)</td>
<td>2014 (405-3261)</td>
<td>.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocyte (GlycoA)</td>
<td>234 (19-520)</td>
<td>122 (0-256)</td>
<td>150 (39-474)</td>
<td>.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocyte (CD66e)</td>
<td>79 (0-287)</td>
<td>8 (0-85)</td>
<td>7 (0-267)</td>
<td>.05</td>
<td>.03</td>
<td>.84</td>
</tr>
<tr>
<td>Monocyte (CD14)</td>
<td>0 (0-253)</td>
<td>0 (0-18)</td>
<td>0 (0-48)</td>
<td>.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cell (CD20)</td>
<td>5 (0-479)</td>
<td>10 (0-30)</td>
<td>3 (0-106)</td>
<td>.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T(_H) cell (CD4)</td>
<td>29 (0-82)</td>
<td>0 (0-20)</td>
<td>13 (0-258)</td>
<td>.01</td>
<td>.008</td>
<td>.11</td>
</tr>
<tr>
<td>T(_S) cell (CD8)</td>
<td>15 (0-133)</td>
<td>0 (0-6)</td>
<td>12 (0-101)</td>
<td>.008</td>
<td>.008</td>
<td>.04</td>
</tr>
<tr>
<td>STBM (NDOG2)</td>
<td>42 (0-89)</td>
<td>2 (0-95)</td>
<td>84 (0-334)</td>
<td>.05</td>
<td>.36</td>
<td>.08</td>
</tr>
<tr>
<td>Endothelial cell (CD62e/CD144)</td>
<td>23 (0-122)</td>
<td>0 (0-59)</td>
<td>11 (0-75)</td>
<td>.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as median number \( \times 10^{8} / \text{L plasma} \) (range).

* differences between all groups (Kruskal-Wallis)

‡ women with preeclampsia vs normal pregnant women (Mann Whitney U, with Bonferroni correction),

† normal pregnant vs nonpregnant women (Mann Whitney U, with Bonferroni correction).
Figure 3. Individual data points and medians for $T_H$ (A), $T_S$ (B), granulocyte (C) and endothelial cell (D) microparticle numbers in women with preeclampsia (PE), normal pregnant women (PR), and nonpregnant women (NP).

Comments

Although the total numbers of circulating microparticles were not significantly altered either in normal pregnancy or in preeclampsia, we found that numbers of microparticles of certain subgroups of microparticles are different in these conditions. In normal pregnancy, the number of
Circulating microparticles

T₃-cell microparticles was decreased as compared with the nonpregnant state, while in preeclampsia numbers of T₃₄-cell, T₅-cell and granulocyte microparticles were increased compared with normal pregnancy. The increased numbers of microparticles of these particular microparticle subgroups in preeclampsia could be a possible mechanism for development of vascular dysfunction in preeclampsia and seem to reflect an altered activation status of the immune system as well as an increased inflammatory response. The increased numbers of lymphocyte microparticles could be released into the maternal circulation from activated lymphocytes, which are present in increased numbers in the placental tissue during preeclampsia [257]. These lymphocyte microparticles could then cause endothelial damage directly or indirectly by inducing microparticle formation through activation of other cells, thus creating a vicious circle. Alternatively, it is possible that cells are activated when passing through the placenta, by an unknown factor that is released from the placenta. Microparticle formation could then be triggered secondarily and result in endothelial dysfunction. In this respect, it is interesting that culture medium, after incubation with placentas from women with preeclampsia, stimulates neutrophil granulocytes to interact with the endothelium [258]. The presence of activated neutrophils in preeclampsia has been reported in several studies (review see [256]). The increased granulocyte microparticle and elastase concentrations in this study confirm this. Both these measures of granulocyte activation also significantly correlated with blood pressure, a measure of disease severity. Another mechanism that could support microparticle formation by neutrophils is their delayed apoptosis in preeclampsia [259] because this could result in elevated numbers of circulating activated neutrophils.

Microparticles derived from T-cells are increased in patients with HIV infection [188], whereas granulocyte microparticles are increased in patients with sepsis and multiple organ dysfunction syndrome [211,210]. Because altered immune and inflammatory responses are involved in these diseases, the increased T-cell and granulocyte microparticle numbers in preeclampsia provide further evidence for involvement of the immune and inflammatory system in the pathogenesis of preeclampsia [256].

A central phenomenon in the vascular dysfunction in preeclampsia is endothelial activation. One could therefore expect endothelial cell microparticle numbers to be increased in this disease. This was not found in this study, although numbers of endothelial cell microparticles tended to be higher in preeclampsia (figure 3D). The numbers of endothelial cell microparticles, however, correlated with numbers of microparticles that were derived from lymphocytes (r = 0.39, P =.04 for T₅₄-cells and r = 0.48, P =.008 for T₅-cells), B cells (r = 0.72, P <.001), granulocytes (r = 0.51, P =.005) and erythrocytes (r = 0.39, P =.04), which indicates a strong relation between the activation status of all these cell types. Two additional explanations, however, should be considered.

First, it is possible that not only the numbers of microparticles are important in the generation of vascular dysfunction in preeclampsia, but also their characteristics. It has been reported that microparticles that result from different stimuli show different characteristics [154]. Thus, their effect on cells may vary as well. It is therefore possible that a subgroup of microparticles with
certain characteristics, formed after a specific stimulus (eg, hypoxia, cytokines, or free radicals), could result in the development of vascular dysfunction, which is observed in preeclampsia. In this respect it is of interest that endothelial cell microparticles, prepared in vitro by stimulation with TNF were procoagulant [167]. The fact that these microparticles in addition exposed several adhesion molecules leads us to the second consideration: that not only the circulating microparticle fraction is important, but also -most likely- the fraction of microparticles that has bound to cells. It is known that microparticles can indeed bind to cells. For example, platelet microparticles bind to neutrophils in a 150:1 ratio, which results in clustering of the neutrophils [197]. Because adhesion of microparticles to cells might induce the observed activation, the bound fraction may prove to be important. No data are available yet on the amount of bound microparticles.

It is generally accepted that the placenta plays an important role in the pathogenesis of preeclampsia. In preeclampsia trophoblast invasion is impaired, which results in the release of an unknown factor from the placenta into the maternal circulation that causes a generalized vascular dysfunction (for review see [255]). Obviously, STBM would have been an excellent candidate for this unknown factor, especially because increased apoptosis of trophoblast in preeclampsia has been reported [260]. We used two antibodies to identify STBM that have previously been used to detect and quantify STBM in normal pregnancy and in preeclampsia. Both antibodies bound to artificially prepared STBM. However, ED822 was unable to detect STBM at low concentrations. In addition, NDOG2 not only bound to a subpopulation of microparticles in normal pregnant women and women with preeclampsia, but also in 8 of the 10 nonpregnant women, 5 of whom had never been pregnant before. In addition, NDOG2 also bound to microparticles that had been isolated from some healthy men (data not shown). Therefore, NDOG2 seems to be non specific for STBM. Thus, we were unable to identify STBM in the maternal circulation in our study. However, because the cellular origin of virtually all microparticles could be established, the fraction of STBM can, at most, be a modest one, in respect to the total number of circulating microparticles. Nevertheless, we can not exclude the possibility that very low numbers of STBM circulate in preeclampsia, which may have a high capacity to damage the vascular endothelium or to activate neutrophils [76].

In conclusion, our results show increased numbers of T-cell and granulocyte microparticles in preeclampsia. It remains to be established whether these microparticles are involved in the pathogenesis of preeclampsia. It is important to identify the mechanisms leading to vascular dysfunction in preeclampsia, since this could provide us with new therapeutical options and ultimately with methods to prevent the disease.

We thank Renée Baak-Pablo, Marianne Schaap and Krista Reijgwart for their excellent laboratory work.