Leukocyte Activation and Circulating Leukocyte-Derived Microparticles in Preeclampsia

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Problem
Preeclampsia shows characteristics of an inflammatory disease including leukocyte activation. Analyses of leukocyte-derived microparticles (MP) and mRNA expression of inflammation-related genes in leukocytes may establish which subgroups of leukocytes contribute to the development of preeclampsia.

Method of Study
Blood samples were obtained from preeclamptic patients, normotensive pregnant and non-pregnant controls. sL-selectin and elastase were measured by ELISA. mRNA was isolated from leukocytes and gene expression was determined by multiplex ligation-dependent probe amplification (MLPA). MP were characterized by flow cytometry.

Results
Altered concentrations of sL-selectin and elastase confirmed leukocyte activation in preeclampsia. These leukocytes showed up-regulation of Nuclear Factor of Kappa light chain gene enhancer in B Cells inhibitor (NFκB-1A) and cyclin-dependent kinase inhibitor (CDKN)-1A compared with normotensive pregnant women. interleukin-1 Receptor Antagonist (IL-1RA) and tumor necrosis factor (TNF)-R1 were increased compared with those in non-pregnant controls. Monocyte-derived MP were elevated in preeclamptic patients compared with pregnant women. The numbers of cytotoxic T-cell-derived and granulocyte-derived MP were elevated compared with those of non-pregnant women.

Conclusion
Leukocytes are activated in preeclampsia. A pro-inflammatory gene expression profile is not prominent, although differences in mRNA expression can be detected. Increased levels of particular subsets of leukocyte-derived MP reflect activation of their parental cells in preeclampsia.

Introduction

Preeclampsia is a pregnancy-specific multisystem disorder occurring in the second half of pregnancy with an incidence in unselected populations of 2.6%.[1] It is a major cause of maternal and fetal morbidity and mortality of which the exact cause remains unclear. Preeclampsia is considered an exaggerated systemic

inflammatory response to pregnancy[2] with activation of many parts of the inflammatory network including leukocyte activation. Indeed, signs of activation of granulocytes, monocytes, and lymphocytes have been reported.

Evidence for the activation of *granulocytes* in preeclampsia is based upon the elevated expression of adhesion molecules and complement-related markers[3–5], the production of reactive oxygen species[3,6] and an increase in soluble markers like elastase and lactoferrin in the plasma of preeclamptic patients.[7,8] Furthermore, granulocyte counts are elevated in preeclamptic patients compared with non-preeclamptic women.[9,10] *Monocytes* from preeclamptic patients express significantly higher levels of cell-surface activation markers (CD11b, CD11c, and CD14) and produce more reactive oxygen species compared with normotensive pregnant women.[5] *T lymphocytes* are also activated in preeclampsia and this is characterized by a predominance of $T_{helper}1$-cell immunity resulting in a more pro-inflammatory cytokine profile.[11,12]

The number of circulating leukocyte-derived microparticles (MP) may also be a marker for the activation of leukocytes. MP are small membrane vesicles, which are budded from the cell surface and released into the circulation during apoptosis or activation of blood cells or endothelial cells. Thus, activation of leukocytes results in the production of leukocyte-derived MP. Indeed, the concentration of T-cell-derived and granulocyte-derived MP were elevated in preeclampsia. In a longitudinal study, we found that monocyte-derived MP were significantly elevated in preeclampsia at 28, 32, and 36 weeks.[13] Furthermore, in preeclampsia, significantly more B-cell-derived MP were present at 36 weeks compared with normotensive pregnancies. However, the patients in this study were longitudinally followed and not matched and there was no non-pregnant control group. In view of these studies, especially the leukocyte-derived MP seem to be important in the development of preeclampsia. Therefore, the present study specifically focuses on these MP and the correlation with differential leukocyte counts and other markers of leukocyte activation and RNA expression.

The production of cytokines is also indicative of the pro-inflammatory state of leukocytes. Changes in cytokine production in preeclampsia have some similarities with those found in sepsis.[14,15] Sepsis can be mimicked by administration of endotoxin to healthy subjects. Isolated leukocytes from these subjects show increased mRNA expression of Macrophage Inflammatory Protein (MIP)-1A, MIP-1B, Interleukin (IL)-8, IL-1β, IL-1 Receptor Antagonist (RA), and Nuclear Factor of Kappa light chain gene enhancer in B Cells inhibitor (NFκB)-1A or IκB inhibitor alpha (IκB).[16] Changes in the levels of plasma proteins encoding genes that also may be differentially expressed in sepsis, have been investigated previously in preeclamptic patients. However, the results from these studies are contradictory.[17–24]

Therefore, in the present study, we determined the activation state of leukocytes by measuring mRNA expression of inflammation-related genes using multiplex ligation-dependent probe amplification (MLPA). In addition, we analyzed circulating numbers of leukocyte-derived MP and subgroups thereof as additional putative markers of leukocyte activation, as well as differential leukocyte counts, and plasma sL-selectin- and elastase concentrations as markers of inflammation and leukocyte activation.

Materials and methods

Patients

The study was approved by the medical ethical committee of the Academic Medical Center. After obtaining written informed consent, blood samples were obtained from preeclamptic patients (n = 10), normotensive pregnant women (n = 10) and non-pregnant women (n = 10). All women were nulliparous. The women were matched for age (±5 years). The preeclamptic patients and normotensive pregnant women were also matched for gestational age (±2 weeks). Inclusion criteria for preeclampsia were: (i) diastolic blood pressure of 110 mmHg or more on any occasion or 90 mmHg or more on two separate occasions at least 4 hr apart, (ii) proteinuria of ≥0.3 gram protein/24 hr, and (iii) symptoms developing after 20 weeks of gestation in previously normotensive women. The control groups consisted of healthy women not using any medication or oral contraceptives. Patient characteristics are presented in Table I.

Collection of Blood Samples

Three blood samples (13.5 mL in total or 4.5 mL each) were taken from the antecubital vein without tourni-

Table I Patient Characteristics

Characteristics	Preeclampsia (n = 10)	Normotensive pregnancy (n = 10)	Non-pregnant women (n = 10)	P	P*
Age (years)	33.1 ± 3.4	30.5 ± 1.6	30.3 ± 1.6	NS	0.04
BMI	26.5 ± 5.2	21.4 ± 1.1	21.9 ± 2.1	0.006	0.02
Blood pressure					
Systolic	164 ± 16	122 ± 9	108 ± 14	0.0001	0.0001
Diastolic	96 ± 8	73 ± 8	68 ± 10	0.0001	0.0001
Proteinuria	7.1 ± 5.5	–	–	–	–
Gestational age (weeks)					
At sampling	28.9 ± 1.9	30.2 ± 3.2	–	NS	–
At delivery	31.3 ± 3.4	39.1 ± 1.6	–	0.0001	–
Birth weight	1267 ± 648	3262 ± 607	–	0.0001	–

All patients were nulliparous and matched for age (±5 years). Preeclamptic patients and normotensive pregnant women were also matched for gestational age (±2 weeks). Data are presented as mean ± S.D. P, statistical difference between preeclamptic patients and normotensive controls; P*, statistical difference between preeclamptic patients and non-pregnant controls.

quet through a 20-gauge needle with a vacutainer system. As the time of sampling may influence the results, blood was collected in the morning between 09:00 and 11:00. The first sample was collected in a 4.5 mL tube containing 0.105 M buffered sodium citrate (Becton Dickinson, San Jose, CA, USA). Within 30 min after collection, cells were removed by centrifugation for 20 min at 1560 g and 20°C. Plasma samples were then divided into 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 0.054 mL (15%) ethylenediaminetetraacetic acid (EDTA; Becton Dickinson) for determination of the total number of leukocytes. The third sample was also collected in a 4.5 mL tube containing EDTA and immediately used for RNA isolation.

ELISA

Plasma concentrations of sL-selectin (sL-selectin; R&D Systems; Abingdon, UK) and elastase (Human PMN elastase; Bender Medsystems; Vienna, Austria) were determined using enzyme-linked immunosorbent assays (ELISA). Assays were performed as described by the manufacturers. The minimal detectable concentration of elastase was 1.98 ng/mL. The intra-assay variation coefficient was 4.8% and inter-assay variation 5.6%. The minimal detectable concentration of sL-selectin was <0.3 ng/mL with an intra-assay variation of 3.7% and an inter-assay variation of 4.2%.

RNA Isolation

Total RNA was isolated from peripheral whole blood using RNeasy columns (Qiagen; Hilden, Germany), according to the protocol of the manufacturer. Total RNA was dissolved in 30 μL RNase-free water (Qiagen; Hilden, Germany) and stored at −20°C until further analysis.

MLPA

Multiplex Ligation-dependent Probe Amplification is an established technique used for the detection of chromosomal abnormalities but can also be applied to study inflammation-related gene-expression. MLPA data proved to correlate well with other established techniques such as quantitative RT-PCR and microarray.[25]

MLPA (kit P009; MRC-Holland, Amsterdam, The Netherlands) was performed with total RNA in a concentration of 40–60 ng RNA/μL. With this MLPA assay, 40 different genes can be determined simultaneously using 40 different probes. The MLPA procedure has been described by us before (Lok et al., 2006). In short, after isolation of RNA, single stranded cDNA was produced with an RT reaction in a high-speed thermal cycler with a heated lid (Biometra Uno II; Goettingen, Germany). After addition of 1.5 μL buffer (1.5 M KCl, 1 mM EDTA, 300 mM Tris–HCl, pH 8.5) and 1.5 μL probe mix (1–4 fmol of each short probe oligonucleotide and each long probe oligonucleotide in

Tris EDTA), hybridization was allowed for 16 hr. The procedure was continued with the addition of a ligase enzyme enabling ligation of different probes. Then amplification of the ligated probes was performed with 32 cycles of a polymerase chain reaction. The PCR-product was stained with ROX-500 (MRC-Holland, Amsterdam, the Netherlands). Samples were purified with Sephadex G-50 (Sigma-Alderich Chemie, Zwijndrecht, The Netherlands) in filter plates (mahvn4550; Millipore; Billerica, USA) and analyzed by capillary electrophoresis on a capillary sequencer (ABI 3100, Applied Biosystems, Warrington, UK). The intensity and size of the different probes were calculated with Genescan and Genotyper software packages (Applied Biosystems). β-2-microglobulin (B2M) was chosen as a reference (household) gene.

Reagents and Assays

Antibodies and dilution

Fluorescein isothiocyanate (FITC)-labeled IgG_1 and phycoerythrin (PE)-labeled IgG_1 and monoclonal antibodies directed against cytotoxic T-cells (anti-CD8-PE), monocytes (anti-CD14-PE) and B-cells (anti-CD20-FITC) were obtained from Becton Dickinson. To detect T_{helper}-cells and granulocytes, anti-CD4-FITC, and anti-CD66e-FITC were purchased from the Central Laboratory of Blood Transfusion (Amsterdam, The Netherlands). Anti-CD61-FITC (anti-GP-IIIa) was obtained from Pharmingen (San Jose, CA, USA) to detect platelets. Allophycocyanin (APC)-conjugated annexin V was purchased from Caltag (Burlingame, CA, USA). 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-CD66e-FITC (1:10), and annexin V-APC (1:40).

Leukocyte counts

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 min at 18,890 g and 20°C to pellet the MP. After centrifugation, 225 μL of the supernatant was removed. The MP pellet and remaining supernatant was resuspended in 225 μL phosphate-buffered saline with citrate (154 mmol/L NaCl, 1.4 mmol/L phosphate, 10.9 mmol/L trisodium citrate, pH 7.4). After centrifugation for 30 min at 18,890 g and 20°C, 225 μL of the supernatant was removed again. The MP pellet was then resuspended after addition of 75 μL phosphate-buffered saline (PBS)-citrate.

Flow Cytometry

Five microliters 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 antibody. The samples were then incubated in the dark for 15 min 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 citrate-containing PBS was added). Samples were analyzed for 1 min in a fluorescence automated cell sorter (FACS Calibur, Becton Dickinson, San Jose, CA, USA) with CellQuest software (Becton Dickinson). Both forward scatter and sideward scatter were set at logarithmic gain. MP were identified on the basis of their size and density and on their capacity to bind annexin V. Annexin V measurements were corrected for auto-fluorescence. The subpopulations of leukocyte-derived MP were identified based on their capacity to bind the cell-specific monoclonal antibodies (CD4, CD8, CD14, CD20 and CD66e). 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: $\text{Number/L} = N \times (100/5) \times (955/57) \times (10^6/250)$, 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, from which approximately 57 (μL) is 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.

Statistical Analysis

Data were analyzed with Statistical Package of the Social Science software for Windows, release 16.0 (SPSS Benelux BV, Gorinchem, The Netherlands). The demographic characteristics of patients are presented as means with standard deviations. The three study groups are compared with ANOVA and Bonferroni *post-hoc* tests. These tests were also used for the MLPA data. Data of the ELISA and flow cytometry were not normally distributed and therefore analyzed with Kruskall–Wallis tests for differences among three groups and Mann–Whitney *U*-tests for differences between two groups. The presence (independent of the extent of expression) of mRNA expression of the different genes was compared with Chi-squared tests. Correlations were analyzed with a Pearsons bivariate two-sided test. Probability values of <0.05 were considered statistically significant.

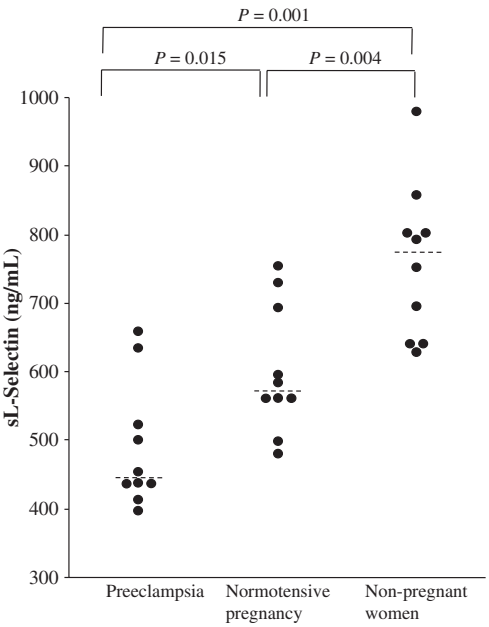

Fig. 2 Concentration of sL-selectin. The concentration of sL-selectin in preeclamptic patients, normotensive pregnant women and non-pregnant controls is presented. Every dot represents one patient. The short interrupted lines represent the median value of each group.

Results

General Markers of Leukocyte Activation in Pregnancy and Preeclampsia

Numbers of circulating leukocytes

As a general marker of inflammation, we determined the total number of leukocytes in maternal blood. Their number was elevated in normotensive pregnant women ($9.4*10^6$/L) compared with non-pregnant controls (6.0×10^6/L, $P = 0.026$). This number was even higher in the preeclamptic patients (12.8×10^6/L, $P = 0.004$ and $P = 0.08$ compared with non-pregnant controls and pregnant women, respectively). This is due to an increase in the number of (neutrophilic) granulocytes, which were significantly elevated in both preeclampsia and normotensive pregnancy compared with non-pregnant women (both $P = 0.001$). The absolute numbers of leukocyte subgroups did not differ between normotensive pregnant and preeclamptic women. The percentages of granulocytes, monocytes, and lymphocytes are shown in Fig. 1.

Levels of sL-selectin in maternal plasma

A widely accepted marker of leukocyte activation is sL-selectin. The median concentration of sL-selectin in cell-free citrated plasma of the preeclamptic patients was 449 ng/mL (range 397–659 ng/mL; Fig. 2). This concentration was significantly decreased compared with both normotensive preg-

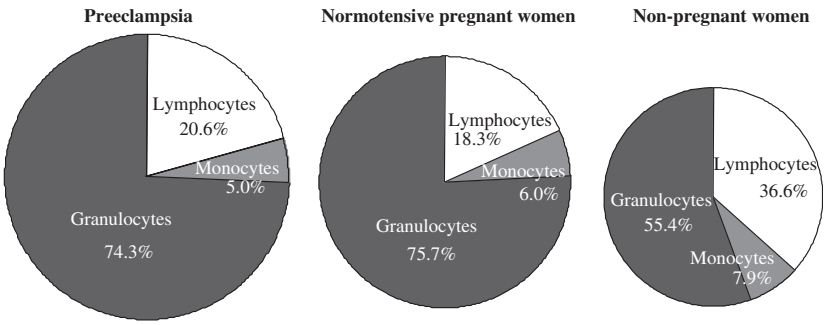

Fig. 1 Leukocyte counts. The percentages of different leukocyte subpopulations are shown for preeclamptic patients, normotensive pregnant women and non-pregnant controls.

Table II Overview of the Presence of Inflammatory Genes in Leukocytes

Genes	Preeclampsia	Normotensive pregnancy	Non-pregnant women
Reference gene Interleukins			
B2M	1	1	1
IL-1α	–	–	–
IL-1β	0.24	0.27[a]	0.16
IL-1RA	0.45[a]	0.40[a]	0.27
IL-2	–	–	–
IL-4(R1)	–	–	–
IL-4(R2)	–	–	–
IL-6	–	–	–
IL-8	0.19	0.14	0.21
IL-10	–	–	–
IL-12(p35)	–	–	–
IL-12(p40)	–	–	–
IL-13	–	–	–
IL-15(R1)	0.03	0.03	0.03
IL-15(R2)	–	–	–
IL-18	–	–	–
Transcription factors/oncogenes			
BMI	0.06	0.05[a]	0.08
MYC	0.30	0.22[a]	0.31
NFκB-1	0.29	0.29	0.26
NFκB-1A	0.45[a]	0.30	0.27
NFκB-2	0.07	0.08	0.08
Enzymes/Enzyme-Inhibitors			
CDKN-1A	0.09[a]	0.07	0.09
GST-P1	0.03[a]	0.03[a]	0.05
PARN	0.10	0.10[a]	0.14
PDE-4B	0.31	0.25	0.25
PTP-1B	0.07	0.07	0.08
PTP-4A	0.80	0.78	0.83
SERP-B9	0.24	0.20	0.25
Other cytokines			
IFNγ	–	–	–
MIF	0.18	0.15[a]	0.20
MCP-1	–	–	–
MCP-2	–	–	–
MIP-1A	0.03	0.02	0.03
MIP-1B	0.09	0.05[a]	0.09
PDGF-B	–	–	0.02
TF	–	–	–
THBS-1	0.06	0.07	0.08
TNF-α	0.03	0.03	0.04
TNF-R1	0.48[a]	0.51[a]	0.36
TNF-β	0.04	0.03	0.04

Table II (Continued)

All probes of the MLPA assay are listed in the second column. Values are relative peak areas compared with B2M. Some genes are not regularly detected (<50% of the samples). The results of the pre-eclamptic patients are in the third column, of the normotensive pregnant patients in the fourth column and of the non-pregnant controls in the fifth column.
IL, interleukin; BMI, B lymphoma; Mo-MLV, (murine leukemia viral oncogene homolog) insertion region; MYC, early-response (proto-oncogene) gene myc; NFκB, nuclear factor of kappa light chain gene enhancer in B cells; NFκB-IA, nuclear factor of kappa light chain gene enhancer in B cells inhibitor alpha (IκB); CDKN-1A, cyclin-dependent kinase inhibitor, GST, glutathione s-transferase; PARN, poly-A specific ribonuclease; PDE, phosphodiesterase; PTP, protein-tyrosine phosphatase, SERP, serine proteinase inhibitor B9; B2M, beta-2- microglobulin; IFN, interferon; MIF, (macrophage) migration inhibitory factor; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; TF, tissue factor; THBS, thrombospondin; TNF, tumor necrosis factor; and TNF-R, TNF-receptor. [a]Significant difference with pregnant or non-pregnant controls.

nant women (575 ng/mL, range 480–756 ng/mL), $P = 0.015$) and the non-pregnant controls (770 ng/mL, range 629–979 ng/mL, $P = 0.001$).

Leukocyte mRNA expression

Of the 40 inflammation-related genes studied by MLPA, 23 genes were expressed in total circulating leukocytes in >50% of the preeclamptic patients, normotensive pregnant women and non-pregnant controls (Table II). The overall expression pattern of these genes, however, did not differ between groups. In preeclampsia, two genes were up-regulated compared with normotensive pregnancy (Fig. 3): NFκB-1A ($P = 0.004$) and CDKN-1A (cyclin-dependent kinase inhibitor, $P = 0.02$). Three genes showed an elevated expression compared with non-pregnant controls: NFκB-1A ($P = 0.001$), IL-1RA, ($P = 0.003$), tumor necrosis factor (TNF)-R1 ($P = 0.004$), whereas the expression of glutathione s transferase-p1 (GST-P1) was decreased ($P = 0.002$). The expression of IL-1RA, TNF-R1, and GST-P1 did not differ between the patients with preeclampsia and normotensive pregnant controls ($P = 0.76$, p = 0.33 and $P = 0.76$ respectively).

Nine genes differed in the extent of mRNA expression between normotensive pregnant women and non-pregnant controls. Three genes were up-regulated: IL-1β ($P = 0.01$), IL-1RA ($P = 0.002$) and TNF-R1 (tumor necrosis factor-receptor 1, $P = 0.001$), and six genes were down-regulated: MYC [early-response (proto-oncogene) gene myc, $P = 0.008$], MIP-1B ($P = 0.005$), PARN (poly-A specific ribonuclease,

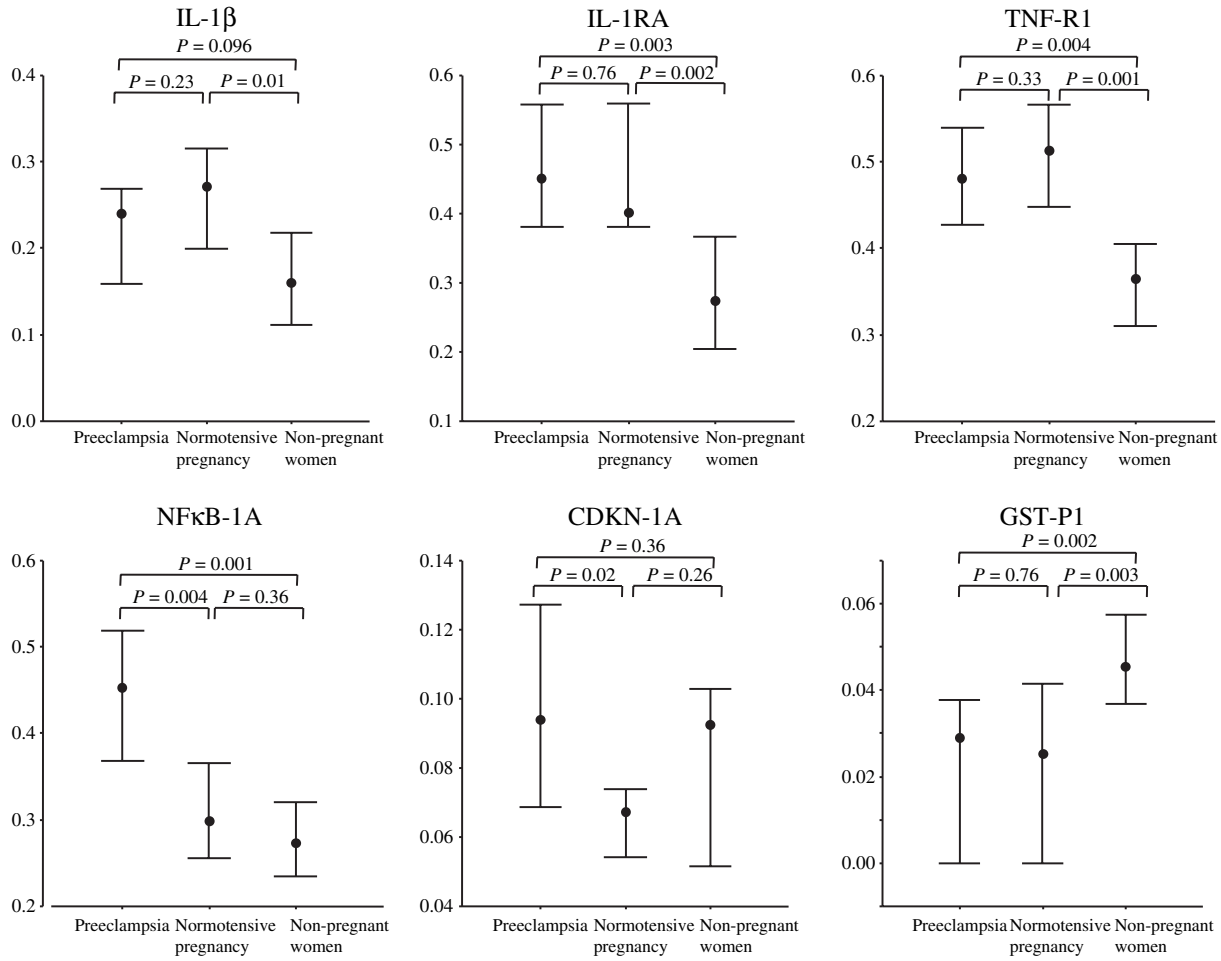

Fig. 3 mRNA expression of interleukin (IL)-1ß, IL-1RA, tumor necrosis factor-receptor 1(TNF-R1), nuclear factor of kappa light chain gene enhancer in B cells inhibitor alpha (NFκB-1A), cyclin-dependent kinase inhibitor (CDKN-1A), and glutathione s-transferase (GST)-P1. The relative mRNA expression compared with B2M expression of several inflammation-related genes differentially expressed in either pregnancy, preeclampsia or both compared with non-pregnant women.

$P = 0.005$), BMI-1 [B lymphoma Mo-MLV (murine leukemia viral oncogene homolog) insertion region 1, $P = 0.003$], MIF (macrophage migration inhibitory factor, $P = 0.001$), and GST-P1 (glutathione s-transferase-p1, $P = 0.003$).

Markers Reflecting Activation of Leukocyte Subsets in Pregnancy and Preeclampsia

Circulating MP

The activation status of leukocytes and subpopulations thereof was determined by measuring the numbers of circulating leukocyte-derived MP. Total numbers of MP and the number of platelet-derived MP (PMP) were decreased in preeclampsia compared with normotensive pregnant women ($P = 0.008$ and $P = 0.004$ respectively) and non-pregnant controls [$P = 0.001$ and $P = 0.001$ respectively, (data not shown)]. In contrast, the total numbers of leukocyte-derived MP were comparable between groups (Fig. 4a). The median total number of leukocyte-derived MP in preeclampsia was 281×10^6, which is 11.5% of the total number of MP (the percentages in pregnant and non-pregnant women were respectively 3.1% and 3.6%). The level of granulocyte-derived MP was elevated in normotensive pregnancy ($P = 0.022$) and preeclampsia ($P = 0.007$) compared with non-pregnant controls and were to be increased in preeclampsia compared with normotensive pregnant women, but this difference

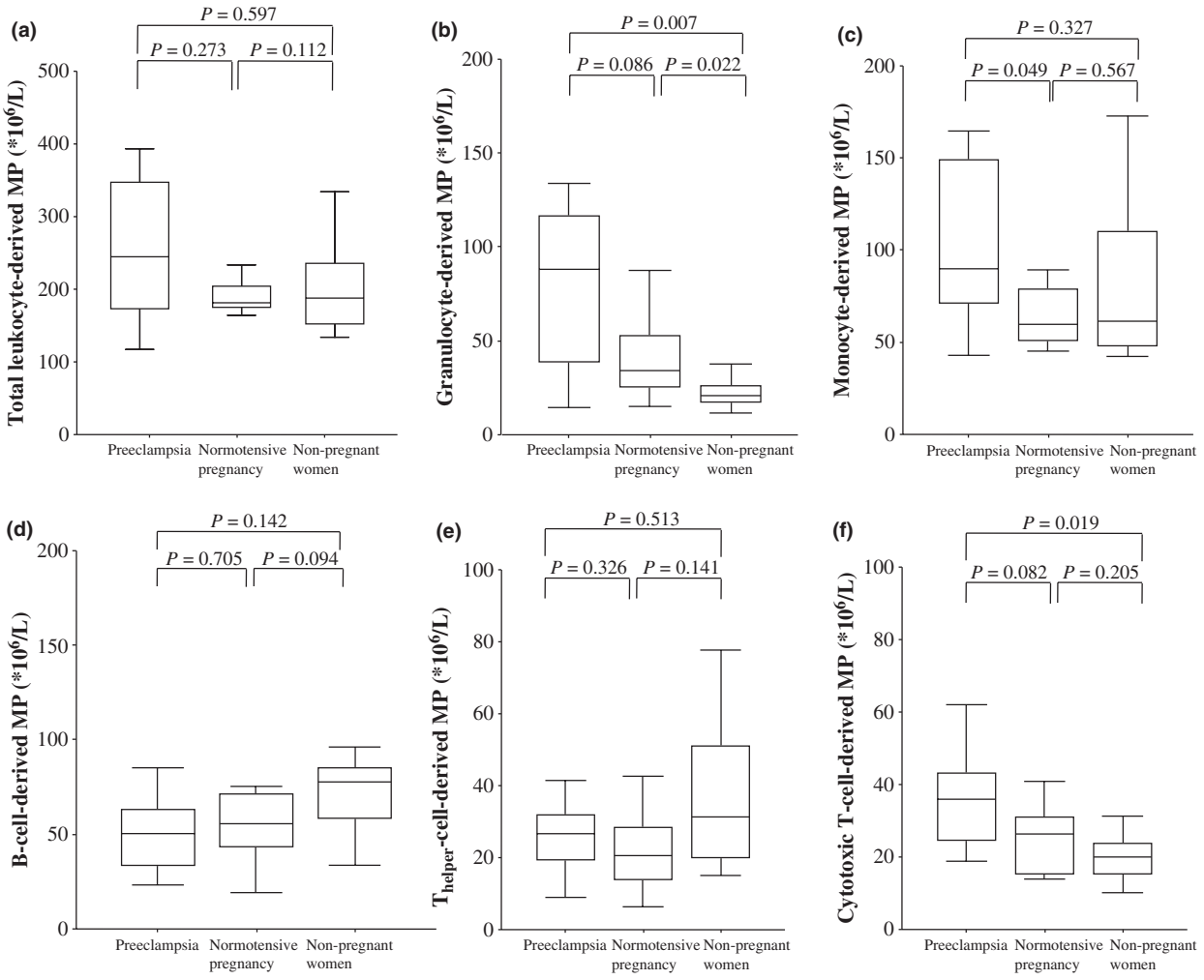

Fig. 4 Numbers of leukocyte-derived MP. This figure presents the total number of leukocyte-derived MP (a) and the subgroups of leukocyte-derived MP: Granulocyte-derived MP (b), Monocyte-derived MP (c), B-cell-derived MP(d), T_{helper}-cell-derived MP (e), and Cytotoxic T-cell-derived MP (f). Data are presented as medians with ranges. One outlier was excluded from the normotensive patients in these data because levels of MP were >2S.D. outside the normal range for MP concentrations.

did not reach statistical significance ($P = 0.086$, Fig. 4b). MP from monocytes were elevated in pre-eclamptic patients compared with normotensive pregnant women ($P = 0.049$), but not when compared with non-pregnant women (Fig. 4c). Representative examples of the dot plots of monocyte-derived MP and granulocyte-derived MP from a non-pregnant woman, a pregnant woman and a preeclamptic patient are shown in Fig. 5a and 5b. The number of B-cell-derived MP did not differ between groups (Fig. 4d) nor did the level of T_{helper}-cell-derived MP (Fig. 4e). The number of cytotoxic T-cell-derived MP was elevated in preeclampsia, but the difference was only significant compared with non-pregnant women ($P = 0.019$, Fig. 4f). There was no difference in the numbers of cytotoxic T-cell-derived MP between the pregnant and non-pregnant controls. Both types of T-cell-derived MP correlated with the number of lymphocytes in the maternal blood in preeclampsia (respectively $r = 0.678$, $P = 0.045$ and $r = 0.681$, $P = 0.044$). Strikingly, the percentages of leukocyte-derived MP differed substantially from the distribution of their parental cells. Based on the numbers of circulating subsets of MP (Fig. 6), especially lymphocytes and monocytes seem to be activated.

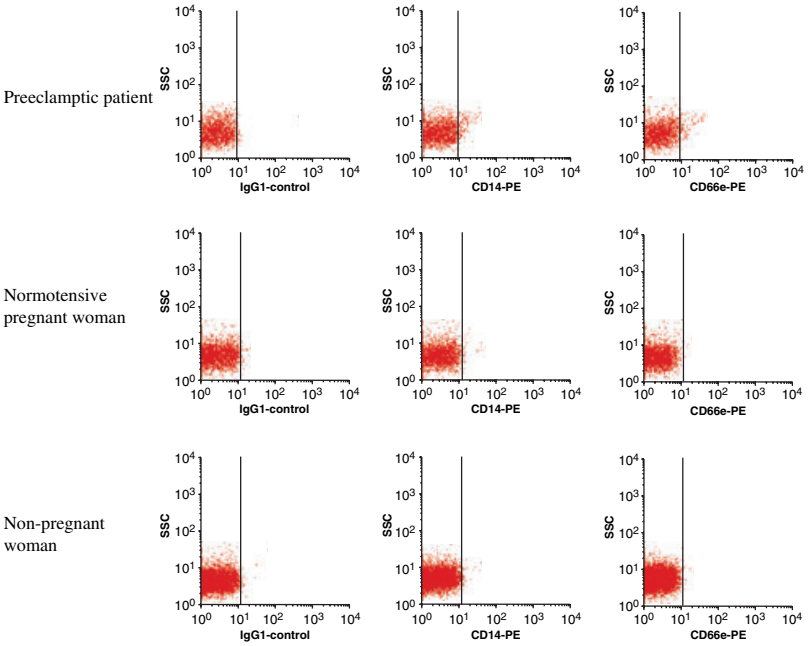

Fig. 5 Dot plots of monocyte-derived microparticles (MP) and granulocyte-derived MP from a non-pregnant woman, a pregnant woman and a preeclamptic patient. Representative dot plots of monocyte-derived MP and granulocyte-derived MP from each study group are provided. The y-axis represents the side-scatter of the MP and the x-axis represents the labeling with anti-CD14 (to identify monocyte-derived MP) and anti-CD66e (to identify granulocyte-derived MP). Furthermore, MP were identified on the basis of their size (forward scatter) and density (sideward scatter) and on their capacity to bind annexin V. Therefore, all dots in the upper right quadrant are monocyte-derived MP and granulocyte-derived MP respectively.

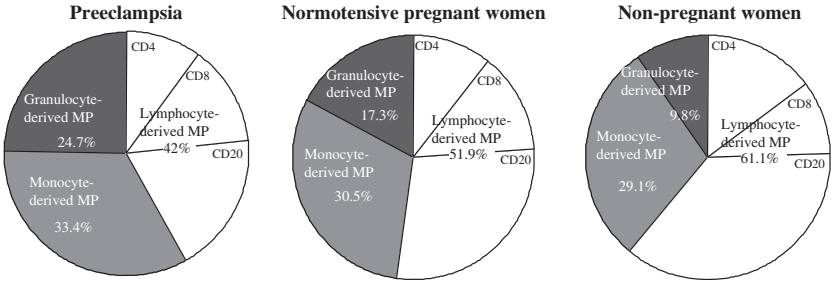

Fig. 6 Percentages of leukocyte-derived microparticles (MP). The fraction of the different leukocyte-derived MP is shown. In contrast to the parental cells (Fig. 1), granulocyte-derived MP compose the smallest fraction of the leukocyte-derived MP, followed by monocyte-derived MP. The largest fraction is composed of lymphocyte-derived MP.

Level of elastase in maternal plasma

To confirm activation of granulocytes in preeclamptic patients, we measured the plasma levels of elastase. The concentration of elastase was significantly elevated in the preeclamptic patients (median 59 ng/mL, range 29–96 ng/mL) compared with pregnant women (33 ng/mL, range 26–66 ng/mL, $P = 0.009$) and non-pregnant controls (28 ng/mL, range 13–40, $P = 0.001$, Fig. 7).

Discussion

In the present study, we investigated with different techniques the extent of activation of leukocytes and subgroups thereof in preeclampsia. An elevated number of leukocytes was present in preeclampsia compared with pregnant women and non-pregnant controls. Plasma levels of sL-selectin were decreased in pregnancy and even more decreased in preeclampsia, and several inflammation-related genes were differentially expressed in preeclamptic patients. Changes in numbers of circulating subsets of leukocyte-derived MP and an elevated concentration of elastase indicated activation of particular subgroups of leukocytes in preeclampsia.

Leukocyte activation in general was confirmed by altered concentrations of sL-selectin in preeclampsia and to a lesser extent in normotensive pregnancy. L-selectin is exposed on leukocytes and is proteolytically cleaved and shed from the cell surface during activation (sL-selectin). Therefore, the concentration of sL-selectin is increased in acute inflammatory processes[26], but may be decreased in chronic inflammatory processes.[27] Decreased concentrations of sL-selectin have also been reported in other vascular diseases including (un)stable angina and acute myo-

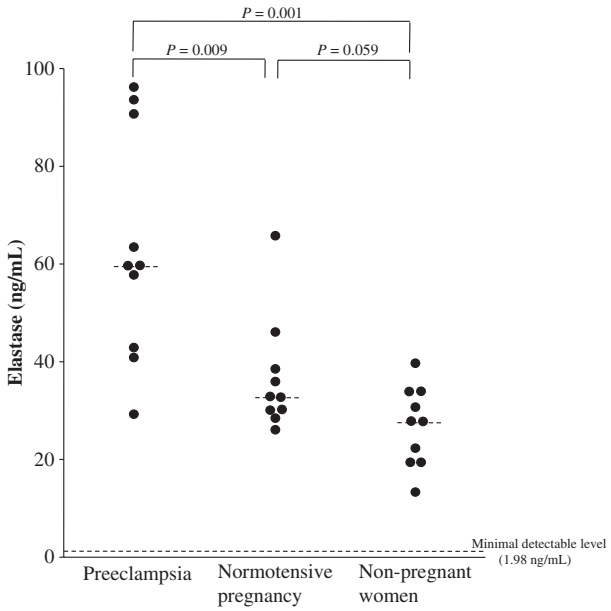

Fig. 7 Plasma concentrations of elastase. The concentrations of elastase in plasma of preeclamptic patients, normotensive pregnant women and non-pregnant controls are shown. Every dot represents one patient. The short interrupted lines represent the median value of each group. The minimal detectable level of elastase is 1.98 ng/mL.

cardial infarction.[27] Based on our present results and those of others[3,28], preeclampsia seems to be a disease characterized by a more chronic type of inflammation.

Data describing gene-expression in circulating leukocytes from the maternal blood to assess the activation status are limited. In the present study, an altered gene profile of inflammation-related and cell-cycle-related genes was found in normotensive pregnancy compared with non-pregnant controls. Nevertheless, no specific predominance of type 2 (e.g. IL-4, IL-5, IL-6 or IL-13) cytokines was present. Also, no down-regulation of type 1 (IL-2, IL-12, TNF) cytokines occurred. These results are in line with previous observations that lymphocytes and monocytes of preeclamptic women only produce cytokines after stimulation *in vitro*.[14] mRNA expression of the pro-inflammatory IL-1β gene in leukocytes was up-regulated in pregnancy, showing that the inflammation is also present in normotensive pregnancy, and that circulating leukocytes may contribute to this inflammation. In preeclampsia, mRNA expression of IL-1β was also elevated, but this increase was not significant compared with non-pregnant women. Moreover, there was also no difference between preeclamptic patients and normotensive pregnant women. An elevated plasma level of IL-1β in normal pregnancy but not in preeclampsia has been reported by others.[19] Endothelial cells are a target for IL-1β. Its effects are modulated by IL-1RA, which is produced by monocytes and macrophages. In the present study, IL-1RA was up-regulated in both pregnancy and preeclampsia compared with non-pregnant controls. Elevated plasma levels of IL-1RA in preeclampsia have been described before.[29] As IL-1RA is an inhibitor of IL-1ß[30], the up-regulation of this gene may be a protective response.

Although we found no differences in the expression of TNF-α between the groups, mRNA for TNF-R1 was up-regulated in pregnancy and preeclampsia. This may suggest that pregnant and preeclamptic women are both more sensitive to the effects of TNF-α. We detected up-regulation of the expression of NFκB-1A in leukocytes from preeclamptic patients. NFκB-1A (or IκB) is an inhibitor of NFκB. NFκB is a transcription factor, which plays an important role in immunomodulation by regulating the expression of a wide variety of genes. NFκB is present in the cytoplasm bound to NFκB-1A. After degradation of NFκB-1A, translocation of NFκB to the nucleus occurs, resulting in pro-inflammatory gene expression. Although up-regulation of NFκB-1A mRNA in pregnancy and preeclampsia seems to be contradictory with the inflammatory hypothesis, circulating leukocytes isolated from septic patients showed a similar decreased expression.[16] Furthermore, it remains uncertain to which extent changes in expression levels of the NFκB-1A gene triggers changes in protein expression, since McCracken et al. reported down-regulation of NFκB-1A protein during pregnancy and preeclampsia.[31]

Cyclin-dependent kinase inhibitor (CDKN)-1A plays a role in the cell cycle. The role of CDKN or its inhibitor in preeclampsia has not been investigated in humans yet. However, pregnant mice, which are heterogeneously deficient for $p57^{Kip}$, [2] i.e. a potent inhibitor of several cyclin/cyclin-dependent kinase complexes, display symptoms similar to preeclampsia.[32] Therefore, the clinical relevance of our finding remains unclear. Finally, the expression of GST-P1 was decreased compared with non-pregnant controls. GST plays a role in the removal of reactive oxygen species. As elevated concentrations of GST are present in plasma from preeclamptic patients, [33] it seems unlikely that GST in plasma from preeclamptic patients originates from circulating leukocytes.

The total number of MP was decreased in preeclampsia, probably because of a decreased platelet count.[34] We also studied the presence of subsets of leukocyte-derived MP to determine whether particular types of leukocytes become activated during preeclampsia and contribute to the inflammatory response.

Granulocyte-derived MP were elevated compared with the non-pregnant state and this has been reported previously.[35] These elevated numbers of granulocyte-derived MP may be because of elevated counts of granulocytes and granulocyte activation, as the number of granulocyte MP correlated with the plasma levels of elastase in the present study. In our longitudinal study, [13] relatively small numbers of granulocyte-derived MP (<0.1%) were detected. This apparent difference with the current data is difficult to explain, because experimental conditions were identical. An important difference, however, was the patient selection. Patients included in the longitudinal study suffered from more severe preeclampsia, as reflected by increased blood pressure and proteinuria. We hypothesized that the severity of preeclampsia may affect the complex interaction between (activated) leukocytes, their MP and the endothelium. There is some evidence to support this hypothesis, as adhesion of granulocytes to endothelial cells from preeclamptic pregnancies is increased compared with granulocyte adhesion to endothelial cells from control pregnancies.[36] Possibly, also the binding of MP from granulocytes to endothelial cells is dependent on the state of the disease. Granulocyte-derived MP are also elevated in patients suffering from multi-organ failure or sepsis compared with healthy controls, and their numbers strongly correlated with plasma levels of elastase.[37] Similarly, we confirmed this correlation in our present study ($r = 0.415$, $P = 0.025$ for the total group). Elastase is a degranulation product of neutrophils, and elevated concentrations have been reported in preeclampsia.[7,8] Elevated levels of monocyte-derived MP were present in preeclampsia compared with normotensive pregnancy thus reflecting activation of monocytes.

T_{helper}-cells and cytotoxic T-cells can be divided into type 1 and type 2 lymphocytes based on their cytokine production profile. Regulatory T cells are important regulators of tolerance induction during pregnancy. The number of regulatory T cells alters during the normal course of pregnancy and even more so in women affected by preeclampsia.[11] Recently, a new subset of CD4-exposing T-cells, Th17 cells, was discovered, which produces IL-17.[38] The role of this subset of T-cells in preeclampsia remains unknown. The balance between the type 1 and type 2 T-cells is of importance in pregnancy and preeclampsia. Preeclampsia is characterized by a predominance of $T_{helper}1$-cell immunity resulting in a more pro-inflammatory cytokine profile.[11,12] In our study, cytotoxic T-cell-derived MP were elevated in preeclampsia compared with the non-pregnant state. This may be the result of a increased number of activated $CD8^+$ cells, i.e. $CD8^+(CD25^+)$ T-cells.[39] The number of CD4-exposing MP did not differ between the groups. By measuring CD4 exposing MP, we measure a composition of MP derived from different CD4-exposing cells such as regulatory T-cells ($CD4^+CD25^{bright}$), naïve T-cells ($CD4^+CD45RA^+$) and T memory cells ($CD4^+CD45RO^+$). It has been shown that the concentration of these cells in preeclampsia differs from the concentration in normal pregnant women; in the study of Darmochwal et al., [39] there was a decrease in $CD4^+CD25^{bright}$ cells and $CD4^+CD45RA^+$ cells and an increase in $CD4^+CD45RO^+$ cells. The net result may had no difference in the number of MP in preeclampsia versus normal pregnancy.

Whether the different subpopulations of leukocyte-derived MP have a role in the development of preeclampsia or merely reflect cell activation remains to be elucidated. Effects of these MP in *in vitro* studies have been reported previously. T-cell-derived MP impair relaxation of mouse aortic rings *in vitro*[40] and leukocyte-derived MP induce the production of the pro-inflammatory cytokines IL-6 and monocyte chemotactic protein (MCP)-1 by endothelial cells.[41,42] On the other hand, we found no direct effect of MP from preeclamptic patients on RNA expression of inflammation-related genes by endothelial cells *in vitro*.[43]

Activation of leukocytes leads to the production and exposure of adhesion molecules. As a consequence, activated cells may quickly bind to other cells within the blood or to the endothelium. To which extent this also applies to MP from such cells, is unknown. Therefore, it cannot be excluded that circulating cells and their MP do not necessarily represent the entire *in vivo* situation.

BMI was significantly elevated in preeclamptic patients. A high BMI is a well-known risk factor for the development of preeclampsia. It may also influence the number of MP.[44,45] However, an effect of obesity on the number of MP has been shown for patients with a mean BMI of >30–40 kg/m^2, which

is higher than the BMI in our preeclamptic patients. An effect on leukocyte-derived MP specifically has never been shown. We did not find a correlation between BMI and the total numbers of MP in pre-eclampsia in our data. Therefore, the impact of difference in BMI between our study groups on the number of MP appears not to be significant.

In conclusion, our findings confirm the occurrence of leukocyte activation in general and leukocyte subgroups in particular in preeclampsia and to a lesser extent in normotensive pregnancy. Changes in leukocyte-derived MP coincide with altered levels of sL-selectin and elastase; however, there is no clear pro-inflammatory mRNA expression profile, although differences in mRNA expression levels of NFkB-1A, CDKN-1A, IL-1RA, TNF-R1 and GST were present in preeclampsia. To what extent activated leukocytes are rapidly removed from the blood, thereby triggering leukocyte production within the bone marrow on one hand and preventing the remaining cells from showing a full-blown pro-inflammatory phenotype on the other hand, remains open for discussion.

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References

1 Morris RK, Cnossen JS, Khan KS, Robson SC, Kleijnen J, Ter Riet G, Mol BW, Van der Post JAM: Serum screening with Down's syndrome markers to predict pre-eclampsia and fetal growth restriction: systematic review and meta-analysis. *HTA* 2008 (In press; http://www.hta.ac.uk/***).

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

3 Sacks GP, Studena K, Sargent K, Redman CW: Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol* 1998; 179:80–86.

4 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.

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

6 Tsukimori K, Nakano H, Wake N: Difference in neutrophil superoxide generation during pregnancy between preeclampsia and essential hypertension. *Hypertension* 2007; 49:1436–1441.

7 Belo L, Santos-Silva A, Caslake M, Cooney J, Pereira-Leite L, Quintanilha A, Rebelo I: Neutrophil activation and C-reactive protein concentration in preeclampsia. *Hypertens Pregnancy* 2003; 22:129–141.

8 Gupta AK, Gebhardt S, Hillermann R, Holzgreve W, Hahn S: Analysis of plasma elastase levels in early and late onset preeclampsia. *Arch Gynecol Obstet* 2006; 273:239–242.

9 Lurie S, Frenkel E, Tuvbin Y: Comparison of the differential distribution of leukocytes in preeclampsia versus uncomplicated pregnancy. *Gynecol Obstet Invest* 1998; 45:229–231.

10 Matsuo K, Ushioda N, Harman CR, Kimura T: Increased leukocyte distribution in the pre-clinical stage of pre-eclampsia. *Int J Gynaecol Obstet* 2007; 96:31–32.

11 Saito S, Shiozaki A, Nakashima A, Sakai M, Sasaki Y: The role of the immune system in preeclampsia. *Mol Aspects Med* 2007; 28:192–209.

12 Sargent IL, Borzychowski AM, Redman WG: NK cells and human pregnancy – an inflammatory view. *Trends Immunol* 2006; 27:399–404.

13 Lok CAR, Van der Post JAM, Sargent IL, Hau CM, Sturk A, Boer K, Nieuwland R: Changes in microparticle numbers and cellular origin during pregnancy and preeclampsia. *Hypertens Pregnancy* 2008; 27:344–360.

14 Veenstra van Nieuwenhoven AL, Bouman A, Moes H, Heineman MJ, de Leij LF, Santema J, Faas MM: Endotoxin-induced cytokine production of monocytes of third-trimester pregnant women compared with women in the follicular phase of the menstrual cycle. *Am J Obstet Gynecol* 2003; 188:1073–1077.

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

16 Spek CA, Verbon A, Aberson H, Pribble JP, McElgunn CJ, Turner T, Axtelle T, Schouten J, Van Der Poll T, Reitsma PH: Treatment with an anti-CD14 monoclonal antibody delays and inhibits lipopolysaccharide-induced gene expression in humans in vivo. *J Clin Immunol* 2003; 23:132–140.

17 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.

18 Koçyigit Y, Atamer Y, Atamer A, Tuzcu A, Akkus Z: Changes in serum levels of leptin, cytokines and lipoprotein in pre-eclamptic and normotensive pregnant women. *Gynecol Endocrinol* 2004; 19:267–273.
19 Conrad KP, Miles TM, Benyo DF: Circulating levels of immunoreactive cytokines in women with preeclampsia. *Am J Reprod Immunol* 1998; 40:102–111.
20 Jonsson Y, Rubèr M, Matthiesen L, Berg G, Nieminen K, Sharma S, Ernerudh J, Ekerfelt C: Cytokine mapping of sera from women with preeclampsia and normal pregnancies. *J Reprod Immunol* 2006; 70:83–91.
21 Daniel Y, Kupferminc MJ, Baram A, Jaffa AJ, Fait G, Wolman I, Lessing JB: Plasma interleukin-12 is elevated in patients with preeclampsia. *Am J Reprod Immunol* 1998; 39:376–380.
22 Adams KM, Mandel LS, Guthrie KA, Atkinson MW: Interleukin-18 in the plasma of women with preeclampsia. *Am J Obstet Gynecol* 2003; 188:1234–1237.
23 Sakai M, Shiozaki A, Sasaki Y, Yoneda S, Saito S: The ratio of interleukin (IL)-18 to IL-12 secreted by peripheral blood mononuclear cells is increased in normal pregnant subjects and decreased in pre-eclamptic patients. *J Reprod Immunol* 2004; 61:133–143.
24 Kauma S, Takacs P, Scordalakes C, Walsh S, Green K, Peng T: Increased endothelial monocyte chemoattractant protein-1 and interleukin-8 in preeclampsia. *Obstet Gynecol* 2002; 100:706–714.
25 Eldering E, Spek CA, Aberson HL, Grummels A, Derks IA, de Vos AF, McElgunn CJ, Schouten JP: Expression profiling via novel multiplex assay allows rapid assessment of gene regulation in defined signalling pathways. *Nucleic Acids Res* 2003; 31:153.
26 Gearing AJ, Newman W: Circulating adhesion molecules in disease. *Immunol Today* 1993; 14:506–512.
27 Haught WH, Mansour M, Rothlein R, Kishimoto TK, Mainolfi EA, Hendricks JB, Hendricks C, Mehta JL: Alterations in circulating intercellular adhesion molecule-1 and L-selectin: further evidence for chronic inflammation in ischemic heart disease. *Am Heart J* 1996; 132:1–8.
28 Chaiworapongsa T, Romero R, Yoshimatsu J, Espinoza J, Kim YM, Park K, Kalache K, Edwin S, Bujold E, Gomez R: Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. *J Matern Fetal Neonatal Med* 2002; 12:19–27.
29 Greer IA, Lyall F, Perera T, Boswell F, Macara LM: Increased concentrations of cytokines interleukin-6 and interleukin-1 receptor antagonist in plasma of women with preeclampsia: a mechanism for endothelial dysfunction? *Obstet Gynecol* 1994; 84:937–940.
30 Arend WP, Welgus HG, Thompson RC, Eisenberg SP: Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *J Clin Invest* 1990; 85:1694–1697.
31 McCracken SA, Drury CL, Lee HS, Morris JM: Pregnancy is associated with suppression of the nuclear factor kappaB/IkappaB activation pathway in peripheral blood mononuclear cells. *J Reprod Immunol* 2003; 58:27–47.
32 Kanayama N, Takahashi K, Matsuura T, Sugimura M, Kobayashi T, Moniwa N, Tomita M, Nakayama K: Deficiency in p57Kip2 expression induces preeclampsia-like symptoms in mice. *Mol Hum Reprod* 2002; 8:1129–1135.
33 Kumtepe Y, Börekçi B, Aksoy H, Altinkaynak K, Ingeç M, Ozdiller O: Measurement of plasma glutathione S-transferase in hepatocellular damage in pre-eclampsia. *J Int Med Res* 2002; 30:483–487.
34 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.
35 VanWijk MJ, Nieuwland R, Boer K, van der Post JA, VanBavel E, Sturk A: Microparticle subpopulations are increased in preeclampsia: possible involvement in vascular dysfunction? *Am J Obstet Gynecol* 2002; 187:450–456.
36 Wang Y, Adair CD, Coe L, Weeks JW, Lewis DF, Alexander JS: Activation of endothelial cells in preeclampsia: increased neutrophil-endothelial adhesion correlates with up-regulation of adhesion molecule P-selectin in human umbilical vein endothelial cells isolated from preeclampsia. *J Soc Gynecol Investig* 1998; 5:237–243.
37 Joop K, Berckmans RJ, Nieuwland R, Berkhout J, Romijn FP, Hack CE, Sturk A: Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. *Thromb Haemost* 2001; 85:810–820.
38 Korn T, Oukka M, Kuchroo V, Bettelli E: Th17 cells: effector T cells with inflammatory properties. *Semin Immunol* 2007; 19:362–371.
39 Darmochwal-Kolarz D, Saito S, Rolinski J, Tabarkiewicz J, Kolarz B, Leszczynska-Gorzelak B, Oleszczuk J: Activated T lymphocytes in pre-eclampsia. *Am J Reprod Immunol* 2007; 58: 39–45.
40 Martin S, Tesse A, Hugel B, Martinez MC, Morel O, Freyssinet JM, Andriantsitohaina R: Shed membrane particles from T lymphocytes impair endothelial

function and regulate endothelial protein expression. *Circulation* 2004; 109:1653–1659.

41 Mesri M, Altieri DC: Endothelial cell activation by leukocyte microparticles. *J Immunol* 1998; 161:4382–4387.

42 Mesri M, Altieri DC: Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J Biol Chem* 1999; 274:23111–23118.

43 Lok CA, Böing AN, Reitsma PH, van der Post JA, van Bavel E, 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.

44 Goichot B, Grunebaum L, Desprez D, Vinzio S, Meyer L, Schlienger JL, Lessard M, Simon C: Circulating procoagulant microparticles in obesity. *Diabetes Metab* 2006; 32:82–85.

45 Esposito K, Ciotola M, Schisano B, Gualdiero R, Sardelli L, Misso L, Giannetti G, Giugliano D: Endothelial microparticles correlate with endothelial dysfunction in obese women. *J Clin Endocrinol Metab* 2006; 91:3676–3679.