Platelet-derived microparticles and coagulation activation in breast cancer patients

Bettina Toth1, Susanne Liebhardt1, Kerstin Steinig1, Nina Ditsch1, Andreas Rank2, Ingo Bauerfeind1, Michael Spannagl3, Klaus Fries1, Armin J. Reininger1

1Department of Obstetrics and Gynecology – Großhadern, Ludwig-Maximilians-University, Munich, Germany; 2Department of Internal Medicine III – Großhadern, Ludwig-Maximilians-University, Munich, Germany; 3Department of Transfusion Medicine and Haemostaseology, Clinic of Anaesthesiology, Ludwig-Maximilians-University, Munich, Germany

Summary
In the mid 1800s Trousseau observed cancer-associated thrombosis, of which the underlying pathogenesis still remains unknown. We performed a prospective study on platelet-derived microparticles (PMP) and their procoagulant potential in breast cancer patients. Fifty-eight breast cancer patients and 13 women with benign breast tumors were included in the study. Microparticles (MP) were examined by electron microscopy and FACS analysis using labels for annexin V (total numbers), CD61 (PMP), CD62P and CD63 (activated platelets), CD62E (endothelial cells), CD45 (leukocytes) as well as CD142 (tissue factor). Prothrombin fragment 1+2 (F1+2) and thrombin generation were measured as blood coagulation markers. Numbers of annexin V+-MP were highest in breast cancer patients with larger tumor size (T2; median = 5,637 x 10^6/l; range = 2,852–8,613) and patients with distant metastases (M1; median = 6,102 x 10^6/l; range = 3,350–7,445), and differed significantly from patients with in-situ tumor (Tis; median = 3,220 x 10^6/l; range = 2,277–4,124; p = 0.019), small tumor size (T1; median = 3,281 x 10^6/l; range = 2,356–4,861; p = 0.043) and women with benign breast tumor (median = 4,108 x 10^6/l; range = 2,530–4,874; p = 0.040). A total of 82.3% of MP were from platelets, 14.6% from endothelial cells and 0.3% from leukocytes. Less than 10% of PMP showed degranulation markers. Larger tumor size (T2) and metastases correlated with high counts of MP and with highest F1+2 levels. Since prothrombin levels and thrombin generation did not parallel MP levels, we speculate that MP act in the microenvironment of tumor tissue and may thus not be an exclusive parameter reflecting in-vivo procoagulant activity.

Keywords
Platelet-derived microparticles, prothrombin fragment 1+2, thrombin generation, electron microscopy, breast cancer

Introduction
Cancer-associated thrombosis is the second leading cause of mortality in affected patients, besides the disease itself (1). Since up to 10% of patients with unknown venous thromboembolism (VTE) can have cancer, idiopathic VTE may be an indicator for occult malignancy (2). The pathogenesis of VTE in cancer is, however, not clearly understood. Several investigators found increased plasma levels of activation markers of platelets and blood coagulation (3–5). There is evidence that procoagulant proteins like tissue factor (TF), plasminogen activator inhibitor type 1 (PAI-1) or cyclooxygenase-2 (COX-2) can be expressed on tumor cells and can thus cause activation of haemostasis (6–8). Furthermore, there has been an association of TF found on cancer cells with hypercoagulability, tumor angiogenesis and progression (9).

At present there is a growing interest to evaluate new markers for coagulation and fibrinolysis, tumor angiogenesis and drug therapy monitoring in cancer patients. Recent investigations suggested a possible influence of circulating microparticles (MP) in...
various malignant diseases, e.g. gastric cancer (10), lung cancer (11), Trousseau’s syndrome (12), colorectal cancer (13) lymphomas (14, 15) and patients with mucinous adenocarcinomas (breast and pancreatic cancer) (16). Such MP can exhibit procoagulant properties, with platelet-derived MP (PMP), in particular, exposing high numbers of binding sites for coagulation factors, thus fostering the formation of tenase- and prothrombinase complexes (17, 18). In addition, PMP are by far the most common MP occurring in human blood and have been found to expose TF, the initiator of coagulation in vivo (18–22), with the possibility of TF transfer to other cells (23, 24). Alternatively, production and expression of TF on the surface of circulating blood cells can also occur after prior binding of MP. In this prospective case-control study, we examined circulating MP from platelet, endothelial cell and leukocyte origin and their procoagulant properties in breast cancer patients and patients with benign breast tumors.

Materials and methods

Study population

Breast cancer patients and patients with benign breast tumors were enrolled in the study if the following confounding factors were absent: hypertension, oral contraception, hormonal treatment, history of cancer or thrombosis, aspirin or low-molecular-weight heparin (LMWH) treatment, history of smoking. Patients with histologically proven breast cancer (n=58) were included before surgical treatment or neoadjuvant chemotherapy and were classified according to the tumor, lymph node, and metastasis (TNM) stage: in-situ tumor (Tis; n=8), tumor size < 2 cm (T1; [27]) and tumor size ≥ 2–5 cm (T2; [16]). With regard to nodal status, most breast cancer patients had negative axillary lymph nodes (n=30). In 14 patients 1–2 axillary nodes and in four patients more than two nodes were found positive. Distant metastases were detected in seven breast cancer patients (M1). In the control group patients with benign breast tumors (n=13) were classified according to the tumor, lymph node, and metastasis status, most breast cancer patients had negative axillary lymph nodes. Informed consent was obtained from all participants, allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study.

Blood sampling and measurements

Blood samples were drawn before surgery from an antecubital vein through a 20-gauge needle without tourniquet use and with sodium citrate (3.8%) anticoagulation. Haemoglobin (g/dl), leukocyte and platelet counts (G/l) were determined using an automated blood cell analyzer (Sysmex KX-21, Sysmex GmbH, Norderstedt, Germany). For MP analysis, platelet-poor plasma was prepared within 15 minutes (min) after blood collection by centrifugation at 1,550 g for 20 min. The plasma was then shock-frozen in liquid nitrogen for 15 min and stored at −80°C until assayed. Serum concentrations of prothrombin fragment 1+2 (F1+2) were measured using an automated immunoassay system (Roche Elecsys 2010, Roche Diagnostics; Mannheim, Germany).

Reagents

Fluorescein isothiocyanate (FITC)-labelled annexin V, phycoerythrin (PE)-labelled annexin V, and IgG-PE were obtained from Immuno Quality Products (Groningen, Netherlands). Anti-CD61-PE and anti-CD142 (tissue factor)-PE antibodies were purchased from BD Biosciences (Heidelberg, Germany), anti-P-selectin-PE (CD62P), anti-CD63-PE, anti-E-Selectin-PE (CD62E), anti-CD45-PE and IgG-FITC antibodies from Immuno notech (Marseille, France). All antibodies and annexin V were diluted with phosphate-buffered saline (PBS; 154 mM NaCl, 1.4 mM phosphate, pH 7.4). Final dilutions were: annexin V-FITC 1:100 (v/v), annexin V-PE 1:200, anti-CD61-PE 1:100, anti-P-selectin-PE 1:100, anti-CD63-PE 1:20, anti-CD142-IFITC 1:10, anti-E-Selectin-PE: 1:20 and anti-CD45-PE: 1:5.

Isolation and analysis of platelet-, endothelial cell- and leukocyte-derived microparticles

MP isolation and analysis were performed as described by Nieuwland et al. (18). A sample of 250 µl frozen plasma was thawed on melting ice for approximately 1 hour. After centrifugation of 250 µl plasma at 17,570 g and 20°C for 30 min, 225 µl of supernatant was discarded. The remaining MP pellet was diluted with 225 µl of phosphate-buffered saline (PBS) containing 10.9 mM trisodium citrate (PBS/citrate buffer). MP were again resuspended and centrifuged for 30 min at 17,570 g and 20°C. The resulting MP pellet was used either for flow cytometry or further analysis (Thrombin generation assay TGA, electron microscopy).

Flow cytometry

After removal of the supernatant (225 µl), 75 µl of PBS/citrate buffer was added to prevent prior activation of platelets and MP. Then the MP pellet was resuspended again. Five µl of the MP suspension was diluted in 35 µl CaCl$_2$ (2.5 mM)-containing PBS; the calcium was added to allow binding of APC-labelled annexin V: Both, annexin V (5 µl) and a cell-specific monoclonal antibody (5 µl) or isotype-matched control antibody (5 µl) were centrifuged for 5 min at 17,570 x g and 20°C to reduce possible debris and then diluted with the CaCl$_2$ (2.5 mM) containing PBS as stated above. Finally, samples were incubated in the dark for 15 min at room temperature. The reaction was stopped with 900 µl calcium buffer (2.5 mM) except for the annexin V control, to which 900 µl citrate-containing PBS was added. MP were analyzed in a FACSscan flow cytometer (Becton Dickinson; Heidelberg, Germany) using the Cell Quest Software (Becton Dickinson; San Jose, CA, USA). Forward scatter (FSC) and side scatter (SSC) were set at a logarithmic gain. Calibration microbeads as well as the distribution of red cells, leukocytes and platelets were used to set the gates for platelets as well as microparticles accordingly. MP were identified on the basis of their size (< 1 µm in diameter), density, and their capacity to bind a cell-specific monoclonal antibody as well as annexin V. We restricted further analysis to annexin V-positive MP, which accounted for approximately 90% of all MP detected (data not shown), and – since annexin V-positivity has been associated with procoagulant activity – the parameter we wanted to examine. Cell-specific labeling with monoclonal antibodies was...
corrected for identical concentrations of isotype-matched control antibodies and annexin V measurements were corrected for autofluorescence. The concentration of MP/l plasma was estimated according to Berckmans et al. (25). Assay variability of flow cytometry was 8%.

**Thrombin generation assay (TGA)**

Experiments were performed with an automated random access coagulation analyzer (ACL 9000, Instrumentation Laboratories, Germany). Optical signals were detected at a wave length of 405 nm. Coagulation was triggered using 25 mM CaCl₂ and recombinant tissue factor (1.44 ng/ml, PT reagent Recombiplastin, Instrumentation Laboratory, Kirchheim, Germany) dissolved in barbitone buffer (Stago, Asnières, France, pH 7.4). The PMP

Table 1: Demographic patient data as well as respective microparticle numbers. Parametrically distributed data are presented as mean ± standard deviation (minimum-maximum); levels of microparticles are presented as median (interquartile range). Circulating MP*: indicates significant differences between patients and women with benign breast tumor; ** indicates significant differences within the breast cancer patients group. p<0.05 = significant.
Electron microscopy
For scanning electron microscopy (SEM) the MP had to be immobilized on a glass cover slip. Thereto the cover slips were coated with 10 µg/ml of von Willebrand factor (VWF) and MP were allowed to sediment and adhere to the immobilized VWF for 1 hour. Attached MP were fixed for 15 min with 2.5% glutaraldehyde, which was then substituted with PBS (pH 7.4). dehydration was performed with a series of ethanol baths with increasing concentration up to 100%. The specimen were then dried with CO₂ above the critical point and sputter-coated with platinum. The MP were examined with a JSM-6300F scanning electron microscope (JEOL, Eching, Germany) at 5 kilovolt beam acceleration. PMP deposition on eight representative SEM images was quantified with the Matrox Inspector morphometry software (version 8.0; Matrox Electronic Systems Ltd., Dorval, Quebec, Canada) via blob analysis and compared between patients and controls, respectively. The coefficient of variance for measuring PMP adhesion to VWF was 13% ($n=15$). 51% of PMP present in platelet-poor plasma as counted with flow cytometry – could be detected bound to the VWF surface (calculated from 4 independent measurements).

Statistical analysis
Parametrically distributed data were expressed as mean (± standard deviation, SD). All other data were presented as the median (Q1-Q3 = interquartile range). Independent variables were analyzed by the Mann-Whitney-U test and Fisher’s exact test. Correlation coefficients (rho) were calculated by using Pearson and Spearman Rho Test. P-values < 0.05 were regarded as statistically significant. Data were examined with SPSS for Windows (release 15.0+16.0).

Results
Study population
There were no statistically significant differences between breast cancer patients and controls for mean age, haemoglobin levels, leucocyte counts, or platelet counts as is shown in Table 1.

Circulating MPs
Annexin V-positive MP mainly originated from platelets (CD61+): 82.3% in breast cancer patients and 79% in controls. MP from activated platelets as indicated by CD63– and CD62P-positivity were found in 6.2%/7.0% (CD63; cancer/control) and 2.4%/1.3% (CD62P), respectively. Whereas MP from activated endothelial cells and leucocyte-derived MP represented 14.6%/10.0% (CD62E+) and 0.3%/3.5% (CD45+) of all MPs, respectively.

The total numbers of circulating MPs (annexin V+) were highest in breast cancer patients with larger tumor size (T2) and distant metastases (M1). They differed significantly between patients with higher as compared to lower tumor stage (Table 1, Fig. 1). Additionally, a significant correlation between numbers of annexin V-positive MP and presence of distant metastases (M1) was observed (p = 0.001, rho = 0.43). By analyzing MP from platelet origin (CD61-exposure) we found the highest counts in breast cancer patients with distant metastases (M1) and also significant differences within the group of breast cancer patients (Table 1, Fig. 2). Parallel to annexin V-positive MP, CD61+ PMP numbers correlated significantly with presence of metastases (p <0.001, rho = 0.40).

Overall, 2.4% of MP (annexin V+) in breast cancer patients and 1.3% in controls exhibited P-selectin on their surface (CD62P+) indicating degranulation of platelet alpha-granula, the difference was statistically significant (p = 0.024). Furthermore, CD62P+ PMP were highest in patients with advanced
breast cancer (M1), and significant differences occurred between patients and women with benign breast tumor as well as within the group of breast cancer patients (all \( p < 0.05 \)) (Table 1). CD62P-positive PMP correlated significantly with presence of metastases \(( p < 0.001, \rho = 0.53 \)). Another platelet activation marker stemming from endosomes, CD63, was detected in 7.5\% of CD61-positive MP in breast cancer patients and 8.9\% in controls. Subpopulations of CD61+ PMP exposing CD63 were highest in patients with distant metastases (M1), without reaching statistical significance.

Only a small subset of annexin V-positive MP expressed TF at their surface: 3.4\% in breast cancer patients versus 2.5\% in controls. The highest numbers of tissue factor-positive MP (CD142+) were found in breast cancer patients with larger tumor size (T2) as well as in patients with distant metastases (M1), without reaching statistical significance (Table 1). Numbers of MP from activated endothelial cells (CD62E+) as well as leukocyte-derived (CD45+) MP were highest in patients with distant metastases and differed significantly between patients and controls as well as within the group of breast cancer patients (all \( p < 0.05 \)) (Table 1). Additionally, CD45+ MP correlated significantly with presence of peripheral metastases \(( p = 0.046, \rho = 0.286 \)), lymph node metastases \(( p = 0.001, \rho = 0.47 \)) as well as levels of CA15–3 \(( p = 0.01, \rho = 0.36 \)).

**Thrombin generation assay and prothrombin fragment 1+2**

Thrombin generation was highest in breast cancer patients with distant metastases (M1) without being statistical significant (Table 1). Prothrombin fragment (F1+2) was highest in patients with larger tumor size (T2) and lowest in patients with benign breast tumor. Significant differences in F1+2 levels were seen between patients with larger tumor size (T2) compared to patients with in situ tumor and women with benign breast tumors (both \( p < 0.04 \)). Prothrombin levels correlated significantly with numbers of CD63+, CD62P+, and CD45+ MP. Additionally, prothrombin levels also correlated with age and presence of lymph node metastases (all \( p < 0.04 \), all \( r < 0.32 \)). In patients with metastatic disease a highly positive correlation between prothrombin levels and CD142+ MP was present \(( p = 0.003, r = 0.997 \)).

**Electron microscopy**

The static sedimentation of MP onto a glass surface coated with VWF and subsequent examination with scanning electron microscopy allowed to assess the morphology, numbers and adhesion properties of the MP. In breast cancer patients 3.5-fold more MP were adherent to VWF than in controls \(( 163 \pm 10.3 \) MP vs. \( 47 \pm 6.5 \) MP per visual field, respectively; mean \( \pm \) SEM; Fig. 3A, B).

**Discussion**

Circulating MP were present in breast cancer patients and in patients with benign breast tumors and were mostly from platelet, and to a lesser degree also from endothelial and leukocyte origin. Less than 10\% of platelet-derived MP originated from activated platelets (CD63+ or CD62P+). Levels of MP from platelets that had undergone degranulation (CD62P+) differed significantly between breast cancer patients and controls and a positive correlation between CD62P+ PMP and prothrombin levels as well as presence of metastases was present. Systemic changes in coagulation activation measured by F1+2 was highest in patients with larger tumor size (T2), but not in breast cancer patients with distant metastases. Thrombin generation was highest in breast cancer patients with distant metastases without reaching significance.

Although an association between cancer and numbers of circulating MP is widely assumed, comparison of data may be difficult due to differences in patient selection. Of note, significantly increased levels of PMP in patients as compared to con-
Circulating microparticles (MPs) can be detected in vivo in various cancer diseases, were shown to be involved in tumor growth in vitro and are known to be procoagulant. However, their pathophysiologic relevance in tumor biology and progression is still unknown.

What does this paper add?

- MPs – mostly platelet-derived – were elevated in breast cancer patients as compared to patients with benign breast tumor and levels were associated with tumor invasiveness.
- Platelet-derived MPs did not parallel prothrombin levels and thrombin formation.
- Thus, MPs may rather reflect local tumor environment conditions than overall in-vivo procoagulant activity.

A recently published pilot study by some of the authors found that leukocyte-derived microparticles (LMP) as well as CEA and CA15–3 levels differed significantly between breast cancer patients and controls, whereas endothelial-derived MP and vWF levels did not. LMP showed an equal specificity-sensitivity profile to the established marker CA15–3 and therefore might have the potential to become a new biomarker in breast cancer patients (29).

Expression of TF on MP may not be an ubiquitous finding in cancer patients considering the discrepancy in findings with patients exhibiting colorectal cancer as compared to breast cancer. Hron et al. (13) found significantly higher numbers of TF-positive MP in patients with colorectal cancer patients as compared to healthy age-matched controls as well as a correlation of TF-positive MP with elevated D-dimer levels. In our group of breast cancer patients, increased levels of circulating TF-positive MP could not be detected, but we found significantly elevated prothrombin fragments F1+2 in the T2 subgroup, indicative of an activation of the clotting system as is connoted by the elevated D-dimer levels. Another explanation for activated coagulation may come from recent findings that TF concentrations as low as 3.6, 8.4, and 10.2 molecules/µm² are sufficient to cause fibrin polymerization on collagen perfused with blood at wall shear rates of 100, 500, and 1000 s⁻¹, respectively. Moreover, the addition of 100 nM TF to whole blood had negligible effect on clotting under static conditions, but caused a 2.5-fold increase in fibrin formation under flow (30). This study gives an estimate of the minute threshold concentrations of surface TF required to trigger coagulation under flow and suggests that already very few TF-positive MP could foster fibrin formation in a microenvironment.

The high levels of MP found in our study that expressed the activation marker phosphatidylserine on their outer membrane, was not paralleled by high values for platelet-degranulation markers. During platelet activation, intracellular granule membrane glycoproteins (GP) become exposed, including P-selectin (CD62P) and gp55 (CD63). Van der Zee et al. (31) suggested that measuring P-selectin- or CD63-exposing PMP may be a feasible and reliable method to assess the platelet activation status in vivo. They showed that both P-selectin- as well as CD63-exposing PMP were increased in patients with peripheral arterial disease and myocardial infarction. In our cancer patients both CD62P+ and CD63+ MP were below 10%. Although CD62P is expressed upon platelet activation by weak or mild agonists, while CD63 is expressed by stronger agonists, the CD63+ MP count was higher than CD62P+ PMP count. A possible explanation could be that P-selectin can dissociate from the platelet surface. Platelets exposing P-selectin are still capable of circulating for days and can subsequently lose their P-selectin (32). P-selectin might also be released from the MP surface. Blann et al. (33) investigated soluble P-selectin in patients with haematological cancers and breast cancers in comparison to controls and found elevated levels of soluble P-selectin in both cancer groups compared to controls. This may fit with the notion that P-selectin in cancer patient plasma exists mainly in its soluble form and that only a minor fraction is membrane-bound on PMP.

In summary, significant differences in levels of circulating PMP exist in breast cancer patients as compared to patients exhibiting benign breast tumors. These MP interfere with the haemostatic balance and may lead to procoagulant changes.
Acknowledgements

We gratefully acknowledge the following colleagues: Andrea Peichl, Marianne Fileki, and Marina Napoleone, respectively (from the Department of Obstetrics and Gynecology, and the Department of Transfusion Medicine – Großhadern, Ludwig-Maximilians-University), for expert technical assistance. Dr. Andreas Crispin (from the Department of Medical Informatics, Biometry and Epidemiology – Großhadern, Ludwig-Maximilians-University) for support in performing the statistical analyses. Dr. Axel Walch and Helga Wehnes (from the GSF – National Research Center for Environment and Health) for help with the SEM.

References