Circulating Microparticles in Breast Cancer Patients: A Comparative Analysis with Established Biomarkers*

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Abstract. *Background: The aim of the present prospective case-control study was to evaluate the putative relevance of circulating microparticles (MP) as a biomarker in breast cancer patients. Materials and Methods: Endothelial cell-(EMP) and leukocyte-derived MP (LMP) were determined by flow cytometry in breast cancer patients (n=41) and healthy controls (n=25) and compared to carcinoembryonic antigen (CEA), cancer antigen (CA)15-3 and von Willebrand factor antigen (vWF) levels by specificity-sensitivity profiles. Results: LMP, CEA and CA15-3 levels differed significantly between breast cancer patients and controls, whereas EMP and vWF did not. The specificity-sensitivity profiles of LMP and CA15-3 were similar. Conclusion: Increasing levels of circulating LMP(CD45+), CEA and CA15-3 correlated with increasing tumor size, thus reflecting disease stage. 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.*

About one million women worldwide are newly diagnosed with breast cancer per year (1). The diagnostic tools to identify women developing breast cancer are mammography, magnetic resonance imaging and core biopsy. So far, prognostic markers in breast cancer consist of histological and biological factors, requiring the examination of tumor tissue (2). For a long time, carcinoembryonic antigen (CEA) was the only circulating biomarker used in patients with breast cancer. Nowadays, cancer antigen (CA)15-3, a serum-based product of the mucin1 gene, is the most widely used biomarker for breast cancer (3). The main applications of CEA and CA15-3 include the surveillance of breast cancer patients as well as therapy monitoring in patients with advanced disease (4).

Recent investigations pointed out a possible clinical relevance of circulating cell-derived microparticles (MP) in different kinds of malignant diseases, including gastric cancer (5), lung cancer (6), Trousseau's syndrome (7), colorectal cancer (8), lymphomas (9, 10), and patients with mucinous adenocarcinomas (breast and pancreatic cancer) (11). However, it is unknown if circulating MP can be used as a tumor marker in different types of cancer.

In this prospective case-control study, the numbers of circulating leukocyte-derived MP (LMP) and endothelial cell-derived MP (EMP) were determined in breast cancer patients. The specificity-sensitivity profiles of circulating LMP and EMP were analyzed and compared to the CEA and CA15-3 profiles.

Recently, levels of von Willebrand Factor antigen (vWF), a marker of endothelial cell activation, has been shown to correlate with breast cancer progression (12). Therefore, the levels of the vWF antigen were also determined and their relation with circulating numbers of EMP was investigated.

Materials and Methods

Study population. Newly diagnosed patients with histologically proven breast cancer (n=41) were included before surgical treatment. Afterwards, the patients were classified according to the tumor, node, metastases (TNM) stages. The study group contained patients with tumor size <2 cm (T1; n=22) and tumor size 2-5 cm (T2; n=13). Most patients showed negative axillary nodes (26), and a few patients presented with 1-2 (10), 3-5 (3) or more positive nodes (2). Distant metastases were detected in six breast cancer

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patients. The healthy controls (n=25) had mammograms without pathological findings within the last 12 months, did not smoke and were not taking oral contraceptives or hormonal treatment. All the participants were Caucasians. Signed informed consent was obtained from all the participants, allowing analysis of all the 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 taken by the same experienced medical student at the same time period (9 to 12 am) by puncture of the antecubital vein without tourniquet through a 20-gauge needle.

For MP analysis, platelet-poor plasma was prepared by centrifugation at 1550xg for 20 minutes within 15 minutes after collection. The plasma was then shock-frozen in liquid nitrogen for 15 minutes and stored at –80˚C until assayed.

The CEA levels were quantified using a microparticle immuno-enzymometric assay (Abbott Laboratories, Chicago, IL, USA) on the AxSYM system.

The serum levels of CA15-3 were determined by an electrochemiluminescent immunoenzymometric assay (Roche Diagnostics, Mannheim, Germany) by the Elecsys system. The serum concentrations of vWF antigen were investigated in randomly selected women of T1 (10), T2 (10) and M1 (6) as well as healthy controls (10) by using latex-amplified immuno-turbidimetry (Dade, Behring, Marburg, Germany). Additionally, hemoglobin (g/dl) and leukocyte counts (Giga (G)/L) were measured in the whole study population.

Reagents for microparticle analysis. Fluorescein isothiocyanate (FITC)-labelled annexin V, phycoerythrin (PE)-labelled annexin V, and IgG-PE were obtained from Immuno Quality Products (Groningen, The Netherlands). Anti-E-selectin-PE was purchased from BD Biosciences (Heidelberg, Germany), IgG-FITC from Immunotech (Marseille, France), and anti-cluster of differentiation (CD)144-FITC from Acris (Hiddenhausen, Germany). Anti-CD45-PE was obtained from Immunotech. All the antibodies and annexin V were diluted with phosphate-buffered saline (PBS; 154 mmol/l NaCl, 1.4 mmol/l phosphate, pH 7.4). Final dilutions were: annexin V-FITC 1:100 (v/v), annexin V-PE 1:200, anti-CD144-FITC 1:20, anti-E-selectin-PE 1:20 and anti-CD45-PE 1:5.

Isolation and identification of EMP and LMP. Isolation and analysis were performed as described by Nieuwland *et al.* (13). In brief, frozen plasma (250 μl) was slowly thawed on melting ice for approximately one hour. After centrifugation at 17,570xg and 20˚C for 30 minutes, 225 μl of MP-free supernatant was taken off.

The remaining MP pellet was diluted with 225 μl of PBS containing 10.9 mmol/L trisodium citrate (PBS/citrate buffer), resuspended and centrifuged again for 30 minutes at 17,570 xg and 20˚C. Afterwards the supernatant (225 μl) was removed, 75 μl of PBS/citrate buffer was added, and the MP pellet was resuspended again. Five μl of the MP suspension was diluted in 35 μl $CaCl_2$ (2.5 mmol/L)-containing PBS. For MP staining, 5 μl Allophycocyanin (APC)-labelled annexin V was added plus 5 μl of a cell-specific monoclonal antibody or isotype-matched control antibodies. For detection of EMP two different antibodies (CD144, CD62E) that are both directed against antigens only expressed on endothelial cells were used. CD144 is directed against vascular endothelial (VE)-cadherin, which is believed to be constitutively exposed on most types of endothelial cells, CD62E (E-selectin) is present only on the surface of activated endothelial cells. The samples were incubated in the dark for 15 minutes at room temperature. The reaction in all samples was stopped with 900 μl calcium buffer (2.5 mmol/L), except for the annexin V control, to which citrate-containing PBS (900 μl) was added.

The MP were analyzed in a FACScan flow cytometer (Becton Dickinson; Heidelberg, Germany) using Cell Quest Software (Becton Dickinson; San Jose, CA, USA). Forward scatter (FSC) and side scatter (SSC) were set at a logarithmic gain. The MP were identified on the basis of their size and density and their capacity to bind a cell-specific monoclonal antibody and annexin V.

Cell-specific labelling 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.* (14).

Statistics. The parametrically distributed data were expressed as mean±standard deviation (SD). All the other data were presented as the median ((Q1-Q3), interquartile range). The independent variables were analyzed by the Mann-Whitney *U*-Test and all the others by using the Chi-Square and Fisher's exact test. *P*-values <0.05 were regarded as statistically significant. The data were examined with SPSS (SPSS, Chicago, Illinois, USA) for Windows (release 15.0).

Results

Study population. The mean age of the breast cancer patients was 59.8±12.7 (34-85) years and did not differ significantly from the controls (57.4±8.7 (42-71) years). The hemoglobin (breast cancer: 13.7±1.1 g/dl (11.3-15.7); controls: 13.7±0.8 g/dl (11.7-14.8)) and leukocyte levels (breast cancer: 7.1±1.8 G/L (4.0-12.4); controls: 6.8±1.9 G/L (3.3-11.8)) also did not differ significantly.

CEA and CA15-3. The CEA levels were elevated in breast cancer patients compared to the controls ($p<0.001$; Table I). There were significant differences in the CEA levels within the study population: the median CEA values from the breast cancer patients with distant metastases (M1) were increased compared to the patients with T1 ($p=0.009$) or the controls ($p<0.001$).

Furthermore, the patients with T1 or T2 had higher CEA levels compared to the controls (T1: $p=0.001$; T2: $p<0.001$). Nine breast cancer patients and one control woman had CEA values above the 95% confidence interval (22% sensitivity, Figure 2).

With regard to CA15-3, differences were also seen between the breast cancer patients and the controls ($p=0.001$) (Table I).

The patients with advanced breast cancer (M1) had elevated CA15-3 levels compared to the patients with T1 ($p=0.042$) or the controls ($p=0.01$). The breast cancer

Table I. *CEA, CA15-3, EMP and LMP in the study population.*

	CEA	CA15-3	Annexin V	CD144	CD62E	CD45
T1	1,3	17,2	3123	1481	466	188
	(0-9)	(6,9-32,2)	(1532-5712)	(817-2581)	(130-1129)	(102-287)
	(0,90-1,78)	(12,90-23,45)	(2211-3937)	(998-1943)	(325-596)	(157-221)
	(0,42-9,02)	(7,22-31,62)	(1583-5588)	(824-2561)	(141-1078)	(105-284)
T2	2,0	16,8	4912	2181	646	249
	(1-56)	(8,3-212,0)	(1648-9665)	(508-3850)	(346-1192)	(48-368)
	(1,35-3,65)	(13,70-31,10)	(2648-8234)	(1697-3227)	(516-894)	(149-318)
	(0,80-56,10)	(8,30-212,00)	(1648-9665)	(508-3850)	(346-1192)	(48-368)
M1	2,95	57,8	6592	2497	944	257
	(1-70)	(7,7-117,0)	(2461-15970)	(1065-4575)	(240-1240)	(126-647)
	(1,90-21,58)	(16,10-91,80)	(3128-9576)	(1304-4329)	(263-1239)	(213-449)
	(1,30-70,40)	(7,70-70,40)	(2461-15970)	(1065-4575)	(240-1240)	(126-647)
Control	0,7	11,97	3411	1674	424	167
	(0-3)	(6,6-95,1)	(1343-6434)	(166-3193)	(192-1542)	(60-310)
	(0,34-1,02)	(9,10-17,67)	(2642-4465)	(391-2196)	(299-1066)	(120-231)
	(0,24-2,97)	(6,60-84,87)	(1503-6329)	(203-3126)	(197-1531)	(62-305)

Median value, (minimum-maximum), (interquartile range), (5%-95% confidence interval) of CEA, CA15-3, Annexin V-, CD144-, CD62E- and CD45-positive microparticles in the study population.

patients with T1 or T2 also had elevated CA15-3 levels compared to the controls (T1: $p=0.014$; T2: $p=0.012$). However, 10 breast cancer patients and one control woman had CA15-3 levels above the 95% confidence interval (24.4% sensitivity, Figure 2).

Annexin V-positive microparticles. A wide range in the numbers of circulating annexin V-binding MP in breast cancer patients was found, without reaching a significant difference between patients and controls (Table I, Figure 1a). The median value of the annexin V-positive MP differed significantly in patients with low tumor size (T1) compared to patients with distant metastases (M1) and patients with high tumor size (T2) ($p=0.017$; $p=0.02$). Altogether, 8 breast cancer patients and one control woman had annexin V-positive MP above 95% confidence interval (22% sensitivity; Figure 2).

Endothelial cell-derived microparticles (EMP). In the breast cancer patients and the controls, CD144-exposing EMP were present, without significant differences (Table I, Figure 1b). The patients with advanced breast cancer (M1) had higher median values of CD144-positive MP compared to the patients with T1 ($p=0.045$). Furthermore, the patients with T2 had elevated CD144-positive MP compared to T1 ($p=0.006$) or healthy controls ($p=0.018$).

Within the study population, only the patients with T2 had significantly higher numbers of CD62E-positive MP compared to the patients with T1 ($p=0.011$).

Elevated numbers of EMP(CD144+) (above 95% confidence interval), but not CD62E-positive EMP, were detected in 5 breast cancer patients compared to one control woman (14.6% sensitivity; Figure 2).

Von Willebrand Factor antigen (vWF). The levels of vWF did not differ between breast cancer patients and controls. However, the breast cancer patients with T2 or M1 had significantly higher vWF levels than the breast cancer patients with T1 ($p=0.019$, $p=0.001$ respectively) and the patients with distant metastases (M1) had higher levels compared to the controls ($p=0.005$). A positive correlation between vWF and CA15-3 levels was present in the breast cancer patients ($p=0.027$, rho=0.432), whereas vWF and numbers of EMP (CD144+, CD62E+) did not correlate.

Leukocyte-derived microparticles (LMP). The numbers of CD45-positive LMP were augmented by increasing tumor size and were highest in the women with advanced breast cancer (M1) (Table I, Figure 1c). Significant differences were present in the breast cancer patients compared to the controls ($p=0.02$). The patients with advanced breast cancer (M1) had higher median values of CD45-positive LMP compared to the patients with T1 ($p=0.028$) or the healthy controls ($p=0.031$). Also the patients with T2 had elevated numbers of LMP compared to the controls ($p=0.04$). No correlation between leukocyte numbers and levels of CD45-positive LMP was detected.

LMP numbers above 95% confidence interval were seen in 5 breast cancer patients compared to one control woman (12.2% sensitivity; Figure 2).

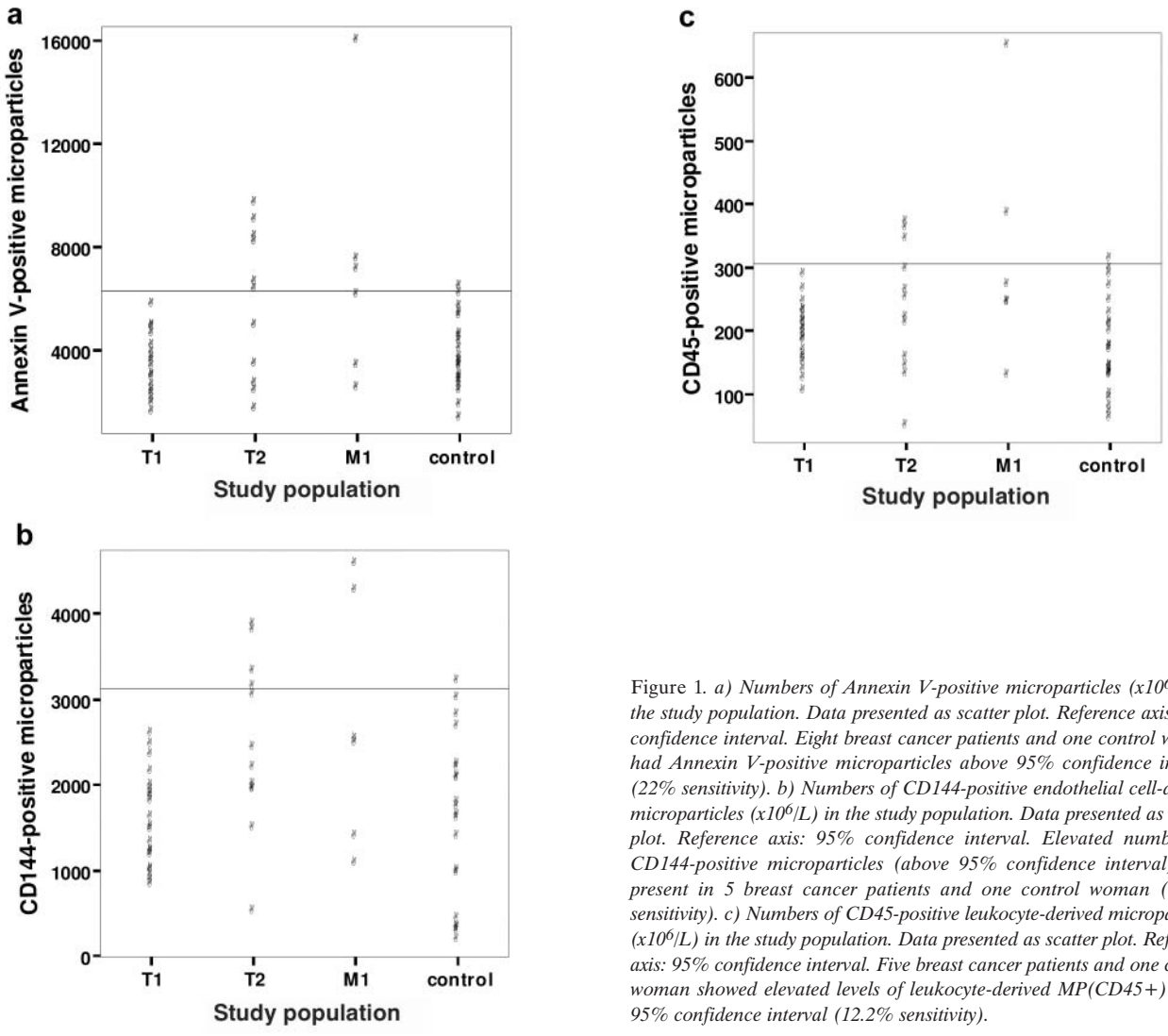


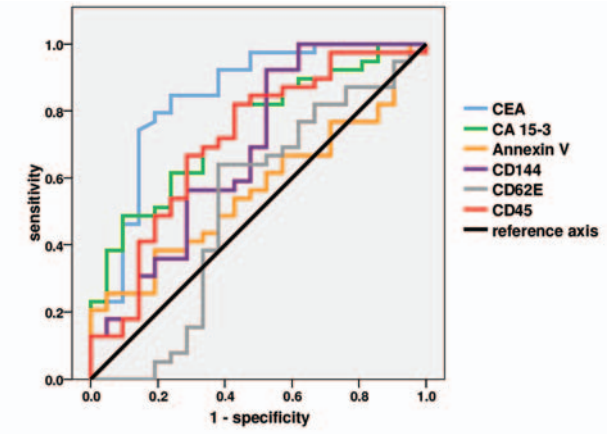
Figure 1. *a) Numbers of Annexin V-positive microparticles (x10^6/L) in the study population. Data presented as scatter plot. Reference axis: 95% confidence interval. Eight breast cancer patients and one control woman had Annexin V-positive microparticles above 95% confidence interval (22% sensitivity). b) Numbers of CD144-positive endothelial cell-derived microparticles (x10^6/L) in the study population. Data presented as scatter plot. Reference axis: 95% confidence interval. Elevated numbers of CD144-positive microparticles (above 95% confidence interval) were present in 5 breast cancer patients and one control woman (14.6% sensitivity). c) Numbers of CD45-positive leukocyte-derived microparticles (x10^6/L) in the study population. Data presented as scatter plot. Reference axis: 95% confidence interval. Five breast cancer patients and one control woman showed elevated levels of leukocyte-derived MP(CD45+) above 95% confidence interval (12.2% sensitivity).*

Discussion

Despite the relatively small size of the study population, our data showed considerable differences in circulating (subpopulations of) cell-derived MP in breast cancer patients. The elevated numbers of CD45-positive MP refelected the disease stage in the breast cancer patients and differed significantly from the healthy controls. The EMP as well as the vWF levels as markers of endothelial cell activation, however, did not differ between the breast cancer patients and controls.

At present, evidence is accumulating that cell-derived vesicles released into the environment by cancer cells, i.e. MP and exosomes, may contribute to cancer progression and tumor outgrowth. Cell-derived vesicles from cancer cells have been studied extensively *in vitro* and especially MP are best known for their ability to initiate coagulation by exposing "cancer procoagulant" or tissue factor, thereby contributing to fibrin formation and promoting tumor growth (15-17). In contrast, exosomes from tumor cells, also called tumor cell-derived exosomes or texosomes, suppress the immune response by exposure of Fas ligand, thus killing T-cells (18). In addition, tumor cell-derived MP and/or exosomes contain angiogenesis-promoting phospholipids, such as sphingomyelin, high concentrations of cytostatics which contribute to multi-drug resistance, and several matrix metalloproteases that degrade the environment (19-22).

So far, no data have been reported on circulating LMP and EMP in breast cancer patients.



Area under the curve

	Area	standard error	asymptotic significance	asymptotic 95% confidence interval	
				upper limit	lower limit
CEA	,846	,057	,000	,733	,958
CA15-3	,746	,064	,002	,620	,872
Annexin V	,565	,074	,407	,420	,711
CD144	,683	,077	,021	,531	,834
CD62E	,519	,087	,810	,348	,690
CD45	,712	,073	,007	,569	,855

Figure 2. *Specificity-sensitivity profiles (ROC curve) of CEA and CA15-3 as well as Annexin V-, CD144-, CD62E- and CD45-positive microparticles. CA15-3 and CD45-positive microparticles showed nearly equal specificity-sensitivity profiles reaching asymptotic significance.*

The elevated numbers of CD144-positive EMP in the patients with T2 and M1 are likely to be associated with endothelial activation *in vivo* and, in addition, may reflect procoagulant changes during tumor development.

Additional evidence of endothelial activation has come from several studies which indicated higher levels of circulating vWF antigen in breast cancer patients compared to women with benign tumors (12) or healthy controls (12, 23, 24). Rohsing *et al.* (12) demonstrated that increased levels of vWF correlated with tumor progression in breast cancer patients. Our present findings further support the relationship between breast cancer and endothelial cell activation.

Kanazawa *et al.* (6) reported elevated levels of soluble (non cell-associated) E-selectin in non-small cell lung cancer patients compared to healthy subjects, suggesting that endothelial cell activation may be a more general phenomenom in cancer patients. Furthermore, Kanazawa *et al.* also found elevated numbers of monocyte-derived MP in cancer patients. The present study also demonstrated that in breast cancer patients LMP are elevated. Their numbers were not related to leukocyte counts, indicating that LMP formation may be affected by cancer or cancer progression. In vitro, LMP transfer tissue factor to developing thrombi (25, 26) and are therefore believed to be involved in thrombus formation (27).

At present, the two most widely investigated clinical tumor markers in breast cancer patients are CEA and CA15-3. In particular CA15-3 is used to detect breast cancer recurrences, evaluate therapeutic response of advanced disease and prognosis. CA15-3 is a highly sensitive marker for distant metastases, especially in bone and liver, but it is not sensitive for locoregional or contralateral breast cancer.

Focusing on the specificity-sensitivity profiles of the established biomarkers CEA and CA15-3 in comparison to circulating MP, LMP(CD45+) in particular might have the potential to become a new biomarker in breast cancer patients.

Further investigations of the numbers of circulating (subpopulations of) MP in breast cancer patients might focus on preoperative evaluation of patients with different disease states, postoperative determination of MP numbers to establish individual baseline values and follow-up of patients to evaluate their prognostic relevance.

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