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Fibrinolytic Activity of Circulating Microvesicles Is Associated with Progression of Breast Cancer

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The fibrinolytic system plays an important role in breast cancer, favoring progression through extracellular-matrix degradation, angiogenesis, apoptosis and cellular proliferation. The expression of urokinase-type plasminogen activator (uPA) in breast cancer tissue is widely recognized as an unfavorable prognostic factor. However, fibrinolytic activity associated with uPA cannot be reliably measured in the blood because of the rapid inhibition of uPA by plasminogen activator inhibitor-1 (PAI-1). By contrast, circulating microvesicles (Mvs) in peripheral blood protect bound enzymes from inhibition. Mvs are extracellular vesicles, released from various types of cells, and their size fluctuates between 100 and 1,000 nm. Mvs carry DNA, RNA, miRNA, and proteins, thereby serving as a source of horizontal communication between cells. We investigated whether fibrinolytic activity on circulating Mvs reflects breast cancer progression. The study population consisted of 13 patients with breast cancer and 13 healthy women. The cancer patients included 4 patients in remission, 3 patients with locally advanced cancer, and 6 with metastatic disease. Mvs were isolated from peripheral blood, quantified by a protein concentration method, and their fibrinolytic potential was measured by their capacity to generate plasmin. Although the quantity of Mvs found in patients with cancer and healthy individuals was similar, plasmin generated on Mvs was twice the amount in patients with metastasis than in healthy women ($P < 0.05$), underlying the value of this distinctive parameter. The data suggest that in breast cancer patients, higher fibrinolytic activity of circulating Mvs could be related to progression and metastasis of breast cancer.

Keywords: breast cancer; cancer progression; fibrinolytic activity; microvesicles; urokinase-type plasminogen activator
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Introduction

The most frequently occurring malignancy among women is breast cancer (Forouzanfar et al. 2011). In Mexico, as in other countries, this disease is the main cause of mortality in women with cancer, and metastasis and tumor growth are the main factors that increase mortality rates (Kamangar et al. 2006). An emerging area of interest

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in the study of breast cancer is its direct interaction with the hemostatic system, because platelets, coagulation and fibrinolysis show cooperative effects that facilitate metastasis and tumor growth.

In the hemostatic system, the activation of platelets enables a concentration of coagulation factors on exposed phospholipids and the formation of thrombin. Thrombin stimulates (through proteinase-activated receptors PAR-1 and PAR-2) the expression of urokinase-type plasminogen activator (uPA) and its receptor (urokinase plasminogen activator surface receptor; uPAR) by native or cancer cells (Lal et al. 2013). The uPA bound to uPAR at the cell membrane transforms plasminogen into plasmin that degrades extracellular matrix (ECM) proteins and also activates matrix metalloproteinases (Andreasen et al. 1997), with a consequent increase in angiogenesis, cellular growth and mobility. This mechanism, known as pericellular proteolysis, participates not only in matrix remodeling (Martin et al. 1993) and inflammatory processes (Evans 1991; Crooks and Hart 2015) but also plays a key role in tumor progression (Sidenius and Blasi 2003).

Breast cancer cells expressing uPA are prone to *in situ* generation of plasmin that extends the tumor burden locally and compromises patients prognosis (Crooks and Hart 2015). Fibrinolysis promotes metastasis through pathways that include ECM degradation and metalloproteinase activation (Tang and Han 2013).

In addition to the effects of fibrinolysis, uPA activates a proteolytic pathway that induces malignant cells to invade vessels, reach the circulatory system and eventually settle and grow in distant organs (Fisher et al. 2001). Moreover, the uPA-uPAR complex has direct effects on angiogenesis, apoptosis suppression, cellular invasion and proliferation. These changes occur through the interaction of uPAR with integrins, G-protein receptors, tyrosine kinases and serine kinases, which all act as activators of intracellular signaling pathways (Blasi and Sidenius 2010).

The prognostic value of uPA has been demonstrated through measurement of protein levels *in situ*. Levels in serum and urine are positively correlated with the stage of the disease, which makes it possible to use uPA as a diagnostic biomarker (Soydinc et al. 2012). High tissue expression levels of uPA and plasminogen activator inhibitor-1 (PAI-1) are also prognostic of breast cancer and are associated with recurrence and with declines in patient survival (Grondahl-Hansen et al. 1993). Nevertheless, prognostic analysis has so far only been applicable to the direct antigenic measurement of these proteins, because locally generated active uPA and plasmin are immediately neutralized by their respective inhibitors (PAI-1 and alpha-2-antiplasmin). This neutralization means that measurement of the fibrinolytic activity in peripheral blood is challenging (Longstaff 2018). Recently, it has been described that membrane fragments released from different types of cells, known as microvesicles (Mvs), trigger fibrinolytic activity (Dejouvencel et al. 2010; Durrieu et al. 2018). Mvs are structures with a diameter of 100-1,000 nm that are formed from plasma membranes. These Mvs can be isolated and concentrated from plasma obtained from circulating blood by differential time/speed centrifugation. This method is intended to separate and concentrate mainly Mvs as opposed to exosomes, which originate from the membranes of intracellular organelles and have a diameter of 30-100 nm. All cells form Mvs, which mediate horizontal cellular communication by carrying proteins, RNA and DNA from the originating cells (Khalyfa et al. 2016).

During fibrinolysis, plasminogen binds to fibrin and its activator and is transformed to plasmin. An important characteristic is that all the proteins participants of the fibrinolytic mechanism are assembled on the fibrin surface. However, other alternative ways of fibrinolysis possibilities have been recently described; Mvs that transport tissue-type plasminogen activator (t-PA) or uPA can generate plasmin through plasminogen activation at their surface (Dejouvencel et al. 2010; Plawinski and Angles-Cano 2013). Fibrinolytic Mvs promote cellular migration, angiogenesis, ECM degradation and a decrease in cellular adhesiveness (Lacroix et al. 2007). Moreover, a cross-talk has been demonstrated between Mvs and platelets/ECM, so that Glu-plasminogen attached to carboxy-terminal Lys in platelet membranes or ECM components is acted by uPA on cell-derived Mvs to yield plasmin, bypassing the need for assembly of the proteins on the same surface (Dejouvencel et al. 2010). These observations suggest that it might be possible to measure fibrinolytic potential in patients with cancer by analysis of Mvs.

Materials and Methods

Study population

The study included 13 female patients with breast cancer and 13 healthy women. Among the 13 patients, four had achieved complete remission within the 6 months prior to enrollment, so they did not have active cancer, three had locally advanced cancer, that means that breast cancer has progressed locally but has not yet spread outside the breast and local lymph nodes (distant metastasis) and six had metastatic disease according to the current TNM staging for breast cancer (Edge and Compton 2010). Breast tumor in all 13 patients were characterized by immunohistochemistry (Table 1) depending on the expression of estrogen and progesterone receptors and the status of the HER-2 receptor. When the expression of the three receptors was undetectable on the tissue, breast cancer was classified as triple negative.

Participants were selected by convenience sampling. Individuals who had an infectious disease, kidney disease, ischemic heart disease, rheumatologic disease, other types of cancer, or thrombosis, or who were under anticoagulant or antifibrinolytic therapy were not included. For all participants, anthropometric measures and past or current relevant medical histories were recorded. In addition, upon enrollment, participants underwent basic laboratory studies,

Table 1. Immunohistochemistry profiles of patients with cancer.

Biomarker	Study group			Total
	Cancer in remission (n = 4)	Locally advanced cancer (n = 3)	Metastatic cancer (n = 6)	
E/P +	3	1	3	7
HER2/neu +	1	2	1	4
Triple negative	0	0	2	2
Total	4	3	6	13

Triple negative immunohistochemistry profile refers to a lack of expression of estrogen, progesterone and HER2/neu receptors in cancer tissue. This condition has a worse prognosis.
E/P, estrogens and/or progesterone; HER2/neu, human epidermal growth factor receptor 2 (CD340).

Table 2. Clinical and biochemical characteristics of patients.

Variable	Study group			
	Healthy women (n = 13)	Cancer in remission (n = 4)	Locally advanced cancer (n = 3)	Metastatic cancer (n = 6)
Age (years)	55.4 ± 4.0	61 ± 5.7	42.3 ± 4.2*	53 ± 7.5
Diabetes	0 (0%)	4 (100%)	3 (100%)	1 (16.7%)
Hypertension	0 (0%)	1 (25%)	3 (100%)	1 (16.7%)
Dyslipidemia	1 (7.7%)	1 (25%)	1 (33.3%)	1 (16.7%)
History of smoking	2 (15.1%)	4 (100%)*	2 (66.7%)	1 (16.7%)
Current smoker	3 (23%)	4 (100%)	3 (100%)	2 (33.3%)
Leucocytes ($\times 10^3/\mu$L)	6.32 ± 3.3	7.4 ± 3.0	6.1 ± 1.3	6.4 ± 2.1
Hemoglobin (g/L)	14.5 ± 1.1	14.6 ± 0.5	13.0 ± 1.4	13.7 ± 1.4
Platelets ($\times 10^3/\mu$L)	264.8 ± 55.1	232.7 ± 43.1	305.3 ± 88.3	212.4 ± 34.3
Creatinine (mg/dL)	0.7 ± 0.2	0.9 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
Glucose (mg/dL)	94.9 ± 9.3	103.3 ± 4.7	89.5 ± 12.0	101.7 ± 22.4

All variables are shown as the mean ± SD (standard deviation) or n (%), depending on the data type.
*$P < 0.05$ for a significant difference compared with the healthy control group in the Chi square test or ANOVA with Dunnett *post hoc* test.

including a complete blood count and a blood-chemistry panel (Table 2). In the group of patients with metastatic disease, two had bone metastasis, two had visceral metastasis (lung and liver) and two had both bone and visceral metastasis.

The nature of the chemotherapy received by patients with locally advanced or metastatic disease was recorded (Table 3). Patients who were receiving chemotherapy had blood sampling for Mvs testing prior to chemotherapy administration.

The American British Cowdray Medical Center's ethics committee approved the protocol, TABC-1.-31. Informed consent was obtained from each participant and the samples were obtained according the principles of Helsinki Declaration.

Proteins

Human plasminogen was isolated according to a published method (Deutsch and Mertz 1970), with modifications as described elsewhere (Fleury and Angles-Cano 1991), and was > 99% pure as assessed by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) and by amino-terminal sequence analysis. Urokinase was obtained from Microbix Biosystems (Mississauga, ON, Canada). Bovine serum albumin (BSA) was obtained from Sigma (St Louis, MO, USA), and aprotinin (Trasylol) was acquired from Bayer (Leverkusen, Germany).

Reagents

The chromogenic substrate S-2251 H-D-Val-Leu-Lys-p-nitroaniline dihydrochloride (Chromogenix) was obtained from Instrumentation Laboratory (Bedford, MA, USA). All other chemicals used herein were obtained from commercial sources and were of the highest purity available. Sodium phosphate dibasic anhydrous (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium chloride (NaCl), sodium azide (NaN_3), ε-aminocaproic acid (EACA), ethylenediaminetetraacetic acid (EDTA), sodium citrate, trizma base, glycerol, SDS, TEMED, 2-mercaptoethanol, calcium chloride, ammonium sulfate, benzamidine hydrochloride,

Table 3. Chemotherapy scheme currently administered to the enrolled patients with active cancer.

Number of patients	Disease stage	Chemotherapy
1	Locally advanced	Cyclophosphamide + docetaxel
2	Locally advanced	Cyclophosphamide + doxorubicin
3	Locally advanced	Docetaxel + trastuzumab
1	Metastatic	Docetaxel + capecitabine
2	Metastatic	Capecitabine
3	Metastatic	Gemcitabine + paclitaxel
4	Metastatic	Docetaxel
5	Metastatic	Gemcitabine + carboplatin
6	Metastatic	Docetaxel

Cyclophosphamide: alkylating agent that interfere with the duplication of DNA and the creation of RNA.
Docetaxel: taxane drug that disrupts the microtubules function in order to stop cell division.
Doxorubicin: anthracycline drug that works by interfering with the function of DNA.
Trastuzumab: monoclonal antibody that binds to the HER2 receptor in order to slow down cell duplication.
Capecitabine: oral anti-cancer drug that is transformed in the body to 5-fluorouracil, which in turn inhibit the synthesis of thymidine monophosphatase stopping the synthesis of DNA.
Gemcitabine: nucleoside analogue drug that works through inhibit DNA synthesis.
Paclitaxel: taxane drug that disrupts the microtubules function in order to stop cell division.
Carboplatin: platinum antineoplastic drug that works through inhibition of DNA synthesis.

HEPES, Tween 20, and Coomassie Brilliant Blue G were purchased from Sigma-Aldrich (St Louis, MO, USA). Lysine Sepharose 4B was obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). The 30% Acrylamide/Bis Solution, 29:1, and Precision Plus Protein All Blue Standards were obtained from Bio-Rad (Hercules, CA, USA). The mouse monoclonal antibodies to human CD41b, CD62P, CD142, CD45, CD62E, CD54, CD106, CD87, CD235a and Annexin V kit, conjugated to one fluorochrome (fluorescein isothiocyanate (FITC), R-phycoerythrin (PE) or allophycocyanin (APC)) were acquired to BD Biosciences (San Jose, CA, USA).

Microvesicle isolation

A blood sample was obtained from each patient after fast of ≥ 8 hours. Venous blood (10 mL) was drawn and placed in a Vacutainer tube containing 0.109 M sodium citrate as anticoagulant (BD Becton Drive, Franklin Lakes, NJ, USA). Plasma was obtained by centrifugation at 1,500 *g* for 15 min at 4°C. A second centrifugation of the separated plasma was performed at 13,000 *g* for 2 min to produce platelet-free plasma. Plasma aliquots of 500 μL were frozen and stored at −80°C until analysis. To separate Mvs, platelet-free plasma was centrifuged at 20,000 *g* for 90 min at 4°C (Beckman, Optima, TLX ultracentrifuge rotor TLA-100.1, tube type Beckman Coulter, Ref No 343776). Pelleted Mvs were washed twice with HEPES buffer (10 mM HEPES, 140 mM NaCl, pH 7.4) using the same procedure, as described previously (Lacroix et al. 2012). The final pellet of Mvs was resuspended in HEPES buffer, then frozen and stored at −80ºC prior to analysis.

Quantification of extracellular vesicles

The concentration of Mvs in each sample was assessed by measuring protein content. Stored frozen Mvs were lysed by thermal shock (five thaw-freeze cycles of +37°C/−196°C using a water bath/liquid nitrogen). The protein concentration of the lysate was determined by measuring the absorbance at 280 nm in an EON spectrophotometer (BioTek Instruments, Winooski, VT, USA).

Fibrinolytic activity in microvesicles

The functional approach of the method detects the activity associated with the components of the fibrinolytic system, showing a broad and complete overview of their activity. We performed a concentration-response relationship to find the Mvs fibrinolytic half maximal effective concentration (EC_{50} = 30 μg/mL). We employed this quantity of Mvs as a fixed concentration in each determination.

We followed the technique described by Lacroix et al. (2007) as plasmin generation test with some differences; the capacity of Mvs to activate plasminogen was determined by placing 12.5 μL of Mvs (corresponding to 30 mg/mL of protein) in a well of a 96-well polystyrene microtiter plate with 12.5 μL of 1 μM plasminogen and 3 mM color-developing reagent in assay buffer (0.75 mM of a chromogenic substrate selective for plasmin S-2251 H-D-Val-Leu-Lys-p-nitroaniline dihydrochloride (Chromogenix), 50 mM Na_2HPO_4/NaH_2PO_4, 80 mM NaCl, 0.01% NaN_3, 2 mg/mL BSA and 0.01% Tween 20, pH 7.4). Reactions were incubated at 37°C, and color development was measured with an EON spectrophotometer (BioTek Instruments).

In this reaction, plasmin was formed from plasmino-

gen by native plasminogen activators bound to the surface of Mvs; this plasmin then cleaved the chromogenic substrate. The rate of p-nitroaniline formation was read at 405 nm and was proportional to the amount of generated plasmin (and therefore to the capacity of the Mvs to activate plasminogen). The results were recorded in units of A405 $\times 10^{-3}$/min/30 μg/mL protein Mvs for plasmin generation, then transformed into IU/mL/30 μg/mL protein Mvs of urokinase by comparison with a reference curve generated by running the assay with $4 \times 10^{-3} - 8.5 \times 10^{-5}$ IU/mL of pure urokinase (from a freshly prepared stock in distilled water at 10,000 IU/mL). Vmax was calculated for each well in the linear part of each curve.

Flow cytometry

Mvs (5×10^8 Mvs per mL/well) were homogeneously resuspended by gentle shaking before staining with an Annexin V apoptosis detection kit as previously described (Gong and Li 2016); briefly, added 5 μL Annexin V-FITC and 5 μL propidium Iodide (PI), gently vortex, incubated for 15 min at 25°C in the dark, diluted with 400 μL of 1X Binding buffer and analyzed within 60 min.

The Mvs subtypes were identified with fluorescent antibodies against the most commonly cited cell surface markers. Mvs were stained according to conventional methods, briefly described, added 20 μL of monoclonal antibodies CD41b-FITC, CD62P-PE, CD142-PE, CD45-APC, CD62E-PE, CD54-APC, CD106-FITC, CD87-PE, uPA-APC or 5 μL CD235a-FITC, gently vortex and incubated for 30 min at 4°C in the dark. The Mvs were then washed with 1 mL of HEPES buffer (10 mM HEPES, 140 mM NaCl; pH 7.4), collected by centrifugation at 20,000 *g* for 90 min at 4°C, and resuspended in 500 μL of the same buffer.

Mvs were identified with a digital cytometer (LSR Fortessa, BD Biosciences, San Jose, CA, USA) with acquisition of 10,000 events for each sample, with the FACS Diva 6.1 program (BD Biosciences). FlowJo v10.0.6 software (FlowJo, Ashland, OR, USA) was used for analysis of the results of the flow cytometry.

Statistical analysis

Categorical or continuous variables are expressed as the mean ± standard deviation (SD) and as the median with interquartile range values, in accordance with their distribution. The Kolmogorov–Smirnov test provided evidence of normal distribution. The Kruskal-Wallis, Student's *t*-test or the Mann-Whitney test was performed to compare the differences between continuous variables according to their distributions. Analysis of variance was used for evaluation of the differences between EV quantity and plasmin generation depending on the stage of breast cancer. Significant differences between categorical variables were evaluated using the Chi-square test.

Statistical calculations were performed using SPSS statistics V21.0 (SPSS, Chicago, IL, USA).

Results

Immunohistochemical profiles in the patients with cancer are shown in Table 1, and the clinical and biochemical characteristics of the 13 patients with breast cancer and the 13 healthy controls are shown in Table 2. Among these variables in the three subgroups of disease, the only significant difference from the control group was the age of patients with locally advanced cancer and the chemotherapy scheme of each patient is shown in Table 3.

Results of the assessments of Mvs protein concentrations, plasmin generation rates and urokinase-equivalent concentrations are presented in Table 4, for participants classified as healthy women, patients in remission, patients with locally advanced cancer and patients with metastatic breast cancer. No significant differences were identified in the protein concentrations of circulating Mvs between any of the groups of patients with breast cancer and the control group. By contrast, the fibrinolytic activity of Mvs was shown to be a distinctive parameter in patients with metastasis.

We further found that in the metastatic breast cancer

Table 4. Protein concentration, plasmin generation and equivalent urokinase concentration of microvesicles.

Biomarker	Study group			
	Healthy women (n = 13) Mean ± SD	Cancer in remission (n = 4) Mean ± SD	Locally advanced cancer (n = 3) Mean ± SD	Metastatic cancer (n = 6) Mean ± SD
Protein concentration (mg/mL)	0.331 ± 0.31	0.111 ± 0.02	0.293 ± 0.21	0.144 ± 0.04
Plasmin Generation ($A_{405} \times 10^{-3}$/min)	7.2 ± 5.5	10.5 ± 2.0	9.0 ± 5.5	14.4 ± 1.8*
Urokinase equivalence (UI $\times 10^{-6}$/mL)	0.49 ± 1.0	1.04 ± 0.6	0.97 ± 1.2	2.65 ± 0.9*

All values are expressed as means ± SD (standard deviation).
*$P < 0.05$ for a significant difference compared with the control group of healthy women in ANOVA, with Dunnett's *post hoc* test.

Table 5. Mean fluorescence intensity of positive microvesicles from healthy women and patients.

Antibody	Mean fluorescence intensity (MFIx10^3)			
	Healthy women (*n* = 13)	**Cancer remission (*n* = 4)**	**Locally advanced cancer (*n* = 3)**	**Metastasis cancer (*n* = 6)**
Anti-CD45 (LCA)	25.5 (0.6-75.3)	49.6 (4.9-90.1)	44.8 (5.3-96.9)	71.8* (46.4-153.9)
Anti-CD41b (Platelet)	2.5 (1.0-15.5)	4.5 (1.5-13.8)	1.0 (0.6-4.8)	3.0 (0.7-8.9)
Anti-CD62P (P-selectine)	2.5 (1.0-27.2)	13.5 (4.1-17.3)	2.9 (1.8-3.7)	3.4 (2.4-7.2)
Anti-CD62E (Endotelium, E-selectin)	1.8 (0.3-13.3)	3.2 (1.4-3.9)	0.9 (0.3-2.9)	1.2 (0.3-3.2)
Anti-CD235a (Glycophorin A)	5.8 (1.0-150.9)	3.9 (3.0-22.3)	3.7 (1.5-9.2)	4.6 (1.7-8.6)
Anti-CD142 (TF)	2.9 (0.5-21.3)	2.8 (2.2-4.4)	2.4 (1.6-5.6)	2.4 (0.7-3.2)
Anti-CD54 (I-CAM)	18.1 (0.6-88.8)	57.3 (3.9-76.6)	59.3 (2.8-153.5)	63.3 (38.5-164.1)
Anti-CD106 (VCAM)	2.9 (0.7-18.9)	4.8 (2.9-14.7)	1.3 (0.7-4.3)	3.5 (0.7-11.3)
Anti-Annexin V (PS)	2.1 (1.0-14.8)	3.9 (1.9-17.1)	1.4 (1.2-4.2)	2.5 (0.9-7.1)
Anti-CD87 (uPAR)	1.4 (0.3-11.7)	2.0 (1.3-2.6)	1.7 (0.5-3.4)	1.1 (0.5-2.7)
Anti-uPA	11.2 (0.9-105.4)	47.2 (3.7-10.9)	40.5 (3.4-64.8)	51.5 (33.5-85.1)

LCA, Leukocyte common antigen; TF, tissue factor; I-CAM, intercellular adhesion molecule 1; VCAM, vascular cell adhesion molecule-1; PS, phosphatidylserine; uPA, urokinase-type plasminogen activator; uPAR, Uuokinase-type plasminogen activator receptor.

*$P < 0.05$ for a significant difference compared with the control group of healthy women in Kruskal-Wallis and Mann-Whitney test.

group, a negative correlation was found between the platelet count (see Table 2) and the Mvs fibrinolytic activity ($r = -0.457$, $P = 0.025$). Platelets and derived Mvs do not bear plasminogen activators but plasminogen bound to C-terminal lysine residues. It is therefore possible that a fibrinolytic crosstalk occurs between platelets and Mvs bearing plasminogen activators. The plasmin formed from bound plasminogen onto platelets could enhance platelet activation and decrease in consequence the platelet count.

Platelets favor tumor cell viability, invasive phenotype angiogenesis and breast cancer tumor growth (Andreasen et al. 1997). In agreement with our data, a negative correlation has previously been observed between the platelet count and the concentration of uPA on Mvs in patients with an advanced stage of cancer (Chaari et al. 2014).

Flow cytometry measurements of mean fluorescence intensity (MFI) are shown in Table 5. All markers studied (CD45, CD235a, CD41b, CD62e, CD54, CD106, CD142, CD62P, annexin V, uPA and CD87) are present in the Mvs of healthy women as well as cancer patients. When comparing each marker between the different study groups, no statistically significant differences were found, except in the CD45, leukocyte common antigen, where the Mvs of patients (remission, local and metastasis, respectively) have 1.9, 1.8 and 2.8 times higher intensity of fluorescence compared to the healthy women group. These data are in agreement with previously reported data indicating that both leukocytes and Mvs from leukocytes have the capacity to generate plasmin (Lacroix et al. 2007, 2012; Chaari et al. 2014; Cointe et al. 2018).

Discussion

Reproducibility in the study of Mvs has been hampered by methodological difficulties in obtaining or counting Mvs, and a lack of standardization of methods. Flow cytometry has been most commonly used to determine the cellular origins of Mvs, but even with this technique, results are affected by the use of different optical systems, and according to MISEV 2018 (Théry et al. 2018) a broad consensus on the specific markers of Mvs subtypes has not yet emerged.

Plasminogen binds directly to all subtypes of Mvs to C-terminal lysine residues of membrane proteins. However, the ability to generate plasmin is only observed in Mvs bearing plasminogen activators such as those formed from endothelial cells and leukocytes (Vallier et al. 2017) and cancer cells.

Mvs are related to different pathologies, including breast cancer. Data have been reported both in its concentration and in the cellular origin (Jansen et al. 2017; Rondon

et al. 2018). This proof-of-concept study allowed us to demonstrate that patients with metastatic breast cancer have a higher Mvs-associated fibrinolytic activity compared with patients in remission. Since the number of Mvs was not significantly different, the originality of our study is in agreement with the comment by Plow and Pluskota (2007): "It's not size, it's the substance." Mvs carry the substance on their surfaces that provides an answer to the role of Mvs in cancer and new insights into the cancer metastatic process.

Altogether, our data suggest that circulating Mvs may be involved in plasmin generation *in vivo* and play thereby a key role in fibrinolysis and pericellular proteolysis. Direct detection and quantification of Mvs fibrinolytic activity in circulating blood could be of potential diagnostic and prognostic value in patients with metastatic breast cancer (Angelucci et al. 2000; Cointe et al. 2018). Future studies should investigate the impact of blocking fibrinolytic activity in breast cancer patients as a potential treatment approach.

In conclusion, this study has shown that patients with metastatic breast cancer had higher fibrinolytic activity associated with leukocytes circulating Mvs than a healthy control group. The level of fibrinolysis is independent of the concentration of Mvs in the blood.

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Conflict of Interest

The authors declare no conflict of interest.

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