Risk factors of thrombosis in cancer : the role of microparticles

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6 Microparticle-associated tissue factor activity: a link between cancer and thrombosis

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Abstract

Background*:* Cancer, in particular mucinous adenocarcinoma, is associated with venous thromboembolism (VTE). Tissue factor (TF), initiator of coagulation, plays a central role in the paradigm that clotting and tumor growth form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa. Expression of TF in tumors is associated with poor differentiation and poor prognosis.

Patient/methods: We investigated the association between clinically manifest VTE and procoagulant properties of circulating microparticles (MP) isolated from blood of unselected pancreatic and breast adenocarcinoma patients, consecutive subjects, who presented with ultrasound or CT-scan confirmed VTE, and healthy subjects.

Results*:* Patients with disseminated breast and pancreatic cancer had significantly increased levels of MP-associated TF activity compared with healthy controls, subjects with idiopathic acute VTE and non-metastatic cancer patients. Patients with both high MP-associated TF-activity and MP-associated epithelial mucin (MUC1) had a lower survival rate at 3–9 months follow-up than those with low TF-activity and no MUC1 expression: the likelihood of survival was 0.42 (95% CI: 0.19–0.94) for an individual with these two predictor variables present, after adjustment for other factors (age cohort, type of cancer, VTE) in the Cox proportional hazards model.

Conclusions*:* Our results suggest an important role for MP-associated TF and MUC1 in the pathogenesis of thrombosis in disseminated mucinous adenocarcinoma patients. Future studies should reveal the mechanism underlying the observed associations.

Introduction

The link between activation of the blood coagulation system and malignancy dates from 1865, when Armand Trousseau first recognized the association between cancer and thrombosis (1). The incidence of thrombosis is high in adenocarcinomas such as ovarian, prostate and gastro-intestinal carcinoma (2,3), but is particularly high (up to 57%) in patients with pancreatic cancer (4–6). However, the cause of this association is still unknown. Thrombosis often precedes the diagnosis of cancer and is associated in cancer patients with a detrimental course of the disease (7). This supports the paradigm that coagulation and tumor growth form a vicious circle, in which hypercoagulability facilitates the aggressive biology of cancer and vice versa. The strikingly poor prognosis of pancreatic cancer, and the high incidence of venous thromboembolism (VTE) support the hypothesis that local or systemic hypercoagulability confer a growth advantage to tumor cells (8).

Although abnormal coagulation profiles have been found in cancer patients, such abnormalities did not correlate with the development of thrombosis (9). Attention has been focused on tissue factor (TF), a transmembrane receptor protein, which is not only the primary initiator of coagulation but also promotes tumor growth, angiogenesis, and metastasis (10–12). Many tumor cells express high levels of TF and in several types of cancer, including breast cancer and pancreatic cancer, TF expression on tumor cells correlated with grade and tumor progression (13–15). Only Kakkar *et al*. (16) reported on the presence of circulating TF antigen in cancer patients, although the significance of this finding has not been clarified.

TF has been demonstrated on circulating microparticles (MP), small membrane vesicles that are released from cells following activation or during apoptosis (17–19). MP have been found in the circulation of healthy subjects (20). Elevated numbers of MP were found in patients with a variety of diseases associated with hypercoagulability and vessel wall injury (21–23). One of the most intriguing questions is whether specific MP express functionally active TF and form a critical determinant in thrombus formation.

We hypothesized that MP may initiate blood coagulation locally or at distant sites via functionally active TF expressed on these MP and that, in part, MP derived from malignant epithelial cells (24,25) may contribute to this process. We are the first to report on elevated MP-TF activity in cancer patients and the association with venous thrombosis (26).

Patients and methods

Cancer patients and controls

We investigated unselected cancer patients with non-resectable locally advanced (*n* = 4) or metastatic (*n* = 19) pancreatic cancer (11 males and 12 females, median age 59 years, range: 42–70), primary breast carcinoma before and 2 months after surgery of the breast tumor (*n* = 10, median age 50 years, range: 28–72), and breast carcinoma patients at the time of presentation of distant metastases (*n* = 17, median age 52 years, range: 28–72). In addition, we investigated 37 healthy subjects (16 males, 21 females, median age 43 years, range: 23–68) and seven subjects (three males, four females, median age 53 years, range: 24–72) who presented with ultrasound or CT-scan confirmed VTE, without a known history of cancer at the time of blood collection. Patients with a primary breast carcinoma had tumors of the histological type 'ductal adenocarcinoma' (one-third of patients had grade I, one-third had grade II and one-third had grade III tumors), and the tumors varied in size between 1 and 5 cm [staging according to the American Joint Committee on Cancer (AJCC) using TNM classification, in which T stands for tumor, N for lymph nodes and M for metastases]. In two of the 10 primary breast carcinoma patients, three axillary lymph nodes that contained tumor cells were found after surgery. In the other eight primary breast cancer patients, axillary lymph nodes did not contain tumor cells. Thus, the group of 10 patients with primary breast carcinoma was classified as AJCC stage I-II patients. The 17 patients with metastatic breast carcinoma were all found to have metastases at distant sites, either in lung, liver and/or bone and were classified as AJCC stage IV patients. The metastases were all established by conventional methods, including chest X-ray, liver ultrasound, bone scintigraphy and CT scan. Of the patients with pancreatic cancer, four patients had metastases in locoregional lymph nodes as well as in the superior mesenteric artery, whereas the other 19 patients had metastases at distance in various different organs. Thus, the pancreatic cancer patients were all classified as having AJCC stage III-IV pancreatic cancer. The pancreatic carcinomas were grade I, II and III respectively, each in about one-third of patients. Two patients with metastatic pancreatic cancer had primary surgery (Whipple procedure) for their tumor more than 1 year ago.

In all patients blood samples were collected before receiving any chemotherapy to preclude these agents affecting the number and type of MP. The number of platelets was always within the normal range and did not differ between the groups. None of the healthy subjects or patients used anti-inflammatory, antihypertensive or antineoplastic agents at the time of blood sampling.

Healthy controls and non-disseminated breast cancer patients were all alive at the end of the study, with a median follow-up of 36 months (32–48). Median survival after collection of the blood samples was 12 months (range: 1–44) in metastatic breast cancer patients and 3 months (range: 1–13) in pancreatic cancer patients.

All individuals signed an institutional review board approved consent. Tumor type was pathologically confirmed in all cancer patients; measurable disease was confirmed by CT scan.

MP isolation

Blood samples were taken from the antecubital vein, without tourniquet, into a 4.5-mL tube containing 0.105 mol L^{-1} citrate (Becton Dickinson, San Jose, CA, USA). Cells were removed by centrifugation for 20 min at 1550 × *g* at room temperature. In the supernatant plasma (source of MP) no platelets could be detected using a Sysmex 2100 Coulter counter and Phase contrast microscopy. The plasma was immediately snap frozen in liquid nitrogen and stored at −80°C for the isolation of MP as previously described (27). For the measurement of MP-associated TF activity (MP-TF activity), MP were washed more extensively to reduce contamination with plasma proteins (0.5% in the final MP preparation) and resuspended in 1/7 of the original volume PBS/citrate. MP prepared from fresh and deep frozen plasma from the same donor did not differ in numbers, phenotype and associated TF activity. Therefore all assays were performed on MP isolated from deep frozen plasma. This allowed comparison of MP from different individuals in the same exercise.

Flow cytometric analysis of MP

Flowcytometric analysis of MP was performed using Allophycocyanin (APC)-labelled AnnexinV and cell-specific monoclonal antibodies (mAb) or isotype-matched control antibodies labelled with phycoerythrin (PE) or fluoresceinisothiocyonate (FITC): anti-CD61-PE (Y2/51, IgG_1) mouse IgG_1-PE (X40) and mouse IgG_1-FITC (X40) from BD Biosciences/Pharmingen (San Jose, CA, USA), anti-CD66e-PE (CLB-gran/10, IH4Fc, IgG_1) from Sanquin (Amsterdam, the Netherlands), anti-CD14-PE (CRIS-6, IgG_1) from Biosource (Camarillo, CA, USA), anti-CD62e-PE (HAE-1f, IgG_1) from Kordia (the Netherlands, Leiden), antiglycophorin A-PE (JC159, IgG_1) from Dako A/S (Denmark, Glostrup), anti-TF-FITC (4508CJ) from American Diagnostics Inc. (Greenwich, CT, USA) and the negative control mouse IgG_1-FITC (clone X40) from BD Biosciences/Pharmingen (San Jose, CA, USA)

for anti-TF-FITC (the thresholds for measurement of MP-TF expression were set based on MP samples incubated with exactly the same concentration of the isotype-matched FITC-labelled mouse IgG_1control antibody) and antihuman epithelial membrane (MUC1) antigen-FITC (B24.1, IgG_1) that recognizes a multiple protein epitope (Biomeda, Foster City, CA, USA). MP were double- or triple-stained with AnnexinV-APC, PE-labelled cell-specific mAb, and anti-MUC1-FITC or anti-TF-FITC mAb, extensively washed to remove non-specific binding and analyzed for 1 min with Cell Quest software (Becton Dickinson, San Jose, CA, USA). The total MP population was defined on the basis of forward scatter and side scatter by which a gate was set to identify the single MP population. Scatter parameters were calibrated using polystyrene microspheres (size: 0.45, 0.70 and 1.09, 2 and 3 micron microspheres). Machine setting was optimized for optimal performance with respect to scatter analysis of small size particles. MP were defined as the population within the gate set on the basis of forward and side scatter, and subsequently the two and three color profiles of the selected populations recorded. The number of MP L^{-1} plasma was calculated as previously described (27).

Confocal laser microscopy of antibody-labelled MP

Isolated MP were incubated with anti-MUC1-FITC and anti-CD61-PE, centrifuged (30 min, 17 570 *g*, 20 °C) and analyzed by confocal laser scanning microscopy (Zeiss LSM 510; Zeiss, Jena, Germany) using established procedures.

Tissue factor activity assay

The TF activity in MP preparations was measured at room temperature by determining the FVII-dependent factor Xa (FXa) generation in the presence of excess negatively charged phospholipids under conditions that the TF concentration is rate limiting. Twenty-five micromolar dioleoylphosphatidylserine DOPS):dioleoylphosphatidylcholine (DOPC) (10:90) vesicles were incubated in 10 mM Hepes, pH 7.45, 137 mM NaCl, 4 mM KCl, 5 mg mL^{-1} ovalbumin, 50 nM hirudin and 6 mM $CaCl_2$ for 15 min. To 100 µL of this solution 20 µL MP-suspension was added and incubated for 15 min before 40 µL 5 nM FVII (or buffer) was added. After 10 min, 25 µL 2.5 mM S2765 was added and the reaction started by adding 40 µL 250 nM FX. The absorbance at 405 nm (expressed in mAbs) was recorded for 90 min, and plotted as a function of time (t) and, after correction for the absorbance in the absence of FVII, as a function of t^2. The slope of the latter curve is a measure for the rate of FXa generation and expressed as mAbs min^{-2} or as fM FXa min^{-1}. In all samples FXa

generation was measured both in the presence and absence of FVII and in the presence and absence of excess (125 μg mL^{-1}) polyclonal rabbit anti-huTF IgG (28) to establish the FVII- and TF-dependence of the FXa generation. Removal of DOPC/DOPS from the reaction mixture did not affect TF activity importantly. The MP-associated TF activity was calculated from the difference in the rate of Xa generation in the absence and presence of anti-TF antibodies and reported as the MP-associated TF-activity (fM Xa min^{-1}) in plasma. DOPS and DOPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Circulating mucins

Circulating mucins Ca 15.3 and Ca 19.9 (normal values ≤ 28 kU L^{-1} and ≤ 37 kU L^{-1}, respectively) were determined in plasma utilizing commercially available immuno-enzymatic assays on an IMx (Abbott Diagnostica, IL, USA). Both assays are routinely performed to measure different isoforms of these mucins.

Statistical analysis

Different groups of individuals (i.e. healthy controls vs. cancer patients, or vs. metastatic cancer patients) were compared with respect to numerical variables (number of total MP and MP subsets and MP-TF activity) by non-parametric Wilcoxon and Mann–Whitney tests. Numerical variables in primary breast cancer patients (preoperative and immediately postoperative) were compared using a paired Student's *t*-test. A Spearman's correlation test was performed to analyze the correlation between MP-TF activity and total number of MP, and MP-TF activity and presence of MUC1-expressing MP. *P* values < 0.05 were considered significant. Kaplan–Meier survival curves were created, groups compared on unadjusted survival by log-rank test and multivariate Cox proportional hazard models constructed. Analyses were performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

MP-associated TF activity in healthy subjects and cancer patients

TF activity associated with MP was studied by measuring FVII-dependent and anti-TF-IgG sensitive generation of FXa in isolated MP (Figure 1). The observation that MP-dependent FX activation was completely FVII and TF dependent indicated the absence of other FX activators such as the so-called cancer procoagulant in the MP preparation (29).

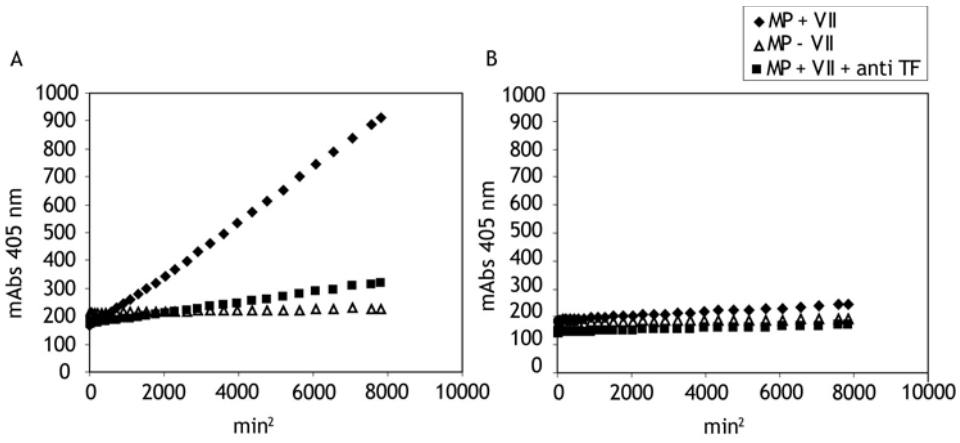


Figure 1. Tissue factor (TF) activity on microparticles (MP). Kinetic assay measuring factor Xa (FXa) formation, expressed as 405 nm absorbance units (mAbs or mAbs 405 nm). MP-associated TF activity in two patients with metastatic pancreatic carcinoma. One patient presented with venous thrombosis of the leg at the time of diagnosis and blood collection (A), while the other patient did not present with thrombosis (B). The slope of the line, which plots the mAbs at 405 nm against min^2 measures the rate of FXa formation (fM min^{-1}). MP + FVII (◆), MP – FVII (△), MP + anti-TF + FVII (■).

In MP isolated from 37 healthy subjects, a mean MP-associated TF activity level of 132 ± 47 fM Xa min^{-1} was found (Figure 2) and TF activities > 273 fM Xa min^{-1} (mean + 3 SD) were considered to be elevated. In patients with primary breast carcinoma, MP-TF activity was slightly elevated in 2/10 patients before and in 0/10 patients after surgery of the primary tumor (Figure 2, C + D). The two patients with elevated MP-TF activity before breast surgery both developed an active tumor following the initial surgery. One patient was diagnosed with a breast carcinoma in the other breast, whereas the other patient developed metastases at distant organs. All other primary breast cancer patients remained disease-free up to 4 years following surgery.

MP-TF activity in the metastatic breast and pancreatic carcinoma patients was increased compared with that in healthy controls ($P < 0.004$). In 5/17 disseminated breast carcinoma (Figure 2E) and in 8/23 pancreatic carcinoma patients (Figure 2F) elevated MP-TF activity was found; there was no correlation between MP-TF activity and the number of MP ($r = -0.22$, $P = 0.170$).

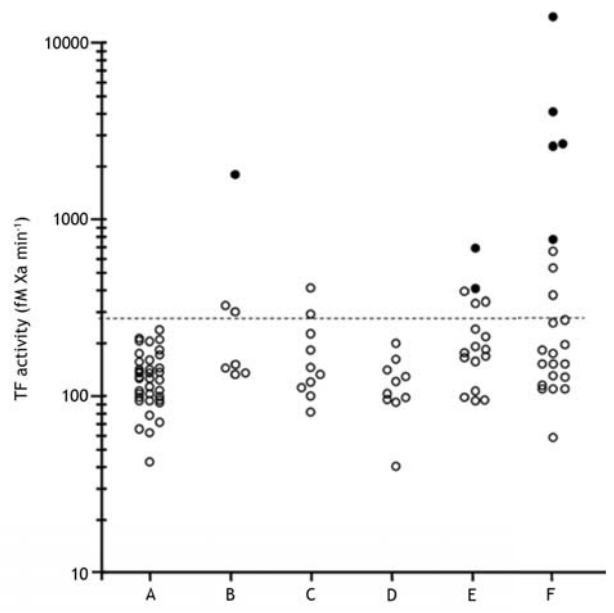


Figure 2. Microparticles (MP)-associated tissue factor (TF) activity in healthy subjects and cancer patients. (A) Thirty-seven healthy subjects. (B) Seven patients with idiopathic VTE without a history of cancer at the time of presentation and collection of blood sample. One of these patients (filled circle) was diagnosed with disseminated mucinous adenocarcinoma within the next month and thus no longer classified as having an idiopathic venous thromboembolism (VTE). (C) and (D) Ten early stage, non-disseminated breast cancer patients before (C) and after (D) surgical removal of the primary breast tumor. (E) Seventeen consecutive patients with metastatic adenocarcinoma of the breast. (F) Twenty-three consecutive patients with locally advanced or at distance metastasized adenocarcinoma of the pancreas. Filled circles (E + F) represent MP-associated TF activity in seven of these breast and pancreatic cancer patients who presented with VTE at the time of blood collection. The dotted line represents the upper limit of the normal range (99th percentile of > 273 fM Xa min^{-1}, i.e. mean plus 3 SD, as measured in 37 healthy subjects).

MP-associated TF activity and venous thrombosis

In the seven AJCC stage III and IV metastatic cancer patients who presented with VTE at the time of referral (six patients with deep VTE and one patient with PE), the mean and median MP-TF activity (Figure 2E,F, filled circles) was 3643 fM Xa min^{-1}, respectively 2620 fM Xa min^{-1}; range: 410–14 180 fM Xa min^{-1}. In the combined group of AJCC stage III and IV metastatic breast and pancreatic cancer patients, who did not develop thrombosis

(Figure 2E,F, open circles), the mean and median MP-TF activity was 209 fM Xa min^{-1} and 170 fM Xa min^{-1}, respectively, range: 59–665 fM Xa min^{-1}. The MP-TF activity per 10^6MP (expressed as fmol min^{-1} 10^{-6}) was up to eighteenfold higher in the disseminated cancer patients with clinically manifest VTE than in cancer patients without VTE ($P < 0.001$).

The median survival of metastatic breast and pancreatic cancer patients who presented with VTE was strikingly short (2 months; range: 1–2) compared with that of metastatic breast (13 months; range: 1–44) and pancreatic cancer patients (4.5 months; range: 1–13) without thrombosis ($P = 0.002$).

MP-TF activity was also measured in seven patients without a known history of cancer at the time of blood collection, who presented with an acute idiopathic thrombosis (two patients with deep vein thromboses and five patients with pulmonary embolisms) (Figure 2B). The median MP-associated TF activity in 6/7 patients was 148 fM Xa min^{-1} (range: 135–331 fM Xa min^{-1}). In the seventh patient with thrombosis of both legs, an elevated MP-TF activity of 1812 fM Xa min^{-1} was found (Figure 2B, filled circle). This patient was diagnosed with a disseminated mucinous adenocarcinoma within the next month, indicating that an elevated MP-TF activity might serve as a predictive marker for the presence of disseminated mucinous adenocarcinomas.

Number and cellular origin of MP

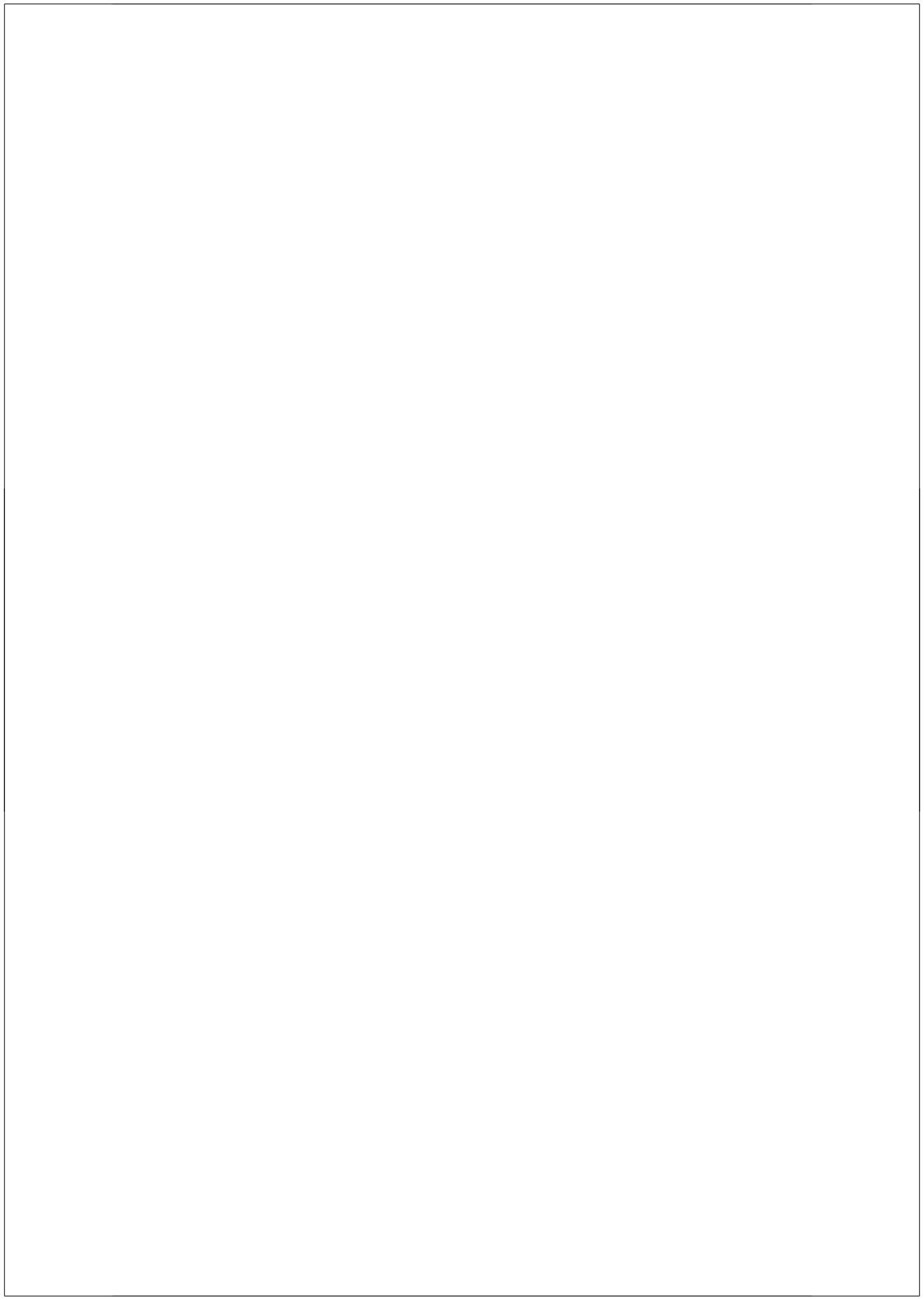
Table 1 shows the concentrations of circulating MP in healthy subjects and patients with cancer measured by two- or three-color flow cytometry. The median concentration of circulating AnnexinV-positive and Annexin V-negative MP together in patients with metastatic breast or pancreatic cancer was significantly higher than that in primary breast cancer patients and about 2-fold higher than that in healthy subjects ($P < 0.001$). In all individuals, > 90% of circulating MP binds to AnnexinV, indicating the presence of phosphatidyl serine on their membranes. Of the $AnnexinV^+$-MP, more than 90% expressed the platelet antigen CD61, whereas < 5% expressed the erythrocyte antigen glycophorin A. In about one-third of metastatic breast and pancreatic cancer patients and in 10% of healthy subjects and primary breast cancer patients the granulocyte antigen CD66e was found. In < 10% of the individuals, MP expressing the monocyte antigen CD14 were found, whereas in < 5% of the individuals, MP expressing the endothelium antigen CD62e were found. TF expressing MP were found in healthy subjects as well as in patients with disseminated breast and pancreatic cancer. We were able to study co-expression of TF antigen with CD61. The number of TF^+-$CD61^+$-MP varied between approximately 200 and 800×10^6 L^{-1} in the healthy subjects and the different groups of patients.

Table 1. Number, cellular origin and composition of microparticles (*10^6 L^{-1}) in healthy subjects and in cancer patients with different stages of adenocarcinoma of the breast or pancreas

	Controls	Breast cancer			Pancreatic cancer
		Early stage		Metastatic	
		Preoperative	Postoperative		
Total annexin V^+MP					
Median	1600	2560	1900	4900#	5600#
Range	720–9000	1650–9000	970–4500	1400–11 000	2350–13 200
Total annexin V^- MP					
Median	310	440	300	470	250
Range	130–650	100–800	130–530	140–1500	110–2100
Annexin V^+CD61^+-MP					
Median	1500	2400	2000	4100#	5600#
Range	700–7100	1300–9000	1000–4500	1300–9300	1800–12 700
Annexin V^+CD66e^+-MP					
Median	31	64	25	104	82
Range	6–490			12–2060	13–1060
(% patients)	(13)	(10)	(10)	(24)	(38)
Annexin V^+TF^+-MP					
Median	255			290	460
Range	130–560			115–2700	240–1550
(% patients)	(22)	< 1	< 1	(29)	(46)
Annexin V^+MUC1^+-MP					
Median		98		410#	310#
Range				50–4100	100–500
(% patients)	< 1	(10)	< 1	(65)	(57)

The total number of microparticles (MP) in cancer patients was compared with that in healthy subjects; #indicates that MP are significantly increased ($P < 0.001$), whereas in all other cases MP are not significantly different from that in healthy subjects. In patients with early stage breast cancer the number of MP slightly decreased after surgical removal of the breast tumor ($P = 0.26$).

In 63% of the disseminated breast and pancreatic cancer patients, epithelial (tumor) cell antigen MUC1-expressing-MP were found. None of the healthy subjects or patients with idiopathic VTE had circulating $MUC1^+$-MP except the patient with VTE who was later diagnosed with a mucinous adenocarcinoma. Only in one patient were $MUC1^+$-MP detected pre- and postoperatively, which could indicate that this particular patient had a higher tumor load than the other patients. Furthermore, in this patient the postoperative blood sample was drawn before the start of adjuvant chemotherapy, which was administered



MP-TF activity, MUC1-positive MP and circulating mucin in relation to presence of VTE in cancer patients

Twenty-one (91%) of the pancreatic cancer patients had elevated CA19.9 (range: 91 to 191 207 kU L^{-1}, normal upper limit 37 kU L^{-1}) and 13 (76%) of the disseminated breast carcinoma patients had elevated CA15.3 (range: 45–638 kU L^{-1}, normal upper limit 28 kU L^{-1}) in their plasma. Similar levels were found before and after MP depletion. These antigens were not detectable in the resuspended MP pellets.

Elevated MP-TF activity correlated with the presence of $MUC1^+$-MP (r = 0.38, P = 0.01). All seven pancreatic cancer patients with elevated MP-TF activity also had $MUC1^+$-MP and five out of seven (71%) presented with thrombosis. Four out of five (80%) of the patients with disseminated breast cancer and elevated MP-TF activity had $MUC1^+$-MP, of whom two presented with thrombosis.

We compared the survival for patients using predictor variables like age, gender, type of cancer, MP-TF activity dichotomized on elevated (> 99th percentile) TF activity > 273 fM Xa min^{-1}, $CD61^+$-MP, $MUC1^+$-MP, and circulating mucins dichotomized on elevated CA15.3 and CA19.9 levels. After stratifying patients by type of cancer, or by age quartile, Kaplan–Meier survival curves were created, and multivariate Cox proportional hazard models constructed. Independent variables included were age, gender, occurrence of venous thrombosis, MP-TF activity, $CD61^+$-MP and $MUC1^+$-MP. In the analyses (overall chi-square of model $P < 0.0001$), the likelihood of survival at 3–9 months follow-up was lower for those with a TF activity > 273 fM Xa min^{-1}, and detectable mucin on MP (RR for survival 0.42, 95% CI: 0.19–0.94; and RR 0.41, 95% CI: 0.19–0.89, respectively) adjusting for the other factors (age cohort, type of cancer, VTE) in the Cox proportional hazards model. In the multivariate model, none of the variables mentioned above, namely age, gender, occurrence of venous thrombosis, $CD61^+$-MP and $MUC1^+$-MP, contributed significantly to the prediction of survival with the exception of MP-TF activity. The cumulative survival curve of patients with pancreatic carcinoma with either elevated or non-elevated MP-associated TF activity is shown in Figure 4; the survival of pancreatic patients with an elevated MP-TF activity was significantly decreased as compared with that in patients with non-elevated MP-TF activity (P = 0.0004).

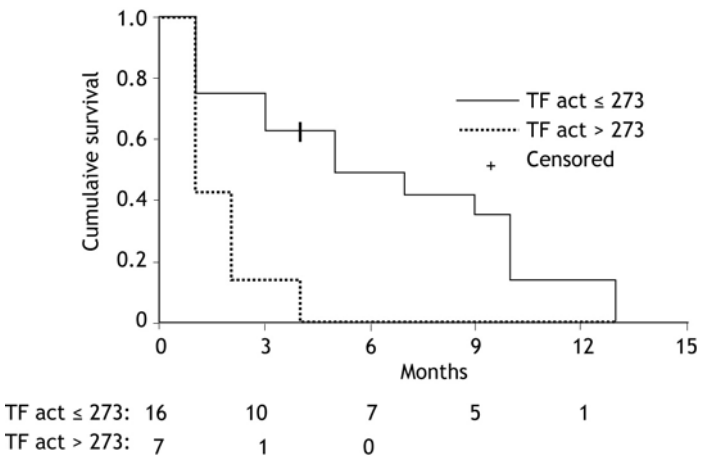


Figure 4. Cumulative survival of pancreatic cancer patients according to the presence of elevated or non-elevated microparticle-tissue factor (MP-TF) activity (> of ≤ 273 fM Xa min^{-1} MP-TF activity). The cross indicates the censored patient still alive at the time of analysis of the survival data. The likelihood of survival was least for pancreatic cancer patients with elevated MP-TF activity (> 273 fM Xa min^{-1}) as compared with those who did not ($P = 0.0004$).

Discussion

This is the first time that MP-TF activity was measured in cancer patients and that the association of MP-TF activity with venous thrombosis is reported. Patients with disseminated breast and pancreatic cancer, who presented with acute VTE, had higher levels of MP-TF activity than healthy subjects, cancer patients without VTE and subjects with idiopathic VTE. MP-TF activity correlated with the presence in the blood of MP expressing the epithelial antigen MUC1. Metastatic breast and pancreatic cancer patients with elevated MP-TF activity and detectable $MUC1^+$-MP had a lower survival rate at follow-up than those with normal MP-TF activity and $MUC1^+$-MP absent, suggesting that the properties of circulating MP are an important determinant of the link between cancer biology and thrombosis.

MP-associated TF activity is a quantitative estimate of the concentration of TF in the MP preparation, which can act as cofactor of FVIIa in FX activation. In contrast, the number of TF^+-MP is not a quantitative estimate of the TF concentration, as the number of TF molecules per MP may vary widely. Also, part of the TF antigen on MP might be

encrypted (not active as cofactor of FVIIa in FX activation, but detectable by the mAb used in the FACS analysis) (30–32).

The observed association between the levels of MP-TF activity and the development of VTE in metastatic adenocarcinoma cancer patients suggests that *in vivo*-generated MP, which can initiate coagulation via the TF-mediated pathway, contribute to the development of thrombosis. Such a hypothesis is supported by the observation that MP expressing TF and PSGL-1, the ligand for P selectin on platelets, accumulated in the developing thrombus in living mice as demonstrated by *in vivo* imaging in real time (33). The elevated MP-associated TF activity observed in pancreatic cancer patients corresponds to TF concentrations sufficient to shorten clot formation of plasma *in vitro* (34). Whether the minute amounts of MP-associated TF activity observed in the plasma of healthy volunteers (0.5–4 fM) are sufficient to stimulate fibrin formation is unknown; TF-dependent FXa generation might be too low to overcome the natural thresholds of the anticoagulant systems (35).

In patients with progressive mucinous cancer tumor-derived $MUC1^+$-MP may display enhanced binding to P and/or L selectin on platelets or other hematopoietic cells or on MP derived from such cells (36,37). Study of the co-expression of MUC1 and platelet antigen CD61 on MP by confocal immunofluorescence microscopy revealed that a small part of circulating MP seemed to result from fusion of cellular vesicles originating from malignant epithelial cells and platelets in patients with disseminated breast and pancreatic adenocarcinoma. Because of the requirement of anionic phospholipids for initiation and propagation of TF-dependent coagulation, it seems likely that only cell- or MP-bound forms of intravascular TF will support thrombin formation. Fusion of these MP may contribute to de-encryption of TF, especially in the presence of negatively charged phospholipids. MP-TF may be derived from tumor cells, but could also be transferred from monocytes to platelets or originate directly from platelet-derived MP (38).

$MUC1^+$-MP were mainly found in patients with disseminated breast and pancreatic cancer, who also had elevated levels of non-MP-bound mucins. Importantly, MUC1 is a glycoprotein that is overexpressed in aberrant forms in epithelial cancers like breast and pancreatic cancers. Mucins may be involved in adhesion and metastasis of tumor cells, whereas cell surface sialylation of tumor cells has been implicated in activation of leucocytes and aggregation of platelets. Wahrenbrock *et al*. (39) suggested that mucins play an important role in the development of VTE in cancer patients and provided a new explanation for the association between mucin-producing carcinomas and a specific

form of hypercoagulability (thrombophlebitis migrans) clinically presenting as platelet microthrombi. Our findings may indicate that besides soluble mucins (39) and tumor cell-bound mucins, also microparticle-bound mucin could play a critical role in the formation of thrombi.

In conclusion, patients with metastatic breast and pancreatic cancer who presented with thrombosis, carry MP that after isolation can initiate blood coagulation *in vitro* because of the presence of active TF on their membranes. The observed association between MP-associated TF-activity and the development of VTE in metastatic cancer patients underscores the possibility that *in vivo*-generated tumor-derived MP initiate coagulation via the TF-mediated pathway. Future studies should confirm our observations and reveal the precise mechanism underlying the observed associations. Greater understanding of the vicious circle between cancer and hypercoagulability may offer new targets for antithrombotic therapy.

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