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Coagulation activation and microparticle-associated coagulant activity in cancer patients

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| ABSTRACT |

**Background**
Cancer increases the risk of venous thromboembolism (VTE). Here, we investigated the contribution of microparticle-dependent procoagulant activity to the prothrombotic state in these patients.

**Methods**
In 43 cancer patients without VTE at study entry and 22 healthy volunteers, markers of *in vivo* and microparticle-dependent coagulation were measured and patients were prospectively followed for 6 months for the development of VTE. Procoagulant activity of microparticles was measured *in vitro* using a tissue factor (TF)-independent phospholipid dependent test, a factor Xa-generation assay with and without anti-TF, and a fibrin generation test (FGT) with and without anti-factor VII(a).

**Results**
Markers of *in vivo* coagulation activation and total number of microparticles at baseline were significantly elevated in cancer patients compared to controls ($F_{1+2}$ 246 vs. 156 pmol/L, thrombin-antithrombin complexes 4.1 vs. 3.0 mg/L, D-dimer 0.76 vs. 0.22 mg/L and $5.53 \times 10^6$ vs. $3.37 \times 10^6$ microparticles/ml). Five patients (11.6%) developed VTE. Patients with VTE had comparable levels of coagulation activation markers and phospholipid-dependent microparticle procoagulant activity. However, median TF-mediated Xa-generation (0.82 vs. 0.21 pg/mL, $p=0.016$) and median VIIa-dependent FGT (13% vs. 0%, $p=0.036$) were higher in the VTE group compared with the non-VTE group.

**Conclusions**
In this exploratory study the overall hypercoagulable state in cancer patients was not associated directly with the microparticle phospholipid-dependent procoagulant activity. However, in the patients who developed VTE within 6 months when compared to those who did not, an increased microparticle procoagulant activity was present already at baseline, suggesting this activity can be used to predict VTE.
INTRODUCTION

Venous thromboembolism (VTE) often complicates the clinical course of cancer (1). It is estimated that almost 15% of all cancer patients develop VTE in the course of their disease (2). Several factors including surgery, central lines, infection, immobilization, chemotherapy, hormonal agents and cancer cells themselves contribute to the hypercoagulable state in cancer patients (3). The exact underlying mechanisms are not yet fully understood, although a number of different mechanisms have been proposed. Most strikingly is the tissue factor (TF)-dependent activation of coagulation. In 1995, a strong association was noticed between malignant disease and elevated levels of circulating non-cell bound TF, the activated form of factor VII (VIIa), thrombin-antithrombin complexes (TAT) and prothrombin fragment F$\text{_{1+2}}$ compared to healthy subjects (4). Since many cancers express high levels of TF (5), this may be a potential source of TF that is associated with the hypercoagulable state in cancer patients. The question remained, however, how tumour-derived TF may enter the blood.

Already in 1981, Dvorak reported that the supernatants of tumour cell lines and ascites fluid contain vesicles carrying coagulant activity. They showed that this coagulant activity was largely removed by centrifugation, and was recovered by reconstitution of the vesicle pellet (6). The same researchers reported later that one explanation for the procoagulant activity of these vesicles may be TF (7). Two decades later, the presence of coagulant TF-bearing microparticles present in blood collected from cancer patients was confirmed by multiple research groups (8-11) but for a long time evidence was lacking to show that these TF-bearing microparticles indeed originate directly from the tumour.

Yu et al were the first to show that tumours release TF into the circulation in mice, and that part of the TF-activity was associated with membrane vesicles (12). A recent study by Davila and co-workers showed that at least part of the circulating TF-bearing microparticles originate from the cancer cells (13). They used a mouse model with a human tumour, and showed that microparticles bearing human TF are present in the mouse blood, demonstrating that a tumour can release TF-bearing microparticles in vivo that can enter the blood. Consistent with this concept, part of the TF-bearing microparticles in plasma samples from pancreatic cancer patients were shown to expose MUC-1, which is a broad-range epithelial marker and under these conditions also stains tumour-derived microparticles. The numbers of the microparticles bearing both TF and MUC-1 decreased more than 10-fold or below detection levels after the tumour had been surgically removed (8).

There is increasing evidence that circulating microparticles are associated with the prothrombotic state in cancer patients. Plasma from cancer patients with VTE contains increased microparticle-associated TF activity compared to patients without VTE (14-16). Also, an association was shown between the risk of developing VTE and the plasma levels of non-cell bound TF and MP-associated TF activity in pancreatic cancer patients (17). Based on these observations, we hypothesize that circulating microparticles contribute to the
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hypercoagulable state in cancer patients in general, and especially in cancer patients prone to
develop VTE. The aim of this study was to study the relationship between in vivo coagulation
activation, microparticle-dependent coagulant activity and VTE in cancer patients.

METHODS

Population
Blood samples were collected from 43 consecutive cancer patients seen at the Department of Medical Oncology of the Academic Medical Center in Amsterdam. Inclusion criteria were age above 18 years and active cancer, and in all patients the diagnosis of cancer was confirmed by pathology. Patients using anticoagulant treatment or with a VTE in the medical history were excluded. The incidence of venous thrombosis was assessed after six months. Furthermore, blood samples were collected from 22 healthy individuals. All patients and healthy individuals signed an informed consent and the protocol was approved by the institutional review board.

Collection of blood samples
Patient blood was collected via a peripheral venous catheter (20-gauge). All blood samples were directly taken from the catheter without the use of a connecting-piece. After discarding the first tube of blood, blood (13.5 mL) was collected into three tubes each containing 0.5 mL 105 mmol/L buffered sodium citrate (BD, Franklin Jakes, NJ). Citrate-anticoagulated blood samples from healthy individuals were taken from the antecubital vein without tourniquet through a 21-gauge needle using a vacutainer system. Within 15 minutes after blood collection, cells were removed by centrifugation for 20 minutes at 1550 x g and 20 °C. Part of the plasma was immediately used for coagulation assays. The remainder was immediately frozen as 0.25 mL aliquots in liquid nitrogen and stored at -80 °C.

Isolation of microparticles for flow cytometry
Isolation of microparticles for flow cytometry was performed as described previously (18). Briefly, a sample of 250 µL frozen plasma was thawed on melting ice for one hour and centrifuged for 30 minutes at 18.890 x g and 20 °C to pellet microparticles. After centrifugation, 225 µL of the supernatant was removed. The pellet was resuspended in the remaining supernatant and then, after another centrifugation step, 225 µL of the supernatant was removed. The microparticle pellet plus remaining supernatant was then resuspended with 75 µL PBS-citrate. Five µL of the microparticle suspension was diluted in 35 µL CaCl₂ (2.5 mmol/L)-containing PBS. Then 5 µL allophycocyanin (APC)-labelled annexin V was added to all tubes plus 5 µL of the cell-specific monoclonal antibody or isotype-matched control antibodies. Samples were analyzed in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software version 4.02 (Becton Dickenson Biosciences, San Jose, CA, USA). The antibodies against fluorescein isothiocyanate (FITC)-labelled IgG, and
phycoerythrin (PE)-labelled IgG, and anti-TF-PE were derived from BD. Anti-CD61-FITC (anti-GP-IIIa) was obtained from Dako (Glostrup, Denmark). Allophycocyanin (APC)-conjugated annexin V was purchased from Caltag (Burlingame, CA, USA).

**STA Procoag PPL assay**

The STA Procoag PPL test is a commercially available activated factor X-based clotting method to measure activity of procoagulant phospholipids (i.e. Procoag PPL). The assay was a gift from Dr. B. Woodhams from Stago (Gennevilliers Cedex, France). This test has been validated in healthy subjects and limited groups of patients (19). Fresh microparticle-containing plasma (25 µL) was mixed with phospholipid-free plasma (25 µL) and pre-heated for 2 minutes at 37 °C in a cuvette. Then, pre-warmed XACT reagent (100 µL; containing activated factor X and calcium) was added and the clotting time was measured on an ACL Top coagulation analyzer (Instrumentation Laboratory Company; Lexington, MA, USA).

**Factor Xa generation assay**

The TF-dependent and total microparticle-dependent coagulant activity were measured using a factor Xa generation assay exactly as described (17). In brief, microparticles were pelleted from plasma (200 µL) by centrifugation (20,000 x g for 15 minutes at 4 °C), washed twice with HBSA buffer (137 mmol/L NaCl, 5.38 mmol/L KCl, 5.55 mmol/L glucose, 10 mmol/L HEPES, 0.1% bovine serum albumin; pH 7.5), and re-suspended in HBSA (200 µL). Samples were incubated with either an anti-human TF-antibody called HTF-1 (4 µg/mL) or a control antibody (mouse IgG: 4 µg/mL) for 15 minutes at 25 °C, and then aliquots (50 µL) were added to duplicate wells of a 96-well plate. Next, 50 µL of HBSA containing FVIIa (10 nmol/L), FX (300 nmol/L) and CaCl₂ (10 mmol/L) was added to each sample and the mixture was incubated for 2 hours at 37 °C. FXa generation was stopped by addition of 25 mmol/L EDTA buffer (25 µL), chromogenic substrate S2765 added (25 µL; 4 mmol/L), and the mixture incubated at 37 °C for 15 minutes. Finally, absorbance was measured at 405 nm using a VERSAmax microplate reader (Molecular Devices Corp.; Sunnyvale, CA, USA). TF activity was calculated by reference to a standard curve generated using relipidated recombinant human TF (0-55 pg/mL). The TF-dependent FXa generation (pg/mL) was determined by subtracting the amount of FXa generated in the presence of HTF-1 from the amount of FXa generated in the presence of the control antibody. Mean TF-dependent FXa generation in healthy controls was 0.21 pg/mL (SD 0.11; interassay CV of 21%).

**Fibrin generation test (FGT)**

The ability of microparticles to generate fibrin was measured directly in plasma in the absence or presence of anti-human FVIIa (Sanquin; Amsterdam, The Netherlands), as described previously (20). After pre-incubation for 5 minutes at 37 °C, clotting was initiated by addition of CaCl₂. Fibrin formation was determined by measuring the optical density ($\lambda = 405$ nm) in duplicate on a spectrophotometer (SPECTRAmax microplate reader; Molecular Devices Corp., Sunnyvale, CA) at 37 °C. We used an anti-factor VII(a) antibody rather than an anti-TF because this antibody
can completely inhibit TF-initiated coagulation at a lower concentration of antibody and provides consistent inhibitory results as compared to some anti-TF antibodies.

Other assays
All other assays were performed as described by the manufacturer. ELISA kits to measure human non-cell bound TF antigen were obtained from American Diagnostica (Greenwich, Conn, USA). This kit measures full length as well as truncated TF. F_{1+2} and TAT were obtained from Enzygnost (Dade Behring; Marburg, Germany) and D-dimer from Innovance (Dade-Behring).

Statistics
Continuous data were expressed as medians with corresponding inter-quartile ranges (IQR). Between group differences were tested with the Mann-Whitney U test. Categorical data are presented as percentages or numbers. All data shown are presented as medians (IQR), unless stated otherwise. P-values ≤0.05 were considered statistically significant. All statistical analyses were performed by using SPSS 15.0.1 (SPSS Inc, Chicago, IL).

RESULTS

Patients
A total of 43 cancer patients and 22 healthy controls were included. The mean age of the cancer patients was 59 ± 12 years and 58% were male. The cancer patients suffered from pancreatic carcinoma (n=13), gastrointestinal carcinoma (n=11), breast carcinoma (n=8), oesophagus carcinoma (n=5), biliary tract carcinoma (n=2) and other types of cancer (n=4). Seven patients had local disease and came for neo-adjuvant therapy, the other patients had locally advanced or metastatic disease. The mean age of the healthy controls was 38 ± 10 years, 32% were men.

Coagulation and microparticles in cancer patients and healthy individuals
Compared to healthy individuals, cancer patients had higher plasma concentrations of F_{1+2}, TAT as well as D-dimer (Table 1; p<0.001 for all). There was no evidence for increased levels of TF antigen in cancer patients, since the levels of non-cell bound TF were comparable to controls. With regard to the microparticles, the numbers of annexin V-binding microparticles were increased in patients compared to controls (p=0.001), but the numbers of TF-bearing microparticles were non-significantly decreased (p=0.186). Subsequently, we tested the microparticle-associated coagulant activity using three different microparticle-based coagulation assays. First, the ability of microparticles to provide a phospholipid surface that propagates coagulation and clot formation was determined in the STA Procoag PPL assay. The clotting time of this assay, which is independent from the presence of TF activity (R.J. Berckmans, pers. commun.), was similar between the cancer patients and controls (p=0.411; Table I). These results were not anticipated considering the higher number of microparticles in plasma samples from cancer patients binding annexin
V. Second, we tested the ability of microparticles to generate factor Xa. There was a trend towards a higher Xa generation capacity associated with microparticles from cancer patients when compared to healthy controls (p=0.066). The TF-dependent Xa generation was comparable between the patients and controls. Similarly, we also found no significant differences between microparticles from patients and controls with regard to fibrin generation in the absence or presence of anti-VIIa (p=0.417 and p=0.349, respectively).

Coagulation in cancer patients developing or not developing VTE

Within six months after blood collection, five patients (12%; 95% CI 4 to 25), three men and two women, developed an objectively confirmed VTE: deep vein thromboses of the leg (n=2), pulmonary embolism (n=2) and vena lienalis thrombosis (n=1). The median time to develop VTE was 3.1 months (range 1.3-4.4) after blood collection. Three of these patients had pancreatic cancer, of whom two patients had locally advanced disease and one patient had metastasized disease. One patient had metastatic gastrointestinal cancer and the remaining patient had locally advanced oesophagus cancer.

The concentrations of F₁+₂, TAT and D-dimer in cancer patients who developed VTE (n=5) were comparable to patients who did not develop VTE within six months (n=38; Table II). Also, plasma concentrations of non-cell bound TF – i.e. microparticle bound as well as truly soluble TF - were comparable in both groups of patients. Taken together, this suggests that at the moment of blood collection the patients prone to develop VTE showed no major signs of increased coagulation activity.

Microparticles and microparticle-initiated coagulation activation in cancer patients developing VTE

As shown in Table 2, no differences were present between cancer patients developing VTE and patients not developing VTE with regard to the numbers of microparticles binding annexin V...
or microparticles bearing TF. Subsequently, we tested the microparticle-associated coagulant activity. The clotting time of the STA procoag PPL test was similar between the two groups of cancer patients (p=0.519; Table II). In contrast, there were marked differences in the ability of microparticles to promote coagulation in the two other assays. First, in patients who developed VTE the microparticles showed a trend towards an increased factor Xa generation, compared to microparticles from patients who did not develop VTE (median 2.16 [interquartile range 0.74-9.4] versus 0.91 [0.64-1.37]; p=0.091). Furthermore, microparticles from patients who developed VTE within 6 months showed a marked increase in the TF-dependent Xa generation compared to microparticles from patients not developing VTE (p=0.016). Similarly, the (microparticle-dependent) clotting of the FGT was markedly faster in plasma samples from patients developing VTE compared to samples from patients not developing VTE (801 s versus 1568 s; p=0.014). Finally, the relative change in the FGT by addition of the anti-VIIa antibody was markedly higher in those patients who developed VTE within 6 months compared to those who did not (13.3% versus 0%, respectively; p=0.036). These data point to a significant contribution of TF-bearing microparticles to the prothrombotic state in cancer patients, and also indicate that this activity may be detectable already before the onset of VTE.

Table 2. Coagulation and microparticles in cancer patients developing VTE

<table>
<thead>
<tr>
<th></th>
<th>Cancer patients + VTE</th>
<th>Cancer patients - VTE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>F$_{1+2}$ (pmol/L)</td>
<td>319 (187-558)</td>
<td>241 (179-312)</td>
<td>0.427</td>
</tr>
<tr>
<td>TAT (mg/L)</td>
<td>5.7 (3.5-13.2)</td>
<td>4.1 (3.2-6.2)</td>
<td>0.290</td>
</tr>
<tr>
<td>D-dimer (mg/L)</td>
<td>0.66 (0.55-7.5)</td>
<td>0.77 (0.38-2.0)</td>
<td>0.326</td>
</tr>
<tr>
<td>sTF (pg/mL)$^1$</td>
<td>145.5 (49.0-369.0)</td>
<td>86.8 (50.7-153.6)</td>
<td>0.445</td>
</tr>
<tr>
<td>MP (Annexin V)$^1$</td>
<td>5.53 x 10$^4$ (5.06-9.84)</td>
<td>5.51 x 10$^4$ (3.27-8.92)</td>
<td>0.629</td>
</tr>
<tr>
<td>MP (TF)$^2$</td>
<td>34.8 x 10$^3$ (1.33-104.0)</td>
<td>10.9 x 10$^3$ (2.67-36.8)</td>
<td>0.672</td>
</tr>
<tr>
<td>PPLT (s)$^3$</td>
<td>73 (68-81)</td>
<td>79 (69-82)</td>
<td>0.519</td>
</tr>
<tr>
<td>Total Xa generation (pg/mL)</td>
<td>2.16 (0.74-9.4)</td>
<td>0.91 (0.64-1.37)</td>
<td>0.091</td>
</tr>
<tr>
<td>TF-dependent Xa generation</td>
<td>0.82 (0.25-6.9)</td>
<td>0.21 (0.04-0.35)</td>
<td>0.016</td>
</tr>
<tr>
<td>FGT (s)$^4$</td>
<td>801 (769-1121)</td>
<td>1568 (1036-3008)</td>
<td>0.014</td>
</tr>
<tr>
<td>TF-dependent FGT (%)</td>
<td>13 (6 - 31)</td>
<td>0 (-12 -11)</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Data are presented as median (IQR); $^1$sTF: non-cell bound TF; $^2$Number/mL; $^3$PPLT: phospholipid dependent clotting time; $^4$FGT: Fibrin Generation Time

| DISCUSSION |

Our present study demonstrates a hypercoagulable state in cancer patients, as reflected by increased plasma levels of coagulation activation markers compared to controls. Although higher levels of microparticles were present in patient plasma samples, this increase was insufficient to shorten the clotting time in a strictly phospholipid-dependent (PPL) based clotting test. Also no differences were present with regard to non-cell bound TF antigen or activity. Our findings on the comparable levels of TF-microparticles and non-cell bound TF are
confirmed by earlier data of Tesselaar et al (15). Although these researchers showed that the TF-microparticle coagulant activity was significantly higher in metastasized cancer patients compared to healthy controls, this result included seven patients with VTE at the moment of blood withdrawal. When these patients are excluded, the microparticle-dependent Xa-generation was comparable between cancer patients and healthy controls. In contrast to our results, myeloma patients were reported to have a higher MP-associated TF activity compared to healthy subjects (11). One possible explanation for this discrepancy is that underlying mechanisms of VTE in patients with solid and haematological cancers may be different.

Within our group of cancer patients, five patients developed VTE within six months. The plasma samples from these five patients showed no clear signs of coagulation activation or differences in numbers of microparticles prior to the VTE compared to the other cancer patients. Also, there were no differences between the levels of non-cell bound TF or the levels of TF-bearing microparticles. Nevertheless, the TF-dependent coagulant activity of microparticles in the plasma samples of these five patients was markedly increased already weeks to months before the onset of VTE, pointing to the prothrombotic nature of these microparticles. Two earlier studies showed that higher levels of MP-associated TF activity are present in plasma samples of cancer patients with VTE compared to patients without VTE, but the blood samples in these studies were collected after the diagnosis of VTE (14;15). This is illustrated by a case report, in which del Conde and coworkers showed that a 55 year old patient with giant-cell lung carcinoma developed eleven major arterial and venous thrombotic events despite antithrombotic therapy. This patient, who suffered from a severe form of “Trousseau’s syndrome”, had an extremely elevated plasma level of TF, which was entirely associated with microparticles. Recently, the microparticle-associated TF activity in plasma samples of patients with pancreatic cancer was suggested to be associated with development of VTE (17). Our present results extend these findings.

The comparison between the cancer patients developing or not developing VTE raises two questions. First, what is the reason for the discrepancy between the microparticle levels and the microparticle procoagulant activity? Possibly, this is due to the much lower sensitivity of TF antigen measurements compared to the clotting activity measurements, implying that low levels of (TF-bearing) microparticles may already significantly affect coagulation. Alternatively, one may speculate that only a minor fraction of the TF exposed by microparticles is in the coagulant form, whereas most of this TF may be in an encrypted form, or that there is an additional factor in the plasma, which modulates the TF-activity, e.g. tissue factor pathway inhibitor. The second discrepancy found, is that there is a clear in vitro hypercoagulable state, while there is no evidence supporting an in vivo hypercoagulable state. These data may fit within the concept proposed by Falati et al., who showed that despite high levels of circulating microparticles no thrombus formation occurs when the vessel wall is intact. Upon vascular damage, however, the circulating TF-bearing microparticles, which also expose the ligand PSGL-1 (P-selectin glycoprotein ligand-1) for P-selectin, can adhere to the damaged vessel wall and thereby initiate thrombus formation.
If true, this may implicate that even high levels of circulating microparticles, even TF-bearing microparticles, are not prothrombotic per se, but additionally require vessel wall damage, e.g. induced by chemotherapy, to develop VTE.

There are several limitations of the present study that should be mentioned. Compared to the cancer patients, the controls were not matched for age or gender, and their blood was collected by venapuncture and not via a venous catheter. Although the differences in plasma concentrations of F$_{1+2}$, TAT and D-dimer are totally in line with the available literature, the pre-analytical variables may have contributed to the observed effects (22-25). Although our findings reached statistical significance and are confirmed using different coagulation assays, the relatively small number of patients and events limits the robustness of our findings and therefore additional studies are necessary.

Taken together, our present findings confirm our hypothesis that measuring the microparticle-associated TF activity in plasma samples of cancer patients may facilitate the identification of cancer patients at risk of developing VTE. In contrast, our present data do not confirm the hypothesis that circulating microparticles have an important contribution to the hypercoagulable state in cancer patients in general.

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AUTHORS CONTRIBUTION

The authors FFvD, RJB, PWK, HRB, AS and RN contributed to the design of the study. FFvD, RJB, AK, NM, and DM were responsible for data collection. The analyses were performed by FFvD. The interpretation of the results and the writing of the manuscript were performed by all authors.

REFERENCE LIST