

# Coagulation activation and microparticle-associated coagulant activity in cancer patients

## An exploratory prospective study

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### Summary

Cancer increases the risk of venous thromboembolism (VTE). Here, we investigated the contribution of microparticle (MP)-dependent procoagulant activity to the prothrombotic state in these patients. In 43 cancer patients without VTE at study entry and 22 healthy volunteers, markers of *in vivo* and MP-dependent coagulation were measured and patients were prospectively followed for six months for the development of VTE. Procoagulant activity of MPs 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). Markers of *in vivo* coagulation activation and total number of MPs at baseline were significantly elevated in cancer patients compared to controls ( $F_{1+2}$  246 vs. 156 pM, 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.  $3.37 \times 10^6$  MPs/ml). Five patients

(11.6%) developed VTE. Patients with VTE had comparable levels of coagulation activation markers and phospholipid-dependent MP 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. In this exploratory study the overall hypercoagulable state in cancer patients was not associated directly with the MP phospholipid-dependent procoagulant activity. However, in the patients who developed VTE within six months when compared to those who did not, an increased MP procoagulant activity was present already at baseline, suggesting this activity can be used to predict VTE.

### Keywords

Cancer, venous thromboembolism, microparticle-dependent coagulant activity

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Received: February 20, 2012

Accepted after major revision: March 29, 2012

Prepublished online: April 26, 2012

doi:10.1160/TH12-02-0099

Thromb Haemost 2012; 108: 160–165

## Introduction

Venous thromboembolism (VTE) often complicates the clinical course of cancer (21). It is estimated that almost 15% of all cancer patients develop VTE in the course of their disease (17). Several factors including surgery, central lines, infection, immobilisation, chemotherapy, hormonal agents and cancer cells themselves contribute to the hypercoagulable state in cancer patients (4). 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  $F_{1+2}$  compared to healthy subjects (11). Since many cancers express high levels of TF (9), 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 (MPs) present in blood collected from cancer patients was confirmed by multiple research groups (1, 10, 13, 25) but for a long time evidence was lacking to show that these TF-bearing MPs 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 (22). A recent study by Davila et al. showed that at least part of the circulating TF-bearing MPs orig-

inate from the cancer cells (5). They used a mouse model with a human tumour, and showed that MPs bearing human TF are present in the mouse blood, demonstrating that a tumour can release TF-bearing MPs *in vivo* that can enter the blood. Consistent with this concept, part of the TF-bearing MPs 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 MPs. The numbers of the MPs bearing both TF and MUC-1 decreased more than 10-fold or below detection levels after the tumour had been surgically removed (25).

There is increasing evidence that circulating MPs are associated with the prothrombotic state in cancer patients. Plasma from cancer patients with VTE contains increased MP-associated TF activity compared to patients without VTE (15, 18, 24). 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 (12). Based on these observations, we hypothesise that circulating MPs contribute to the 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, MP-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 mM buffered sodium citrate (BD, Franklin Lakes, NJ, USA). 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 (min) after blood collection, cells were removed by centrifugation for 20 min at 1,550 x g and 20°C. Part of the plasma was immediately used for coagulation assays. The re-

mainder was immediately frozen as 0.25 ml aliquots in liquid nitrogen and stored at -80°C.

### Isolation of MPs for flow cytometry

Isolation of MPs for flow cytometry was performed as described previously (2). Briefly, a sample of 250 µl frozen plasma was thawed on melting ice for 1 hour (h) and centrifuged for 30 min at 18,890 x g and 20°C to pellet MPs. 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 MP pellet plus remaining supernatant was then resuspended with 75 µl phosphate-buffered saline (PBS)-citrate. Five µl of the MP suspension was diluted in 35 µl CaCl<sub>2</sub> (2.5 mM)-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 analysed in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software version 4.02 (Becton Dickinson Biosciences, San Jose, CA, USA). The antibodies against fluorescein isothiocyanate (FITC)-labelled IgG<sub>1</sub> and phycoerythrin (PE)-labelled IgG<sub>1</sub> 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 (FX)-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 (20). Fresh MP-containing plasma (25 µl) was mixed with phospholipid-free plasma (25 µl) and pre-heated for 2 min at 37°C in a cuvette. Then, pre-warmed XACT reagent (100 µl; containing activated FX and calcium) was added and the clotting time was measured on an ACL Top coagulation analyser (Instrumentation Laboratory Company; Lexington, MA, USA).

### FXa generation assay

The TF-dependent and total MP-dependent coagulant activity were measured using a FXa generation assay exactly as described (12). In brief, MPs were pelleted from plasma (200 µl) by centrifugation (20,000 x g for 15 min at 4°C), washed twice with HBSA (Hepes buffered saline albumin) buffer (137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, 10 mM 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 min 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 nM), FX (300 nM) and CaCl<sub>2</sub> (10 mM) was added to each sample and the mixture was incubated for 2 h at 37°C. FXa generation was stopped by addition of 25 mM EDTA buffer (25 µl), chromogenic substrate S2765 added (25 µl; 4 mM), and the mixture incubated at 37°C for 15 min. Finally, absorbance was measured at 405 nm using a VER-SAmax 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; inter-assay coefficient of variation of 21%).

### Fibrin generation test (FGT)

The ability of MPs to generate fibrin was measured directly in plasma in the absence or presence of anti-human FVIIa (Sanquin; Amsterdam, The Netherlands), as described previously (3). After pre-incubation for 5 min at 37°C, clotting was initiated by addition of CaCl<sub>2</sub>. 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, USA) at 37°C. We used an anti-FVII(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, CT, USA). This kit measures full length as well as truncated TF. F<sub>1+2</sub> 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 interquartile 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  $\leq 0.05$  were considered

statistically significant. All statistical analyses were performed by using SPSS 15.0.1 (SPSS Inc, Chicago, IL, USA).

## Results

### Patients

A total of 43 cancer patients and 22 healthy controls were included. The mean age of the cancer patients was  $59 \pm 12$  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  $38 \pm 10$  years, 32% were men.

### Coagulation and MPs in cancer patients and healthy individuals

Compared to healthy individuals, cancer patients had higher plasma concentrations of F<sub>1+2</sub>, 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 MPs, the numbers of annexin V-binding MPs were increased in patients compared to controls ( $p = 0.001$ ), but the numbers of TF-bearing MPs were non-significantly decreased ( $p = 0.186$ ). Subsequently, we tested the MP-associated coagulant activity using three different MP-based coagulation assays. First, the ability of MPs 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, personal communication), was similar between the cancer patients and controls ( $p = 0.411$ ; ► Table 1). These results were not anticipated considering the higher number of MPs in plasma samples from cancer patients binding annexin V. Second, we tested the ability of MPs to generate FXa. There was a trend towards a higher FXa generation capacity associated with MPs from cancer patients when compared to healthy controls ( $p = 0.066$ ). The TF-dependent FXa generation was comparable between the patients and controls. Similarly, we also found no significant differences between MPs from patients and controls with regard to fibrin generation in the absence or presence of anti-FVIIa ( $p = 0.417$  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

**Table 1: Coagulation and microparticles in cancer patients and healthy individuals.**

|   | Cancer patients                       | Healthy individuals                   | P-value |
|---|---------------------------------------|---------------------------------------|---------|
| F <sub>1+2</sub> (pM)                     | 246<br>(181–319)                      | 156<br>(139–198)                      | <0.001  |
| TAT (mg/l)                                | 4.1<br>(3.2–7.8)                      | 3.0<br>(2.5–3.4)                      | <0.001  |
| D-dimer (mg/l)                            | 0.76<br>(0.40–2.26)                   | 0.22<br>(0.17–0.54)                   | <0.001  |
| sTF (pg/ml) <sup>1</sup>                  | 92.6<br>(20.7–170.9)                  | 66.0<br>(30.4–124.9)                  | 0.163   |
| MP (Annexin V <sup>+</sup> ) <sup>2</sup> | 5.53 x 10 <sup>6</sup><br>(3.33–8.72) | 3.37 x 10 <sup>6</sup><br>(2.94–4.90) | 0.001   |
| MP (TF <sup>+</sup> ) <sup>2</sup>        | 11.0 x 10 <sup>3</sup><br>(2.7–38.6)  | 39.8 x 10 <sup>3</sup><br>(0.0–29.7)  | 0.186   |
| PPLT (s) <sup>3</sup>                     | 78<br>(69–82)                         | 78<br>(73–85)                         | 0.411   |
| Total Xa generation (pg/ml)               | 0.98<br>(0.64–1.43)                   | 0.75<br>(0.54–0.95)                   | 0.066   |
| TF-dependent Xa generation (pg/ml)        | 0.21<br>(0.05–0.36)                   | 0.26<br>(0.06–0.41)                   | 0.899   |
| FGT (s) <sup>4</sup>                      | 1386<br>(968–2852)                    | 1299<br>(930–1776)                    | 0.417   |
| TF-dependent FGT (%)                      | 0<br>(-13–9)                          | -6<br>(-12–1)                         | 0.349   |

Data are presented as median (IQR); <sup>1</sup>sTF: non-cell bound TF; <sup>2</sup>Number/ml; <sup>3</sup>PPLT: phospholipid-dependent clotting time; <sup>4</sup>FGT: fibrin generation time.

**Table 2: Coagulation and microparticles in cancer patients developing VTE.**

|   | Cancer patients + VTE                  | Cancer patients – VTE                 | P-value |
|---|--|---------------------------------------|---------|
| F <sub>1+2</sub> (pM)                     | 319 <sup>1</sup><br>(187–558)          | 241<br>(179–312)                      | 0.427   |
| TAT (mg/l)                                | 5.7<br>(3.5–13.7)                      | 4.1<br>(3.2–6.2)                      | 0.290   |
| D-dimer (mg/l)                            | 0.66<br>(0.55–7.5)                     | 0.77<br>(0.38–2.0)                    | 0.326   |
| sTF (pg/ml) <sup>1</sup>                  | 145.5<br>(49.0–369.0)                  | 86.8<br>(50.7–153.6)                  | 0.445   |
| MP (Annexin V <sup>+</sup> ) <sup>1</sup> | 5.53 x 10 <sup>6</sup><br>(5.06–9.84)  | 5.51 x 10 <sup>6</sup><br>(3.27–8.92) | 0.629   |
| MP (TF <sup>+</sup> ) <sup>2</sup>        | 34.8 x 10 <sup>3</sup><br>(1.33–104.0) | 10.9 x 10 <sup>3</sup><br>(2.67–36.8) | 0.672   |
| PPLT (s) <sup>3</sup>                     | 73<br>(68–81)                          | 79<br>(69–82)                         | 0.519   |
| Total Xa generation (pg/ml)               | 2.16<br>(0.74–9.4)                     | 0.91<br>(0.64–1.37)                   | 0.091   |
| TF-dependent Xa generation                | 0.82<br>(0.25–6.9)                     | 0.21<br>(0.04–0.35)                   | 0.016   |
| FGT (s) <sup>4</sup>                      | 801<br>(769–1121)                      | 1568<br>(1036–3008)                   | 0.014   |
| TF-dependent FGT (%)                      | 13<br>(6–31)                           | 0<br>(-12–11)                         | 0.036   |

Data are presented as median (IQR); <sup>1</sup>sTF: non-cell bound TF; <sup>2</sup>Number/ml; <sup>3</sup>PPLT: phospholipid dependent clotting time; <sup>4</sup>FGT: fibrin generation time.

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 metastasised disease. One patient had metastatic gastrointestinal cancer and the remaining patient had locally advanced oesophagus cancer.

The concentrations of  $F_{1+2}$ , 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 2). Also, plasma concentrations of non-cell bound TF – i.e. MP 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.

### MPs and MP-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 MPs binding annexin V or MPs bearing TF. Subsequently, we tested the MP-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 2). In contrast, there were marked differences in the ability of MPs to promote coagulation in the two other assays. First, in patients who developed VTE the MPs showed a trend towards an increased FXa generation, compared to MPs from patients who did not develop VTE (median 2.16 [IQR 0.74–9.4] vs. 0.91 [0.64–1.37]; p=0.091). Furthermore, MPs from patients who developed VTE within six months showed a marked increase in the TF-dependent FXa generation compared to MPs from patients not developing VTE (p=0.016). Similarly, the (MP-dependent) clotting of the FGT was markedly faster in plasma samples from patients developing VTE compared to samples from patients not developing VTE (801 seconds vs. 1,568 seconds; p=0.014). Finally, the relative change in the FGT by addition of the anti-FVIIa antibody was markedly higher in those patients who developed VTE within six months compared to those who did not (13.3% vs. 0%, respectively; p=0.036). These data point to a significant contribution of TF-bearing MPs to the prothrombotic state in cancer patients, and also indicate that this activity may be detectable already before the onset of VTE.

## 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 MPs were present in patient plasma samples, this increase was in-

sufficient 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-MPs and non-cell bound TF are confirmed by earlier data of Tesselaar et al. (18). Although these researchers showed that the TF-MP coagulant activity was significantly higher in metastasised 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 MP-dependent FXa-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 (1). 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 MPs 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 MPs. Nevertheless, the TF-dependent coagulant activity of MPs 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 MPs. 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 (18, 24). This is illustrated by a case report, in which del Conde et al. showed that a 55-year-old patient with giant-cell lung carcinoma developed 11 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 MPs. Recently, the MP-associated TF activity in plasma samples of patients with pancreatic cancer was suggested to be associated with development of VTE (12). 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 MP levels and the MP 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) MPs may already significantly affect coagulation. Alternatively, one may speculate that only a minor fraction of the TF exposed by MPs 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 MPs no thrombus formation occurs when the vessel wall is intact. Upon vascular damage, however, the circulating TF-bearing

MPs, 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 (8). If true, this may implicate that even high levels of circulating MPs, even TF-bearing MPs, 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 (14, 16, 19, 23). 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 MP-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 MPs have an important contribution to the hypercoagulable state in cancer patients in general.

#### Acknowledgements

We kindly like to thank Marianne Schaap, Anita Grootemaat, Anita Böing and Laura Splint for technical assistance. Also, we thank the Trombose Stichting Nederland for their financial support (TSN 2010–2).

#### Conflicts of interest

None declared.

#### What is known about this topic?

- Venous thrombosis is a frequently occurring complication in cancer patients.
- Cancer patients exhibit increased coagulation activation compared to healthy subjects.
- Cancer patients have increased levels of microparticles (MPs).
- Cancer patients presenting with venous thrombosis have higher levels of tissue factor bearing MPs compared to cancer patients without venous thrombosis.

#### What does this paper add?

- Although elevated levels of MPs are present in blood from cancer patients, such MPs are not necessarily associated with an increased procoagulant activity.
- Cancer patients who will or who will not develop venous thrombosis show no differences in concentrations of coagulation activation markers or MP numbers.
- Cancer patients who will develop venous thrombosis do have an increased tissue factor / factor VIIa coagulant activity already months before the onset of venous thrombosis when compared to cancer patients who will not develop venous thrombosis.

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