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Extracellular vesicles exposing tissue factor for the prediction of venous thromboembolism in patients with cancer: A prospective cohort study

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ABSTRACT

Introduction: The procoagulant activity of extracellular vesicles (EV) exposing tissue factor (TF) is a promising biomarker for venous thromboembolism (VTE) in cancer patients. We evaluated an in-house EV-TF activity assay (the fibrin generation test) for the prediction of cancer-associated VTE. We also compared the results with the fibrin generation tests to an EV-TF-dependent factor Xa generation assay in samples from pancreatic cancer patients.

Materials and methods: Data collected in a multinational, prospective cohort study were used. Patients with various types of advanced cancer were enrolled if chemotherapy was scheduled or started in the previous 3 months. Patients were followed for 6 months for the occurrence of VTE. The fibrin generation test was performed at baseline to measure EV-TF procoagulant activity.

Results: The fibrin generation test was performed in 648 patients with advanced cancer. The mean age was 62 years; 58% had distant metastasis. Forty patients (6.1%) developed VTE. Overall, a high fibrin generation test result was associated with a two-fold increased risk for VTE (HR 2.0; 95%-CI, 1.1–3.6). The association was stronger in patients with pancreatic cancer (HR 4.1; 95%-CI, 0.91–19) than in those with other tumor types (HR 1.5; 95%-CI, 0.72–3.1). Correlation between the FGT and the TF-dependent factor Xa generation assay in patients with pancreatic cancer was poor (Spearman's $R = 0.35$).

Conclusion: This study shows that a high EV-TF procoagulant activity as measured by the fibrin generation test is associated with an increased risk of VTE in cancer patients, in particular in those with pancreatic cancer. Future studies should aim to further improve the feasibility and accuracy of EV-TF activity assays.

1. Introduction

Venous thromboembolism (VTE), which encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE), is a frequent complication in cancer patients with an estimated overall incidence rate of about 4 per 100 person-years, although the rate may vary substantially dependent on cancer type and stage [1]. Nevertheless, current guidelines recommend against routine thromboprophylaxis in ambulatory cancer patients, because the risk of VTE is deemed too low to justify the burden and risks of daily subcutaneous injections with low-molecular-weight heparin [2–4].

Risk stratification tools may aid in selecting high-risk patients in whom the benefits of thromboprophylaxis outweigh the risks. With that aim, the predictive performance of various prediction scores and biomarkers has been and are being evaluated in patients with cancer. One biomarker that has gained attention is the coagulant activity of extracellular vesicles (EV) exposing the procoagulant protein tissue factor (TF).

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Extracellular vesicles (EV) are submicron vesicles that are shed by various types of cells, including tumor cells [5]. Tumor-derived EV exposing TF (EV-TF) have been proposed to contribute to cancer-associated VTE [6]. The transmembrane protein TF, which is the main initiator of the coagulation pathway, is overexpressed in various types of cancer and can promote tumor growth and metastasis [7–9].

When tumor cells release EV-TF into the blood, these TF-positive EVs can activate coagulation and predispose cancer patients to VTE. Not surprisingly, TF is strongly upregulated in cancer types that are associated with a high risk of VTE, such as pancreatic cancer [10]. Since EV-TF are thought to be causally implicated in thrombus formation, they are potential markers of the risk of VTE in cancer patients.

Evidence for the performance of the EV-TF procoagulant activity in predicting VTE in cancer patients has been conflicting [11–15], but the association appears to be strongest in patients with pancreatic cancer [13,15]. We have previously developed an in-house assay, the fibrin generation test, which measures the procoagulant activity of EV-TF in fresh citrated plasma [16]. In a large, prospective cohort study, we sought to (1) evaluate the performance of this fibrin generation test in predicting VTE in patients with different types of cancer, (2) evaluate its performance in predicting VTE specifically in patients with pancreatic cancer, and (3) compare the results of the fibrin generation test to another EV-TF activity assay, namely the TF-dependent factor Xa generation test in samples from pancreatic cancer patients.

2. Methods

2.1. Study design and eligible patients

Data were collected in a multinational, prospective cohort study that was design to evaluate clinical predictors and biomarkers for VTE in cancer patients. Patients were enrolled between July 2008 and February 2016 in seven centers in The Netherlands, Italy, Mexico, and France. Ambulatory patients were eligible if they had locally advanced or metastatic cancer of the esophagus, lung, colon, pancreas, breast, stomach, ovaries, prostate, or bladder. Patients were to be scheduled for chemotherapy within 7 days, or had to have started chemotherapy in the previous 3 months. Exclusion criteria included the use of anticoagulants and adjuvant chemotherapy. The study was approved by the institutional review boards of all participating hospitals. Included patients provided written informed consent. The study was registered in ClinicalTrials.gov (identification number: NCT02095925).

2.2. Study procedures and outcome

At baseline, healthcare professionals collected clinical and laboratory data using a standardized case report form. Blood was collected in 0.109 M citrated plastic tubes *via* antecubital vein puncture or from a peripheral catheter directly after placement. The first tube was discarded. Within 1 h after blood withdrawal, platelet poor plasma was prepared by centrifugation at 1560 × *g* for 20 min at 20 °C. Fresh plasma was immediately used for the fibrin generation test, while the remaining plasma was snap frozen and stored at −80 °C.

Patients were followed for a maximum of 180 days by clinic visits or through telephone and/or chart review. The primary outcome was the composite of objectively confirmed symptomatic or incidental PE, distal or proximal leg DVT, or non-catheter-related upper extremity DVT, or symptomatic catheter-related upper extremity DVT. VTE was considered incidental if diagnosed on imaging performed for other reasons than suspected VTE. Imaging reports of all potential outcomes were verified by two of the authors who were blinded to possible predictors of VTE, including biomarker results. Deaths were not adjudicated routinely for fatal PE, but PE was considered to be fatal only if the autopsy confirmed PE or in case of an objective test positive for PE prior to death.

The targeted sample size of the original study was 800 to 1000 patients based on the assumption of a 5% to 6% absolute risk of VTE during 6 months follow-up. This would result in 50 observed events, enabling robust multivariable modelling of the Khorana score. The present analysis focuses on the group of patients in whom the fibrin generation test was measured.

2.3. Fibrin generation test

In six of seven centers, the fibrin generation test was performed on fresh, platelet poor plasma within 1 h after blood withdrawal. This test was developed to measure the procoagulant activity of EV-TF as previously described [16]. Briefly, samples were incubated for 5 min at 37 °C in duplicate with either saline (control) or an anti-human factor VIIa antibody (Sanquin, Amsterdam, The Netherlands) to block TF/factor VIIa-mediated coagulation. After recalcification, the time to fibrin formation (½ max) was measured using optical densitometry (λ = 405 nm) for a maximum of 1 h (SPECTRAmax microplate reader, Molecular Devices, Sunnyvale, CA). The procoagulant activity of the EV-TF was expressed as the relative prolongation in the time to fibrin formation in the samples incubated with the anti-factor VIIa antibody compared to the saline-incubated samples. A fibrin generation test result of 13% inhibition by anti-human factor VIIa or more was considered to indicate a high EV-TF activity. This pre-defined cut-off was based on the median time to fibrin generation test in a previous exploratory study [12]. Prior to start of the study, the six participating centers were provided with reference plasma samples that were used to confirm the reproducibility of the assay.

2.4. Tissue factor dependent factor Xa generation assay

The factor Xa generation assay has been previously described in detail [17].

2.5. Statistical analyses

2.5.1. Fibrin generation test

The discriminatory performance of the continuous fibrin generation test result in predicting VTE was evaluated by calculating the area under the receiver operating characteristic (ROC)-curve. The 95% confidence intervals (CIs) were estimated using DeLong's method.

For the dichotomized fibrin generation test result at the pre-defined cut-off of 13%, we evaluated the incidences in patients with a “low” and “high” fibrin generation test result, *i.e.* < 13% or ≥13% prolongation of the time to fibrin formation in the presence of anti-human factor VIIa. The cumulative incidence of VTE at 180 days in patients with a high and low fibrin generation test result were estimated in a competing risk analysis. Time to VTE was censored when patients underwent curative cancer surgery, started therapeutic anticoagulation for other reasons than VTE, were lost to follow-up, or at the end of 6-month follow-up, while death was treated as a competing risk. The 95% CIs were calculated using Choudhury's method [18]. The difference in cumulative VTE incidence between patients with a low and high fibrin generation test result was assessed by Gray's test and quantified by estimating a subdistribution hazard ratio (SHR) with 95% CI using the competing risk regression model proposed by Fine and Gray [19]. A multivariable analysis adjusting for age, sex, distant metastasis, D-dimer levels (log-transformed), and the Khorana score was performed to evaluate whether the fibrin generation test remained predictive conditional on these variables. To explore potential bias associated with missing data, a sensitivity analysis using multiple imputation (20×) for missing fibrin generation test results was performed. We also performed a sensitivity analysis in which events occurring in the first 3 months were considered.

2.5.2. Fibrin generation test in pancreatic cancer vs. other tumor types

Previous studies have shown that the procoagulant activity of EV-TF

is higher in patients with pancreatic cancer than in those with other types of cancer, and that higher EV-TF procoagulant activity may be a predictor of VTE and poor survival particularly in these patients [20]. Therefore, the discriminatory performance of the continuous and dichotomized fibrin generation test result was evaluated separately in patients with pancreatic cancer and those with other types of cancer in the present study. The difference in area under the ROC-curve between these two groups was assessed using DeLong's test.

2.5.3. Fibrin generation test vs TF-dependent factor Xa generation assay

In a subsequent analysis of patients with pancreatic cancer, the fibrin generation test was compared to the TF-dependent factor Xa generation assay. The correlation between the (log-transformed) assay results was plotted and assessed by Pearson's correlation coefficient. The discriminatory performance of the factor Xa generation assay in predicting VTE was assessed by calculating the area under the ROC-curve for the continuous test result and the SHR for the dichotomized test result (positivity cut-off 0.5 pg/mL).

A *P*-value below 0.05 was considered statistically significant. All analyses were performed in R, versions 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria; www.R-project.org), in particular using the *cmprsk* v2.2-7 for the competing risk analyses and *pROC* v1.8 for the ROC-curve analyses.

3. Results

The fibrin generation test was performed in 648 of 855 cancer patients (76%) enrolled in six centers. The mean age was 62 years, 57% were male, and 58% had distant metastasis. Most patients were enrolled during chemotherapy (*N* = 473; 72%). Patient characteristics are summarized in Table 1.

During the 6-month follow-up period, 40 patients (6.1%) developed VTE of whom 21 had PE only, 14 proximal DVT only, 2 PE with concomitant DVT, 2 upper extremity DVT, and 1 isolated distal DVT. Twenty-five events (63%) were symptomatic. Two events (5%) were fatal. The cumulative incidence at 180 days was 6.6% (95% CI, 4.8 to 8.8) in the competing risk analysis. The median time to VTE was 52 days (interquartile range [IQR], 34 to 106). During a median follow-up of 180 days (IQR, 97 to 180), 3 patients were lost to follow-up (0.5%) and 154 (24%) died.

Table 1
Patient characteristics.

Characteristic	All patients (*N* = 648)	No VTE (*N* = 608)	VTE (*N* = 40)	*P*-value
Mean age (SD), years	62 (11)	62 (11)	64 (11)	0.50
Male, *n* (%)	367 (57)	344 (57)	23 (58)	1.0
Mean body mass index (SD), kg/m^2	25 (4)	25 (4)	24 (4)	0.91
WHO performance status ≥ 2, *n* (%)	49 (7.6)	46 (8.1)	3 (8.1)	1.0
Previous VTE, *n* (%)	11 (1.7)	11 (1.8)	0 (0)	0.82
Antiplatelet therapy	87 (13)	86 (15)	1 (2.6)	0.06
Tumor type, *n* (%)				0.001
Esophagus	158 (24)	146 (24)	12 (30)	
Lung	120 (19)	113 (19)	7 (18)	
Colorectal	115 (18)	108 (18)	7 (18)	
Pancreas	100 (15)	90 (15)	10 (25)	
Breast	84 (13)	84 (14)	0 (0)	
Gastric	31 (4.8)	31 (5.1)	0 (0)	
Ovarian	19 (2.9)	18 (3.0)	1 (2.5)	
Prostate	13 (2.0)	13 (2.1)	0 (0)	
Bladder	8 (1.2)	5 (0.8)	3 (7.5)	
Cancer stage, *n* (%)				0.13
Stage II	38 (5.9)	38 (6.3)	0 (0)	
Stage III	236 (36)	224 (37)	12 (30)	
Stage IV	373 (58)	345 (57)	28 (70)	
Neoadjuvant chemotherapy, *n* (%)	125 (19)	122 (30)	3 (10)	0.037

Abbreviations: EV, extracellular vesicles; FOLFIRINOX, 5-fluorouracil, irinotecan, and oxaliplatin; IQR, interquartile range; SD, standard deviation; TF, tissue factor.

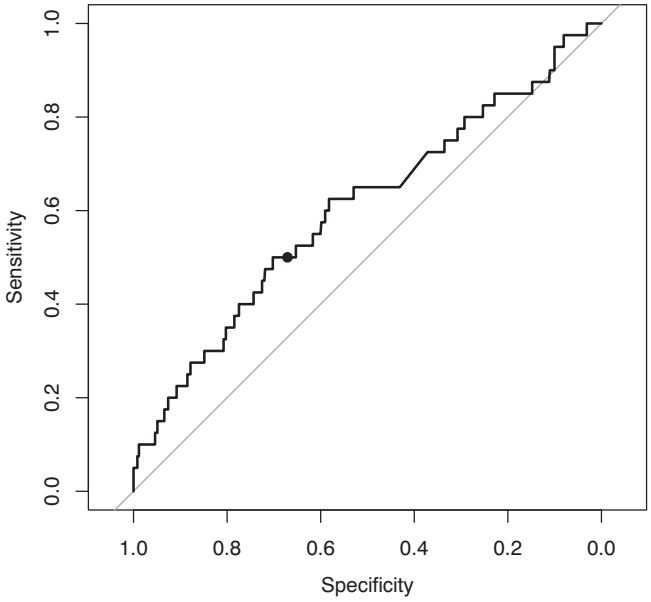

Fig. 1. Receiver operating characteristic curve of fibrin generation test result.
Fig. 1 legend. The dot marks the sensitivity and specificity at the pre-defined 13% cut-off. The sensitivity and specificity of the fibrin generation test were 50% (95% CI, 35 to 65) and 67% (95% CI, 63 to 71), respectively.

3.1. EV-TF activity measured with the fibrin generation test

The median fibrin generation test result was 3.3% overall (IQR, −8.0 to 21), indicating that in half of patients the time to fibrin generation was prolonged by 3.3% or more in the presence of the anti-factor VIIa antibody. The area under the ROC-curve of the fibrin generation test, reflecting its discriminatory performance in predicting VTE, was 0.60 (95% CI, 0.50 to 0.70; Fig. 1). When only considering the 27 events occurring in the first 3 months, the area under the ROC-curve was 0.65 (95% CI, 0.54 to 0.76).

Of the 220 patients (34%) with a high fibrin generation test result, 20 (9.0%) developed VTE (cumulative incidence at 180 days, 9.7%; 95% CI, 6.1 to 14) compared to 20 of 428 patients (4.9%) with a low fibrin generation test result (cumulative incidence at 180 days, 5.2%; 95% CI, 3.2 to 7.7). This corresponded to a SHR of 2.0 (95% CI, 1.1 to 3.6; Fig. 2A). The association did not change in a multivariable analysis adjusted for age, sex, presence of distant metastasis, D-dimer levels, and the Khorana score (SHR 1.9; 95% CI, 0.99 to 3.8; Supplementary Table), when only events occurring in the first 3 months were considered (SHR, 2.4; 95% CI, 1.1 to 5.2), nor in the sensitivity analysis using multiple imputation for missing values (SHR 1.8; 95% CI, 1.02 to 3.3).

3.2. Fibrin generation test in patients with pancreatic cancer vs. other types of cancer

The area under the ROC-curve of the fibrin generation test was 0.68 (95% CI, 0.52 to 0.84) in pancreatic cancer patients compared to 0.56 (95% CI, 0.44 to 0.68) in patients with other types of cancer (*P* = 0.24 for difference). Fifty of 100 patients (50%) with pancreatic cancer had a high fibrin generation test result, compared to 170 of 548 patients (31%) with other types of cancer (*P* < 0.001). Among patients with pancreatic cancer, a high fibrin generation test result was associated with a 4-fold increased risk of VTE (SHR 4.1; 95% CI, 0.91 to 19;

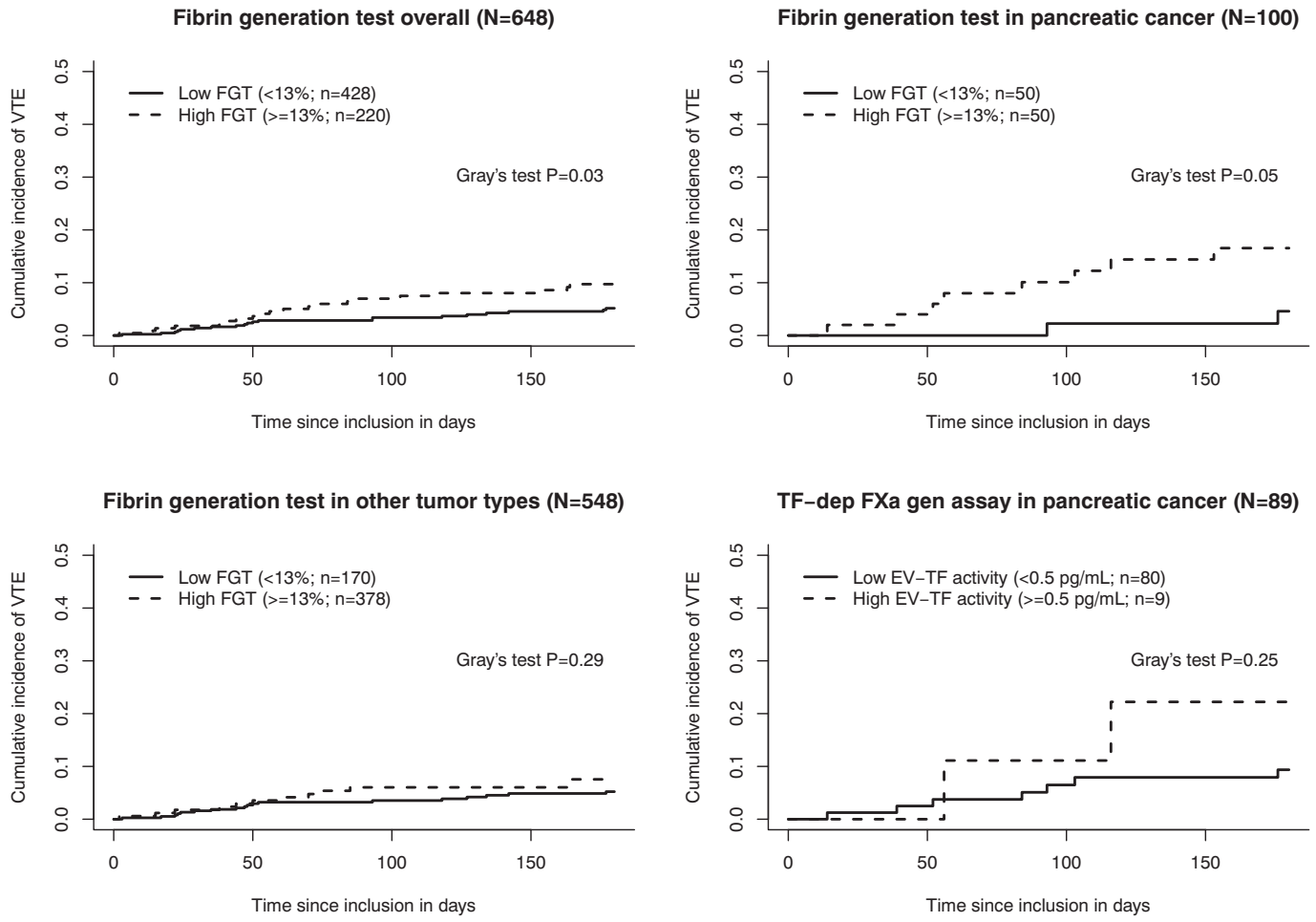

Fig. 2. A–D. Cumulative incidence of venous thromboembolism in patients with a low and high fibrin generation test result or EV-TF activity. Abbreviations: EV, extracellular vesicles; FGT, fibrin generation test; TF, tissue factor.

Fig. 2B), whereas it was associated with a 1.5-fold increased risk in patients with other types of cancer (SHR 1.5; 95% CI, 0.72 to 3.1; Fig. 2C). The subdistribution hazard ratios in patients with esophageal (N = 158), lung (N = 120), and colorectal cancer (N = 115) were 1.7 (95% CI, 0.57 to 5.3), 1.0 (95% CI, 0.19 to 5.1), and 0.91 (95% CI, 0.18 to 4.7), respectively. The cumulative incidence of VTE at 180 days was 17% (95% CI, 7.6 to 28) in pancreatic cancer patients with a high fibrin generation test and 7.6% (95% CI, 4.1 to 12) in the patients with other types of cancer who had a high fibrin generation test.

3.2.1. Fibrin generation test vs. TF-dependent factor Xa generation assay

Plasma of 89 with pancreatic cancer was available for comparison of the fibrin generation with the TF-dependent factor Xa generation assay. Nine of these patients (10%) developed VTE over the 6-month follow-up period, corresponding to a cumulative incidence of 11% (95% CI, 5.2 to 19) at 180 days. No patients were lost to follow-up and 66 (74%) died.

The median fibrin generation test result in patients with pancreatic cancer was 15% (IQR, 0 to 46) and the median TF-dependent factor Xa generation assay result was 0 pg/mL (IQR, 0 to 0.05). There was a significant, but poor positive correlation between the results of the fibrin generation test and the TF-dependent factor Xa generation assay (Spearman correlation coefficient $r = 0.35$; $P < 0.001$; Fig. 3). When patients without any measurable EV-TF activity were excluded from the analysis, the correlation increased substantially (Spearman rho correlation coefficient $r = 0.63$; $P < 0.001$; Supplemental Fig. 1). A total of 48 patients (54%) had a high fibrin generation test result and 9 patients (10%) had a weak to strong EV-TF activity. The 9 patients with a weak to strong EV-TF activity also had a high fibrin generation test result.

The area under the ROC-curve was 0.62 (95% CI, 0.45 to 0.79) for the fibrin generation test and 0.50 (95% CI, 0.29 to 0.72) for the TF-dependent factor Xa generation assay (P = 0.42; Supplemental Fig. 2). Two of 9 patients (22%) with a weak to strong EV-TF activity based on the TF-dependent factor Xa generation assay developed VTE (cumulative incidence at 180 days, 22%; 95% CI, 2.4 to 54), compared to 7 of 80 patients (9%) with no EV-TF activity (8.8%; 95% CI 4.1 to 17; Fig. 2D), translating into a SHR of 2.5 (95% CI, 0.55–11).

4. Discussion

In the present study, high EV-TF procoagulant activity as assessed by the fibrin generation test was associated with a two-fold higher risk of VTE in patients with various types of advanced cancer. This association was predominantly driven by the strong association between a high EV-TF activity and VTE occurrence in patients with pancreatic cancer in whom there was a non-significant four-fold increased risk. Correlation between EV-TF activity measured by the fibrin generation test and the more commonly used TF-dependent Xa assay was poor.

High EV-TF activity as measured by the fibrin generation test was not significantly associated with VTE among patients with various types of cancer other than pancreatic cancer. This finding is in line with a prospective cohort study by Thaler and colleagues, who evaluated 358 cancer patients for the risk of VTE using a chromogenic TF-dependent factor Xa end-point assay [15]. They found that EV-TF activity was not

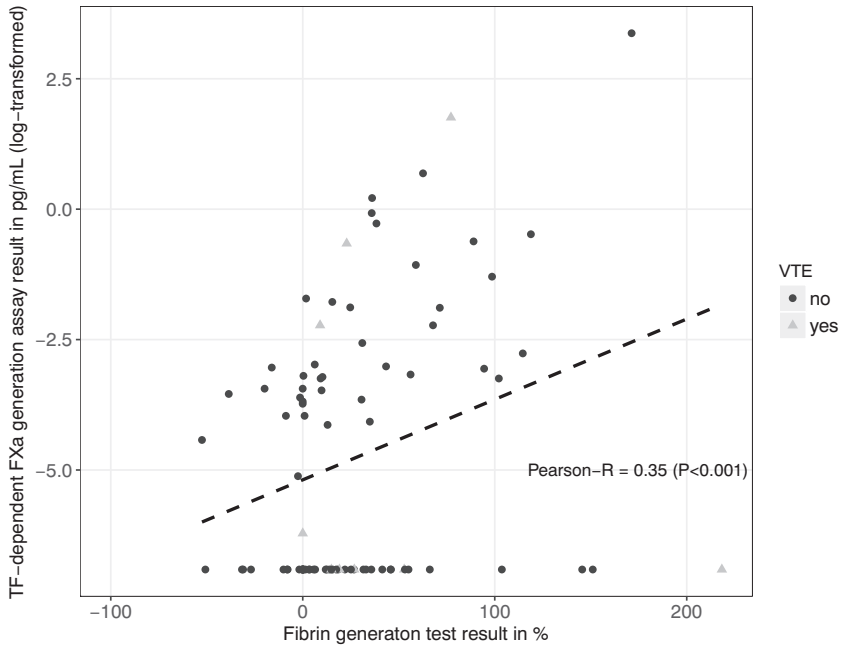

Fig. 3. Correlation between fibrin generation test and tissue factor-dependent factor Xa generation assay.
Abbreviations: FXa, factor Xa; TF, tissue factor; VTE, venous thromboembolism.
To enable log-transformation of EV-TF activity in the TF-dependent factor Xa generation assay, 0.001 pg/mL was added to test results of 0 pg/mL.

predictive of VTE in patients with brain, stomach, and colorectal cancer, whereas EV-TF activity appeared to be predictive of VTE in patients with pancreatic cancer for the end-point assay. Similarly, higher EV-TF activity was associated with VTE occurrence in a retrospective study of 117 patients with pancreaticobiliary tumors [13]. Taken together, these studies indicate that EV-TF activity remains a promising marker for subsequent VTE in patients with pancreatic but not other types of cancer. Interestingly, EV-TF activity remained a significant predictor of cancer-associated VTE conditional on the Khorana score, which indicates that EV-TF activity could be a valuable addition to this well-known prediction score.

TF concentrations in plasma under physiological and pathological conditions are often low, which makes TF measurement in plasma challenging. Over the past two decades, various quantitative (antigen-based) and qualitative (activity-based) assays have been used to measure EV-TF. In general, quantitative assays, such as enzyme-linked immunosorbent assays and flow cytometry, tend to have a low sensitivity and specificity and measure both cryptic (non-coagulant) and non-cryptic (coagulant) TF, which hampers the interpretation and usefulness of the results [21,22]. In the present study, we focused exclusively on a TF activity-based assay, essentially a plasma recalcification test, called the fibrin generation test.

Some limitations of the fibrin generation test need to be acknowledged. Performing the test can be a logistical challenge. The test should be performed on fresh plasma as soon as possible, but no later than 60 min following blood withdrawal mainly to circumvent platelet activation and subsequent release of platelet vesicles. In clinical practice, this procedure will put a strain on laboratory personnel because they must be readily available to perform the assay. The extent to which various pre-analytical factors, such as withdrawal technique (vein puncture *vs.* catheter), centrifugation protocol, and citrate tubes (plastic *vs.* glass) influence the test result, remains largely unknown [23]. For example, we used platelet-poor plasma for the test, which contains cell debris, apoptotic bodies, and platelets, which all can contribute to the coagulant activity. Moreover, we observed that the average variation in the fibrin generation test result between the duplicate samples gradually increased with longer times to fibrin generation (data not shown). Future refinements in the testing protocol may therefore focus on improving reproducibility and explore the possibility of freezing the samples before (batch) analysis. Taken together, this indicates that the test is currently not suitable for use in clinical practice. Yet, despite these limitations, this study was conducted at six centers, indicating the potential feasibility of this test. The correlation between the fibrin generation test and a more commonly used in-house TF-dependent Xa generation assay to measure EV-TF activity appeared to be poor, for reasons that are currently unclear. It has to be noted that the fibrin generation test is to be performed on fresh plasma, while the TF-dependent Xa assay generation assay was performed on isolated EVs after freeze-thawing.

Strengths of the present study include the relatively large sample size, multicenter design, minimal loss to follow-up, and central verification of outcome events. A few limitations also merit consideration. The majority of patients were enrolled during chemotherapy and it is unknown to what extent chemotherapy may influence EV-TF activity. Reitter and colleagues showed that the levels of several coagulation markers may change in time during chemotherapy [24]. The same could hold true for EV-TF activity, although no difference in the overall discriminatory performance of the fibrin generation test was observed between patients enrolled prior to chemotherapy and those enrolled during chemotherapy (data not shown). Enrolment of patients during chemotherapy may also have resulted in selection of a low risk group, since the risk of VTE is usually highest in the first months after diagnosis and treatment initiation. Our VTE risk estimate may therefore be a conservative one, also because deaths were not routinely adjudicated for fatal VTE. We were unable to assess the influence of various pre-analytical factors that may have influenced the test results, such as blood collection and exact time to centrifugation. The fibrin generation test was not performed in all patients due to logistical problems or hemolytic blood samples. However, it is unlikely that this introduced bias, since the sensitivity analysis using multiple imputation yielded similar results. Finally, the low number of events in the subgroup of patients with pancreatic cancer may not have provided enough power to detect significant differences thereby increasing the risk of type II errors.

In a cross-sectional study, Tesselaar and colleagues were the first to link EV-TF activity to cancer and thrombosis [25]. Now, a decade later,

it remains challenging to accurately measure EV-TF and, consequently, to use the results in clinical practice. Only Zwicker and colleagues explored whether EV-TF levels measured by impedance flow cytometry could be used to risk stratify cancer patients in a small phase 2 trial [26], but despite the encouraging results, it appears no further steps have been undertaken. Yet, the results of the present study support the premise that EV-TF activity can be a possibly useful predictor of VTE in patients with selected tumor types. This biomarker, either as a stand-alone test or in combination with a clinical risk score, may aid clinicians in deciding about thromboprophylaxis in patients at high risk of VTE. The two-fold increased risk of VTE in patients with high EV-TF activity is in the same range as the associations observed in studies evaluating other biomarkers, such as D-dimer, prothrombin fragment 1 + 2, endogenous thrombin potential, and factor VIII [27–30]. Future studies should focus on improving the feasibility and accuracy of the various activity-based assays and exploring its use in the combination with other biomarkers or clinical scores.

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Disclosures

None of the authors have potential conflicts of interest to declare.

Author contributions

MDN, AK, PWK, RJB, HRB, and RN designed the study. NvE, MDN, GC, IM, and HMO contributed to data acquisition. NvE performed the statistical analysis. NvE, YH, HRM, NM, and RN interpreted the data and wrote the first draft. MDN, GC, AK, IM, HMO, PWK, and RJB revised the manuscript for important intellectual content. All authors approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2018.04.009.

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