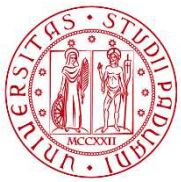


UNIVERSITÀ DEGLI STUDI DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI MEDICINA

Direttore: Prof. Fabrizio Fabris

Ph.D. course in Clinical and Experimental Sciences

Curriculum: Hematological And Geriatric Sciences

XXX cycle

PhD Thesis

A bit deeper on hypercoagulability and cancer: focus on longitudinal trend of procoagulant microvesicles in gastro-intestinal malignancy

Coordinator: Prof. Paolo Angeli

Supervisor: Prof. Paolo Simioni

Co-Supervisor: Dott. Luca Spiezia

Ph.D. student: Elena Campello

ACADEMIC YEAR 2016/2017

INDEX:

ABSTRACT:

Introduction. It is fully recognized that cancer patients are at significant risk of developing thrombotic events (VTE). The prevention of such complications is of the utmost importance from a clinical point of view, seeing as they play a considerable part in the morbidity and mortality of these patients. The main issue is that the pathogenesis of the cancer-associated coagulopathy is complex and multifactorial. To assess the level of risk and identification of patients at high risk for thrombosis, the guidelines recommend including the detection of plasma thrombotic markers in "score systems" combining clinical and biological markers. However, current scores have been shown to perform poorly in predicting VTE in cancer patients. Thus, the identification of novel biomarkers associated with the grade of hypercoagulability in individual malignancies is required to drive the development of cancer type–specific scoring systems with improved predictive value.

Aim of the study. We conducted a longitudinal cohort study to evaluate the trend of several coagulation parameters in patients with gastro-intestinal cancer with particular focus on circulating microvesicles (MVs) and MV-tissue factor (TF) activity. Our primary outcome was the description of coagulation fluctuations over a 6-month period following cancer diagnosis and the secondary outcome was the association between coagulation parameters and the occurrence of VTE complications.

Material and Methods. Patients with a new diagnosis of gastro-intestinal cancer who underwent surgery were consecutively enrolled at the Padua University Hospital. Exclusion criteria were: cancer recurrence, severe liver or renal failure, Karnofsky Performance Status <60%, recent venous/arterial thromboembolism, pregnancy/puerperium, overt/recent sepsis. Longitudinal blood samples were collected at baseline, 7 days after surgery, 1 and 6 months after surgery. Each patient was followed for at least 6 months and for a maximum of 12 months. The primary outcome was the evaluation of coagulative parameters trend over a 6-month period following the diagnosis. Coagulation test performed included: factor (F)VIII and fibrinogen levels; D-Dimer and plasminogen activator inhibitor (PAI-1) antigen; hereditary thrombophilia; thromboelastometry; contact activation system (FXIIa-C1-inhibitor (C1INH), FXIa-C1INH and KAL-C1INH complexes); MV-TF activity; circulating MVs. Clinical outcomes recorded during the follow-up were: i) surgical radicality (i.e. complete or incomplete); ii) cancer severity (i.e. localized or advanced cancer); iii) any thrombotic event including superficial vein thrombosis, symptomatic or asymptomatic VTE or thrombosis in unusual sites. The secondary outcome was the association between the coagulative profile at the different time-points and the clinical outcomes.

Results. Ninety-three patients (25 with pancreatic, 33 with colon and 35 with gastroesophageal cancer) were enrolled. The median clinical follow-up was 6 months [6-8.5] for pancreatic, 8 months [7-11] for colon, and 6.5 months for gastric cancer [6-9.25]. VTE incidence rate was 9.21 [95%CI 3.37-20.4] per 100 person-years for pancreatic cancer, 6.69 [95%CI 2.13-16.2] per 100 person-years for colon and 10.4 [95%CI 4.24-21.7] per 100 person-years for gastric cancer. The subgroup of pancreatic cancer at baseline showed increased levels of FVIII, D-Dimer, PAI-1 antigen, MV-TF activity and circulating MVs compared with the other cancer subtypes.

In the overall cancer population, baseline contact system complexes were increased compared to levels measured in a reference healthy population, and pancreatic and gastric cancers showed the highest activation. Patients receiving chemotherapy at the 6-month time point showed significantly higher levels of FXIIa-C1INH and kallikrein-C1INH complexes compared to patients without chemotherapy.

In a multivariate model, levels of MV-TF activity were independent predictors of incomplete surgical resection (OR 2.25 [1.25-7.0]) and cancer severity (OR 1.87 [1.20-3.8]).

We observed that the majority of MVs detected were small (diameter 0.2-0.4 μm). Moreover, we confirmed that PS-negative MVs are the majority and thus PS is not the most suitable marker to detect the total number of MVs. MV-TF activity correlated with PS+MVs big and small, with endothelial MVs and big tumour MVs. Endothelial MVs, as well as MV-TF activity, showed a positive association with surgical radicality and cancer severity (OR 1.19 [1.04-1.36] and 1.30 [1.05-1.6], respectively).

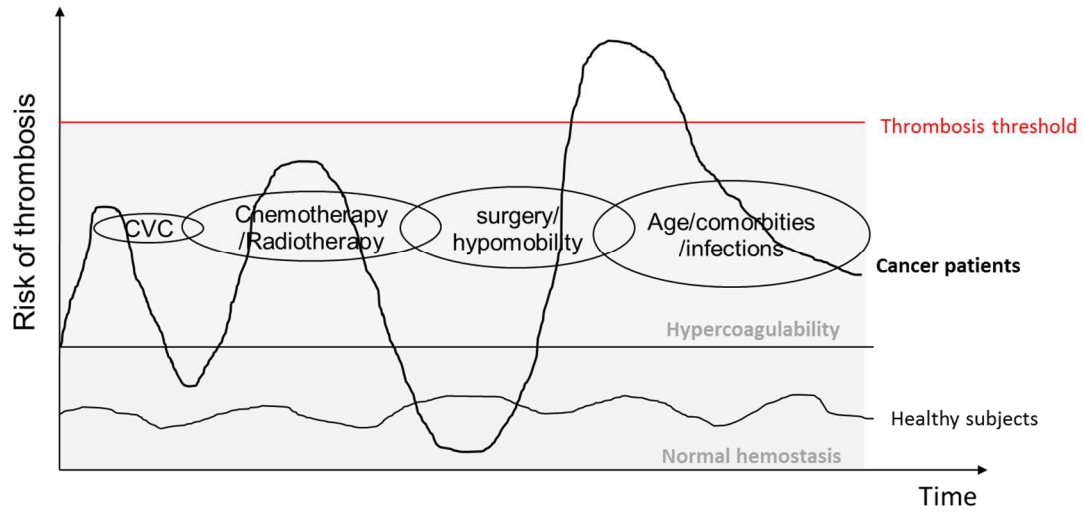
Levels of MV-TF activity ≥0.19 pg/mL conveyed a 2.38 [1.81-4.11] HR for VTE occurrence. Furthermore, baseline levels of FXIa >0.61 nM conveyed a 1.66 [1.02-2.9] HR to develop VTE over a median follow-up period of 6 months after diagnosis. This prediction model was adjusted for age, sex, BMI, cancer type, severity, and surgical radicality.

Conclusions. Hypercoagulability in gastro-intestinal cancer is mainly mediated by high levels of FVIII, increased levels of complexes derived from the activation of the contact system, high MV-TF activity and increased levels of PS+MVs, endothelial and tumour MVs. Pancreatic cancer showed the most hypercoagulable profile. The prothrombotic factors remained altered up to 6 months after surgical resection of the neoplasm even in patients with surgical radicality, indicating that cancer-associated hypercoagulability persists months after tumour removal. Increased MV-TF activity and endothelial MVs are independent predictors of advanced disease and incomplete surgical resection. Finally, baseline increased levels of MV-TF activity and FXIa were independent predictors of VTE occurrence over the 6 months following cancer diagnosis. As TF is upregulated in cancer, it seems reasonable to hypothesize that concomitant activation of both the intrinsic and extrinsic pathways may act synergistically to produce a highly prothrombotic state in cancer.

1. Introduction

Patients with cancer are at increased risk to develop venous thromboembolism (VTE), an association that is commonly known as Trousseau's syndrome (1, 2). The clinical manifestations of cancer-associated VTE include deep vein thrombosis (DVT) and pulmonary embolism (PE), as well as visceral or splanchnic vein thrombosis (3). Indeed, cancer is a strong and independent risk factor for VTE (4, 5). It has been estimated that ~20% of all first VTE events are associated with cancer (6). Additionally, cancer-associated thrombosis is also linked to a worse prognosis, and VTE is the second leading cause of death in cancer (6). Cancer induces a systemic hypercoagulable state that elevates the baseline thrombotic threshold of affected patients. This hypercoagulable state is the result of a complex interplay among cancer cells, host cells and the coagulation system as part of the host response to cancer. Moreover, several compounding risk factors for VTE usually coexist in cancer patients which may be classified as patient-, tumour- or treatment-related, may additively exceed the threshold for clinically overt thrombosis (Figure 1).

Figure 1: Longitudinal risk of thrombosis in a patient with cancer. Normal individuals maintain a hemostatic equilibrium whereas cancer patients are typically in a pre-thrombotic state, and at risk of developing overt thrombosis.



1.1 Epidemiology of cancer-associated thrombosis

In 1865, Armand Trousseau, a French physician, was one of the first to describe an association between thrombosis and cancer (1). Although the association had already been reported earlier in 1823 by Jean Baptiste Bouillaud (7), the condition was later called Trousseau syndrome, perhaps because of the irony of Trousseau diagnosing the disease on himself and dying from it in 1867. Since then, many studies have confirmed the association between cancer and venous thrombosis and demonstrated that the incidence of VTE in cancer patients is high and it has risen steadily over the past few decades (6).

Overall, cancer accounts for an estimated 18 % (95 % confidence interval [CI]: 13.4–22.6 %) of the total number of VTE cases (5). According to the results of a well-known, recent registry, the Registro Informatizado de Enfermedad TromboEmbólica (RIETE), which included >35,000 consecutive symptomatic venous thrombosis patients from 2001 to 2011, active cancer was reported in 6075 patients (17%) (8). Moreover, the frequency of VTE in cancer patients admitted to hospital ranges from 2 % to 12 % (9, 10). Based on hospitalization rates for VTE, the incidence rate for cancer-associated VTE was estimated 8 patients per 1,000 patients/year (11). According to a recent UK cohort study that included 6,592 cancer-associated VTEs, the incidence rate of first VTE in patients with active cancer was 5.8 (95 % CI, 5.7-6.0) per 100 person-years and the overall incidence rate for recurrence was 9.6 (95 % CI, 8.8-10.4) per 100 person-years (12). On the other hand, cancer patients have a several-fold increased risk of venous thrombosis compared with the general population or patients without cancer, with relative risks (RRs) ranging from 4 to 7 (4, 11, 13). Particularly, the risk of VTE was increased sevenfold in patients with cancer compared with patients without (Odds Ratio [OR] 6.7; 95% CI: 5.2-8.6). By linkage of 4 United Kingdom databases, Walker and coworkers estimated the RR of VTE in cancer versus age-matched non-cancer controls from the general population to be 4.7 (hazard ratio [HR] 4.7; 95% CI: 4.5-4.9 (4). Similar results were reported from a Danish population-based cohort of 57,591 incident cancer cases that were followed over time for venous thrombosis occurrence, together with 287,476 individuals without cancer from the general population. Non-cancer controls were matched for age, gender, and county of residence. After adjustment for comorbid conditions, the risk of VTE was also 4.7 times higher in cancer patients

compared with the non-cancer subjects (RR 4.7; 95% CI: 4.3-5.1) (11). More recently it was found that the incidence rate of VTE was 54-times higher than that reported in the non-cancer cohort (5,800 vs 107/100,000 person-years) considering the active-cancer cohort (the denominator consisted of active-cancer time periods in contrast to any time after the first cancer diagnosis) (12). Although these RRs demonstrate a strong association between cancer and venous thrombosis, absolute risks are clinically more meaningful when trying to convey a patient's actual risk of venous thrombosis. The reported absolute risk (cumulative incidence) of VTE in cancer patients varies widely (1%-8%) depending on patient population, duration of follow-up, calendar period, and the method of detecting and reporting venous thrombotic events (6). In the Vienna Cancer and Thrombosis Study (CATS)–a large, prospective observational study–the cumulative incidence of VTE among cancer patients over the median observation period of approximately 19 months was 7.4% (14).

In a large registry study, cancer was found to be the strongest independent risk factor for all-cause and PE-related mortality in patients with VTE (8). In the same study, 3% of deaths in cancer patients were PE-related versus 1% of deaths in non-cancer patients ($p < 0.001$) (8). Additionally, survival rates tend to be significantly lower and prognosis significantly worse in cancer patients with VTE relative to those without (15-17). For instance, in a Danish registry study, patients with cancer diagnosed concurrently with VTE had a significantly lower one-year survival rate (12 % vs 36 %; $p < 0.001$) and significantly more distant metastases (prevalence ratio [95 % CI]: 1.26 [1.13 to 1.40]) compared with cancer patients without VTE (17).

In addition to the increased risk of VTE in cancer patients and the worse prognosis linked to cancer-associated VTE, healthcare costs are approximately 40 % to 50 % higher in cancer patients with VTE as compared with cancer patients without VTE (18, 19). These increased healthcare costs are primarily driven by higher inpatient and outpatient costs (19, 20). In a population-based, longitudinal cohort study, after adjusting for cofactors including age, sex, and cancer type and stage, the direct five-year healthcare costs were significantly higher in cancer patients with VTE versus cancer patients without VTE (approximately $49,000 vs $27,000 in 2013 US dollars; $p \leq 0.001$) (20). Figure 2 and Table 1 and 2 summarize the epidemiology and burden of VTE in cancer.

Figure 2. Pooled rates of venous thromboembolism per 1,000 person-years (p-ys) for studies where follow-up started at the time of cancer diagnosis. Numbers in brackets refer to the number of studies that contributed to the pooled estimate (from Horsted F. et al. 2012 (21)).

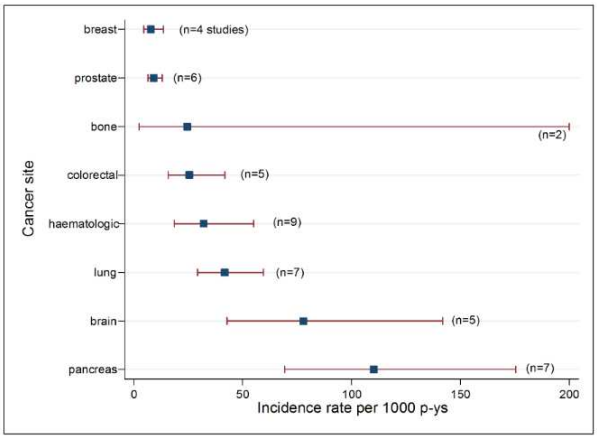


Table 1: **Risk of venous thromboembolism (VTE) in cancer overall with pooled incidence rates and 95% confidence intervals (CI) obtained from random effects meta-analysis** (21)**.**

First author (year)[ref]	Total participants	Total person-years of follow-up	Total VTE	Incidence rate/1000 person-years (95%CI)	Average follow-up (months)
Average risk					
Blom (2006)(22)	66,329	31,867	815	25.6 (23.9-27.4)	6
Chew (2006)(23)	235,149	389,150	3,775	9.7 (9.4-10.0)	20
Cronin-Fenton (2010)(24)	57,591	127,492	1,023	8.0 (7.6-9.5)	27
Pooled incidence rate				**12.6 (7.0-22.6)**	
Heterogeneity (I^2 =99.7%)					
High risk					
Sallah (2002)(9)	1,041	2,256	81	35.9 (28.9-44.7)	26
Otten (2004)(25)	206	133.1	15	112.7 (67.9-186.9)	8
Ay (2009)(26)	821	1,126	62	55.1 (42.9-70.6)	16
Hall (2009)(27)	14,214	9,249	489	52.9 (48.4-57.8)	8
Connolly (2010)(28)	4,405	904,5	93	102.8 (83.9-126.0)	2
Abdel-Razaq (2010)(29)	606	111.0	21	189.2 (123.4-290.2)	2
Di Nisio (2010)(30)	1,921	1,281	39	30.5 (22.3-41.7)	8
Reeves (2010)(31)	176	17.2	2	116.3 (29.1-464.9)	1
Pooled incidence rate				**68.0 (48.0, 96.4)**	
Heterogeneity (I^2 =93.4%)					

Table 2: Incidence rate (95 % CI) of first VTE per 100 person-years by cancer type and age among patients with active cancer (from Cohen et al. 2017 (12)).

Age	Pancreas	Stomach	Brain	Ovary	Lung	Haematologic	Colon	Breast
<18	-	-	-	-	-	**0.7** (0.3–1.6)	-	-
18-29	-	-	-	-	-	**1.5** (0.6–3.1)	-	-
30-39	**25.8** (7.0–66.1)	-	**5.7** (2.5–11.3)	**5.7** (2.5–11.3)	**14.5** (5.8–29.8)	**3.0** (1.7–4.9)	**7.2** (2.9–14.8)	**2.4** (1.4-3.8)
40-49	**17.1** (8.5–30.5)	**12.6** (6.1–23.2)	**10.5** (6.7–15.7)	**10.5** (6.7–15.7)	**9.2** (5.9–13.7)	**2.9** (2.0–4.1)	**6.1** (4.1–8.8)	**2.0** (1.5–2.6)
50-59	**21.4** (16.0–28.2)	**13.2** (8.7–19.1)	**14.5** (10.3–19.8)	**14.5** (10.3–19.8)	**11.4** (9.5–13.6)	**4.7** (3.8–5.7)	**5.4** (4.3–6.7)	**2.4** (1.9–2.9)
60-69	**17.1** (13.6–21.4)	**11.0** (8.2–14.5)	**16.3** (12.2–21.4)	**16.3** (12.2–21.4)	**11.0** (9.8–12.4)	**4.7** (4.0–5.4)	**6.7** (5.8–7.7)	**3.3** (2.8–4.0)
70-79	**12.3** (9.6–15.5)	**13.1** (10.7–15.9)	**15.3** (10.6–21.3)	**15.3** (10.6–21.3)	**10.0** (8.9–11.2)	**4.6** (3.9–5.3)	**7.4** (6.6–8.3)	**4.3** (3.5–5.2)
80-89	**11.8** (8.5–16.0)	**7.4** (5.3–10.0)	**7.0** (2.6–15.3)	**7.0** (2.6–15.3)	**8.4** (7.1–9.9)	**5.4** (4.5–6.3)	**6.5** (5.6–7.6)	**4.7** (3.7–5.9)
>89	-	**4.2** (1.2–10.9)	-	-	**6.3** (3.3–11.0)	**2.8** (1.4–5.2)	**7.6** (5.0–11.1)	**6.1** (3.9–9.0)
Total >18	**14.6** (12.9–16.5)	**10.8** (9.5–12.3)	**12.1** (10.3–14.0)	**12.1** (10.3–14.0)	**10.1** (9.5–10.8)	**4.5** (4.1–4.8)	**6.7** (6.3–7.2)	**3.2** (2.9–3.4)

1.2 Risk factors for venous thrombosis in cancer patients

Cancer is a heterogeneous disease, and its different types and stages should be taken into account when determining the risk of VTE. Also several patient- and treatment-associated factors are known to increase the risk of thrombosis. Particularly, when considering cancer patients, risk factors for developing VTE can be grouped into tumour-related factors, patient-related factors and treatment-related factors (3, 6, 32) (Table 3).

Table 3: Overall risk factors for cancer-associated thrombosis.

Cancer-related	Treatment-related	Patient-related
- Anatomical site of cancer - Advanced stage of cancer - Initial period after cancer diagnosis	- Major surgery - Hospitalization - Chemotherapy and hormonal therapy - Anti-angiogenic therapy - Erythropoiesis stimulating agents - Central venous catheters - Blood transfusions	- Older age - Female gender - Race (black ethnicity) - Common comorbidities (diabetes, obesity, previous VTE, atherosclerosis, inflammation…) - Hereditary trombophilia

1.2.1 Cancer-related risk factors

Tumour-related factors include:

- primary site
- grade
- cancer stage
- time since diagnosis

Extensive work has been published on type of malignancy and subsequent risk of venous thrombosis. A 2006 large registry study assessing 20 of the most common cancers reported the highest incidence of concurrently diagnosed VTE in patients with metastatic cancers of the *pancreas*, *stomach*, *lung*, *uterus*, *bladder*, and *kidney* (15). In general, the types of cancer most often associated with VTE are gastric and pancreatic cancers (3, 4, 6, 11, 15, 32-34). Other cancers associated with a high risk of VTE include brain, gynaecological cancers, lung, renal, bladder, bone, and haematological malignancies (4, 6, 11, 13, 15, 32-34). Relatively low risks are generally seen in patients with *breast* or *prostate* cancer.

Although VTE incidence per type of cancer varies in different studies, a clear positive association can be observed with 1-year relative mortality of the cancer type, as a measure of the biological aggressiveness of the cancer, and the associated thrombogenic potential. Patients with metastatic

cancers have a significantly greater risk of developing VTE relative to patients with localised cancer (13, 15, 16, 32). In the above mentioned CATS, the cumulative probabilities of VTE after six months in cancer patients with local, regional, or distant stage cancer were 2.1 %, 6.5 %, and 6.0% (p = 0.002) (35). Interestingly enough, the cumulative probability was similar in patients with regional or distant stage cancers while patients with local stage cancer had a much lower VTE risk (35). Finally, the histological grading is also an important risk marker. Once again, in CATS the rate of VTE was roughly twice as high in patients with high-grade tumours compared with those with low-grade tumours (HR [95 % CI], 2.0 [1.1–3.5]; p = 0.015), after adjusting for cofactors including distant metastases, sex, and age (36). There is also an observed time-dependent association between VTE and cancer, with most VTE events occurring in the first few months after cancer diagnosis and decreasing thereafter (4, 6, 13, 15, 16, 33). In the MEGA study, the risk of venous thrombosis was highest in the first 3 months after cancer diagnosis (OR 53.5; 95% CI: 8.6-334.3), was decreased but still high in the period between 3 and 12 months (OR 14.3; 95% CI: 5.8-35.2), and decreased to almost no elevated risk 10 years after cancer diagnosis (13). This phenomenon has been shown for all types of cancer in the large follow-up study by linkage of 4 United Kingdom databases (4). This change in risk over time highlights why follow-up studies regarding VTE incidence in cancer patients need to start at time of cancer diagnosis. If follow-up is started at a later point, the incidence will be lower, and studies cannot be compared directly. From a pathophysiological point of view, there are several possible explanations for the increased risk of VTE in the first few months after diagnosis compared with the period thereafter. First, several cancer treatment modalities increase the risk of VTE, inducing a high risk directly after diagnosis and start of treatment. Second, a proportion of treated cancer patients will go into remission, leading to a reduced thrombotic risk thereafter. A third explanation is that a proportion of cancer patients will succumb to the disease over time. The occurrence of such a competing event (death) will prevent thrombotic events from being observed (6).

1.2.2 Treatment-related risk factors

Treatment-related factors include both mechanical causes (surgery and central venous catheters) and pharmacologic agents, such as chemotherapeutic and hormonal agents, antiangiogenic and erythropoiesis-stimulating agents (3, 5, 6, 11, 13, 32, 33). In particular, platinum-based chemotherapies (e.g. cisplatin) and anti-angiogenesis treatments (e.g. bevacizumab) are frequently associated with VTE (33, 37-39). Results from a clinical trial in advanced gastroesophageal cancer patients showed varying rates of VTE for either 1 of 4 epirubicin/platinum/fluoropyrimidine combination regimens during treatment until 30 days after the last treatment cycle. A higher cumulative incidence of VTE was observed in patients receiving a cisplatin-containing combination regimen (12.2%) compared with oxaliplatin (6.5%) (40). According to the results of 2 meta-analyses of clinical trials, patients treated with cisplatin or bevacizumab therapy were at significantly higher risk for VTE relative to patients treated with non-cisplatin or non- bevacizumab therapies (RR [95 % CI]: 1.67 [1.25–2.23] and 1.33 [1.13–1.56], respectively) (41, 42). Other treatments associated with VTE in cancer patients include thalidomide, hormonal therapy, erythropoiesis-stimulating agents, and red blood cell or platelet transfusions (6, 13, 32-34). In a randomized trial in postmenopausal women with node-positive primary operable breast cancer (with positive estrogenic and progesterone receptor status), the cumulative incidence of VTE was assessed for women randomized to 2 years of tamoxifen or to tamoxifen (2 years) plus chemotherapy for 6 months. The cumulative incidence in the tamoxifen only group was 2.6% vs 13.6% in the combined treatment group (43). Moreover, two systematic reviews of randomized controlled trials demonstrated that cancer patients treated with erythropoiesis-stimulating agents in addition to red blood cell transfusions had an increased risk of VTE over patients not additionally treated with these compounds (with a RR of 1.7) (44, 45). Additionally, surgery is a well-known risk factor for VTE, both in cancer and non-cancer population. In cancer patients, risk of 90-day postoperative VTE is reported to be twice as high as in non-cancer patients (46).

1.2.3 Catheter-related thrombosis

Central venous catheters (CVCs) have been shown to facilitate chemotherapy, transfusions, antibiotics, and parenteral nutrition delivery. Furthermore, they allow readily available venous access for laboratory testing and have been shown to improve patients' quality of life and reduce health care costs by allowing patients to receive parenteral therapy at home (47). All CVCs are designed to have their catheter tip dwelling at the junction of the superior vena cava and the right atrium within the central venous system (48). CVCs can be classified as tunneled or non-tunneled catheters, peripherally inserted central catheter (PICC), implanted ports and dialysis catheters (48). Different types of CVCs have distinctive features including varying numbers of lumens (single, double or triple) or the addition of valves which prevent reflux of blood back into the lumen of the catheter. Clinicians need to use the smallest-diameter catheter that will fulfil the patient's need (e.g. multiple lumens might be required for chemotherapy infusion in cancer patients) and ensure that the CVC is removed when it is no longer used to minimize the risk of any associated complications (47). Risk factors for catheter-related thrombosis can be divided into those that are intrinsic to the CVC (or its insertion) and those extrinsic or related to patient's characteristics (Table 4). The largest systematic review and meta-analysis on this topic have shown that the type of CVC (PICC > implanted ports), its location (e.g. junction of the superior vena cava and the right atrium) and the insertion site (femoral > subclavian > jugular) are important intrinsic predictors of thrombosis (49). Other possible risk factors include CVC diameter and left-sided insertions (50). Cancer, particularly extensive or metastatic disease, has consistently been shown to be one of the strongest risk factors for catheter-related thrombosis (51, 52). A systematic review of 64 studies evaluating the risk of upper extremity DVT in 29,503 adults who had a CVC, showed that the mean rates of superior arm vein thrombosis were 4.9% overall, 6.7% in patients with cancer, and 13.9% in patients admitted to critical care (51). Finally, a systematic review of 10 studies of 1000 cancer patients with a CVC found that those with Factor V Leiden or prothrombin gene mutation had a >4-fold greater odds of catheter-related thrombosis (53).

Table 4: Risk factors associated with catheter-related thrombosis (From Rajasekha and Streiff 2017 (54)).

Device-related	Patient-related	Treatment-related
- Multiple insertion attempts - Catheter insertion site (femoral.>jugular>subclavian) - Large catheter size to vein diameter ratio - Catheter subtype (peripherally-inserted [PICC]>centrally-inserted>implanted port) - Catheter infection - Improper catheter position (not at atriocaval junction) - No. of lumens and catheter size (6F triple-lumen>5F double-lumen>4F single-lumen) -Material (polyethylene/polyvinylchloride >silicone/polyurethane) - Previous catheter	- Malignancy (metastatic>localized) - Recent trauma/surgery - Previous VTE - End-stage renal disease - Critically ill patients - Older age - Systemic or catheter-related infection - Immobilization - Inherited thrombophilia	- Ongoing cancer therapy - Radiation therapy - Bolus (vs diluted) chemotherapy infusions - Antiangiogenic agents and platinum therapy - Erythrocyte stimulating agents - Parenteral nutrition - Surgery

1.2.4 Patient-related risk factors

Several traditional risk factors for thrombosis are additionally present in many cancer patients such as older age (≥65), prolonged immobility, obesity, prior history of VTE, and comorbidities (4, 15, 33, 34). In the California Cancer Registry study in colorectal cancer patients, a significant predictor of VTE during the first year after cancer diagnosis was the presence of >3 comorbid conditions (HR 2.0; 95% CI: 1.7-2.3) (55). Not all studies have replicated the association between advanced age or obesity and the increased risk for VTE in cancer patients, whereas the occurrence of varicose veins and prior VTE has also been associated with VTE in cancer patients (32, 37, 56, 57). Finally, prothrombotic mutations are additionally reported to influence the risk of thrombosis in cancer patients. For

example, there appears to be a correlation between the factor V Leiden mutation and VTE risk (13, 53). Cancer patients with factor V Leiden were reported to have a twofold increased risk of VTE compared with cancer-free non carriers (adjusted OR 2.2; 95% CI: 0.3-17.8) (13, 58). Similarly, in a prospective study conducted on women with breast cancer undergoing adjuvant tamoxifen treatment, patients who developed VTE were significantly more likely to have a factor V Leiden mutation compared with patients without VTE (59).

1.3 Clinical presentation of cancer-associated thrombosis

Bilateral DVT seems to be more common among cancer patients than in non-cancer patients (6). A study showed that rates of symptomatic bilateral lower limb DVT, symptomatic ilio-caval thrombosis, and upper limb DVT were higher in cancer patients compared with cancer-free patients (8.5% vs 4.6%, 22.6% vs 14.0%, and 9.9% vs 4.8%, respectively) (60). However, rates of PE and symptomatic proximal DVT were similar. The relatively high incidence of upper limb DVT in cancer patients can explained, at least in part, by the frequent use of a CVC, as previously mentioned (51, 52). Furthermore, cancer has been commonly reported in rare forms of thrombosis such as Budd-Chiari syndrome, extrahepatic portal vein obstruction, and mesenteric vein thrombosis (61).

1.3.1 Recurrent venous thrombosis and cancer

Cancer patients are associated with a roughly two- to threefold increased risk of recurrent VTE compared with non-cancer patients (6, 62) (Table 5). Prandoni et al. followed 355 consecutive patients with a first DVT for 8 years and found a twofold risk of recurrent DVT in cancer compared with non-cancer patients (HR 1.7; 95% CI: 1.3-2.3) (62). Moreover, in a prospective cohort study including 842 DVT patients the 12-month cumulative incidence of recurrent VTE was found to be 20.7% in cancer patients on conventional anticoagulant treatment vs 6.8% in non-cancer patients on anticoagulant treatment (63). Recurrence appeared to be related to extent of disease, classified according to the tumour node metastasis (TNM) classification. The highest recurrence rates in patients with extensive vs moderately or less extensive cancer, which again reflects the apparent correlation between aggressiveness of cancer and thrombogenic potential (6). A study conducted within the

above-mentioned RIETE enrolled patients with symptomatic acute VTE and studied the 3-month outcomes: 3805 out of the 18,883 participants had been diagnosed with active cancer. The study found a RR for recurrent PE of 2.0 and for recurrent DVT of 2.4 in patients with a cancer diagnosis >3 months before their first thrombotic event (64). At the moment, not much is known about the risk of recurrent VTE for different types of cancer and previous studies have yielded contradictory results (63, 64).

Table 5: Incidence rate (IR, 95 % CI) of recurrent VTE per 100 person years by VTE type of first VTE among patients with active cancer (from Cohen et al. 2017 (12)).

Time at risk of recurrent VTE	Recurrent VTE IR (95%CI)	Recurrence after first PE IR (95%CI)	Recurrence after first DVT IR (95%CI)
<180 days	22.1 (19.9-24.4)	24.0 (20.8-27.6)	20.2 (17.3-23.4)
180-365 days	7.9 (6.2-9.8)	7.3 (5.1-10.0)	8.4 (6.1-11.4)
1-<2 years	6.6 (5.3-8.2)	7.1 (5.1-9.5)	6.2 (4.4-8.4)
2-<3 years	4.1 (2.8-5.8)	4.2 (2.4-7.0)	3.9 (2.2-6.4)
3-<4 years	3.2 (1.8-5.1)	3.1 (1.3-6.5)	3.2 (1.4-6.0)
4-<5 years	2.5 (1.2-4.8)	2.0 (0.4-5.9)	2.9 (1.1-6.3)
Total	9.6 (8.8-10.4)	10.5 (9.3-11.7)	8.8 (7.8-9.8)

1.4 Role of biomarkers in cancer

As stated by Strimbu and Tavel: “The term biomarker refers to a broad subcategory of medical signs observed outside the patient that can be measured accurately and reproducibly” (65). It is hypothesized that biomarkers of a prothrombotic state can be used to identify patients at increased risk for primary and recurrent VTE (39, 66, 67). Haematologic biomarkers associated with an increased risk of VTE in cancer patients include elevated platelet and/or leukocyte counts and low

haemoglobin levels (Table 6). An elevated platelet count is strongly and independently associated with VTE in cancer patients (56, 66), with one study reporting a 3.5-fold increased risk of VTE in patients with platelet count ≥ 443×109/L (68).

Other biomarkers independently associated with the presence of VTE in cancer patients in CATS include elevated plasma levels of soluble P-selectin (sP-selectin), prothrombin fragment 1+2 (F1+2), and D-dimer, as well as increased thrombin generation potential (32, 68-71). P-selectin, a cell adhesion molecule primarily found in endothelial cells and platelets, is believed to mediate the adhesion of leukocytes, platelets, and cancer cells in inflammation, thrombosis, and cancer cell growth/metastasis (72). F1+2 is considered a specific in vivo marker of thrombin generation; D-dimer is the primary degradation product of cross-linked fibrin and reflects the global activation of the haemostatic and fibrinolytic system (73, 74). In patients with colorectal cancer, the presence of preoperative positive D-dimer was associated with an increased risk of VTE during the first year post-surgery in comparison with patients negative for D-dimer pre-surgery (HR [95 % CI]: 6.53 [1.58–27.0]; p = 0.009) (75). Another biomarker linked to an increased risk of cancer-associated VTE is C-reactive protein (CRP) (37), a marker of systemic inflammation (76). However, not all studies have been able to replicate the association between CRP and cancer-associated VTE (77). There are very few studies aimed the assessment of the longitudinal fluctuations in cancer-associated VTE biomarkers. A recent longitudinal study conducted over six months in patients with colorectal, lung, pancreatic, or brain cancer reported that cancer patients who developed VTE consistently showed significantly elevated haemostasis biomarker levels, including sP-selectin and D-dimer, throughout the entire 250-day observation period versus those patients who did not develop VTE (78). As there was no continuous increase in D-dimer levels prior to VTE occurrence (62), the added value of continuous biomarker monitoring, rather than a single measurement, in improving VTE prediction and patient care is not clear.

Table 6. Possible biomarkers of cancer-associated thrombosis

Haematologic biomarkers	Leukocytosis, Thrombocytosis, Anaemia
P-Selectin	Increased circulating soluble P-Selectin
Microvesicles-tissue factor activity	Increased in pancreatic cancer
D-dimer	
Prothrombin fragment 1 + 2	
Thrombin generation potential	
C- reactive protein	

1.5 Risk assessment models of cancer-associated thrombosis

Based on the aforementioned risk factors, models were developed to stratify VTE risk in cancer patients. The availability of predictive models can facilitate the management of cancer-associated thrombosis by allowing the identification of patients most at risk for thrombosis who may benefit from early thromboprophylaxis (6, 79). The first was developed by Khorana specifically for cancer patients undergoing chemotherapy (66). This model is based on five predictive variables: cancer site, platelet count, haemoglobin levels or the use of erythropoiesis-stimulating agents, leukocyte count, and body mass index. This model uses a simple scoring system, is based on readily accessible baseline clinical and laboratory data, and has been shown to accurately predict the short-term risk of symptomatic VTE in patients undergoing chemotherapy-based treatments. The Khorana score has been validated in both prospective and retrospective observational studies (39, 80-82) (Table 7). In the CATS the score was expanded by adding two laboratory biomarkers (i.e., P-selectin and D-dimer), and the prediction of VTE was considerably improved (80) (Table 7). More recently, another modified Khorana risk assessment score (i.e., the Protecht score) was designed by adding platinum- or gemcitabine-based chemotherapy to the five predictive variables for identifying high-risk cancer patients in a post-hoc analysis of the Protecht clinical trial (82). In the setting of multiple myeloma, Palumbo et al. published a risk assessment model for the prevention of thalidomide and lenalidomide-

associated thrombosis (83). Finally, the Ottawa Score was developed to identify among patients with cancer and thrombosis those at highest risk of recurrent VTE, who may benefit from prolonged anticoagulant treatment (84, 85). The recent guidelines for prevention and treatment of VTE in cancer patients released by the American Society of Clinical Oncology (ASCO) remark that the thrombotic risk prediction cannot rely on the results of a single test. Instead, to assess the level of risk and identification of patients at high risk for thrombosis, the guidelines recommend including the detection of plasma thrombotic markers in "score systems" combining clinical and biological markers (79, 86) (Table 8).

Table 7. Predictive model for chemotherapy-associated venous thromboembolism.

Khorana VTE risk assessment score		**Points**
Site of cancer	- very high risk (stomach, pancreas)	**2**
	- high risk (lung, lymphoma, gynaecologic, genitourinary)	**1**
Platelet count	≥ 350 x 10^9/L	**1**
Haemoglobin	< 100 g/L or use of red blood cell growth factors	**1**
Leukocyte count	> 11 × 10^9/L	**1**
BMI	≥ 35 kg/m^2	**1**
Vienna VTE risk assessment score		
sP-selectin	≥ 53.1 ng/mL	**1**
D-dimer	≥ 1.44 µg/mL	**1**
0 score, low risk		
1-2 score, intermediate risk		
≥ 3, high risk.		

Table 8. Modified Khorana Score recommended by American Society of clinical Oncology (ASCO) (86).

Characteristics		**Points**
Site of cancer	- very high risk (stomach, pancreas, brain)	**2**
	- high risk (lung, lymphoma, gynaecologic, bladder, testicular, renal)	**1**
Platelet count	≥ 350 x 10^9/L	**1**
Haemoglobin	< 100 g/L or use of red blood cell growth factors	**1**
Leukocyte count	> 11 × 10^9/L	**1**

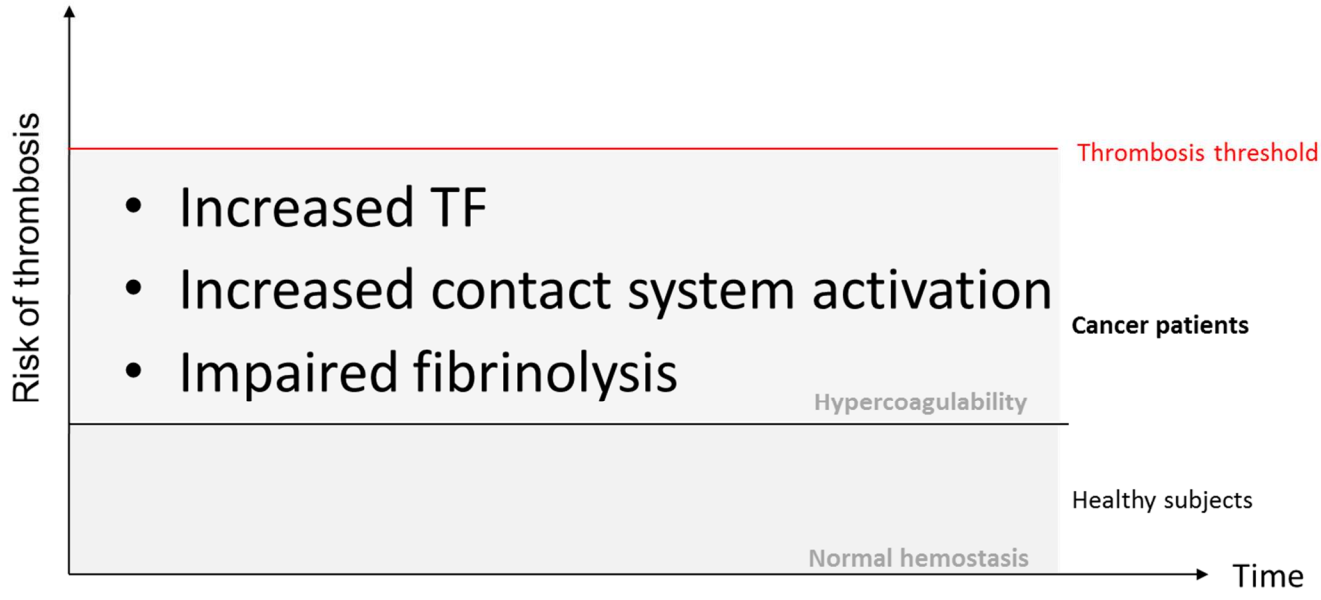
BMI ≥ 35 kg/m^2	**1**

0 score, low risk
1-2 score, intermediate risk
≥ 3, high risk.

1.6 Mechanisms of thrombosis in cancer

The pathogenesis of cancer-associated VTE is multifactorial and likely involves multiple overlapping pathways. All aspects of Virchow's triad of venous stasis, hypercoagulability, and vessel wall injury (32, 87, 88) may all play a role in cancer-associated VTE, with the increased immobility, chemotherapy- and surgery-induced endothelial damage, and a cancer-induced state of hypercoagulability. Suggested mechanisms for cancer-associated hypercoagulability include direct coagulation pathway activation, inhibition of fibrinolytic activity, and induction of inflammatory responses (32, 79, 89-91) (Figure 3).

Figure 3: Main mechanisms involved in cancer-related hypercoagulability.



TF: tissue factor.

1.6.1 Direct coagulation activation

Tumour cells themselves have been shown to contribute to the hypercoagulable state via the production of procoagulant factors (Figure 4). First, a positive correlation was observed between serum concentration levels of cancer procoagulant (CP), a cysteine proteinase believed to be produced by tumour cells and to direct activate coagulation factor (F)X (independently from FVII) (92) and fibrinogen in patients with gastrointestinal adenocarcinomas (93).

Among procoagulant proteins, the main mechanism by which cancer induces fibrin formation is suggested to be through an upregulation of tissue factor (TF) and through the production of TF-positive microvesicles (MVs). Tissue factor (TF) is a transmembrane receptor that binds plasma factor VII/VIIa and triggers blood coagulation following vascular injury and in various diseases (94-96). Cancer cells are well known to express TF and release TF-positive MVs (96-99). TF expression increases with histologic grade in different cancer types, including pancreatic cancer (98, 100, 101). Patients with cancer have been showed to have elevated plasma TF concentration compared to individuals without cancer (102). In addition, in one study TF expression in tumour cells was significantly correlated with the development of VTE in women with ovarian cancer (103). Two studies have reported a correlation between the level of TF in pancreatic and brain tumours and VTE (98, 101). In addition to this role in cancer-associated thrombosis, there have been reports relating to TF involvement in tumour growth and metastasis (104, 105). In fact, elevated TF expression has been reported to be associated also to the tumour endothelium. Activated oncogenes (K-ras, EGFR, PML-RARA, and MET) or inactivated tumour suppressors (eg, p53 or PTEN) lead to an increase in TF levels and activity, which presumably promotes not only hypercoagulability but also tumour aggressiveness and angiogenesis (2). Furthermore, a mutation in EGFR gene renders cancer cells hypersensitive to the action of coagulation proteins, such as TF, which is ultimately responsible for creating a favourable microenvironment for tumour growth (106). These data confirm the existence of a symbiotic relationship between cancer and thrombosis, where cancer cells support clot formation, and in turn, clotting proteins support cancer growth and dissemination (79).

Other prothrombotic abnormalities observed in cancer patients may be related to the pathogenesis of cancer-associated hypercoagulability. For instance, an increase in plasma levels of von Willebrand

factor and factor VIII, as well as a reduction in protein S were reported in multiple myeloma patients (107, 108). Furthermore, prostate cancer cells express long chain polyphosphates on their surface, which one study showed can initiate coagulation through a factor XII-dependent pathway (109).

1.6.2 Inhibition of fibrinolytic activity

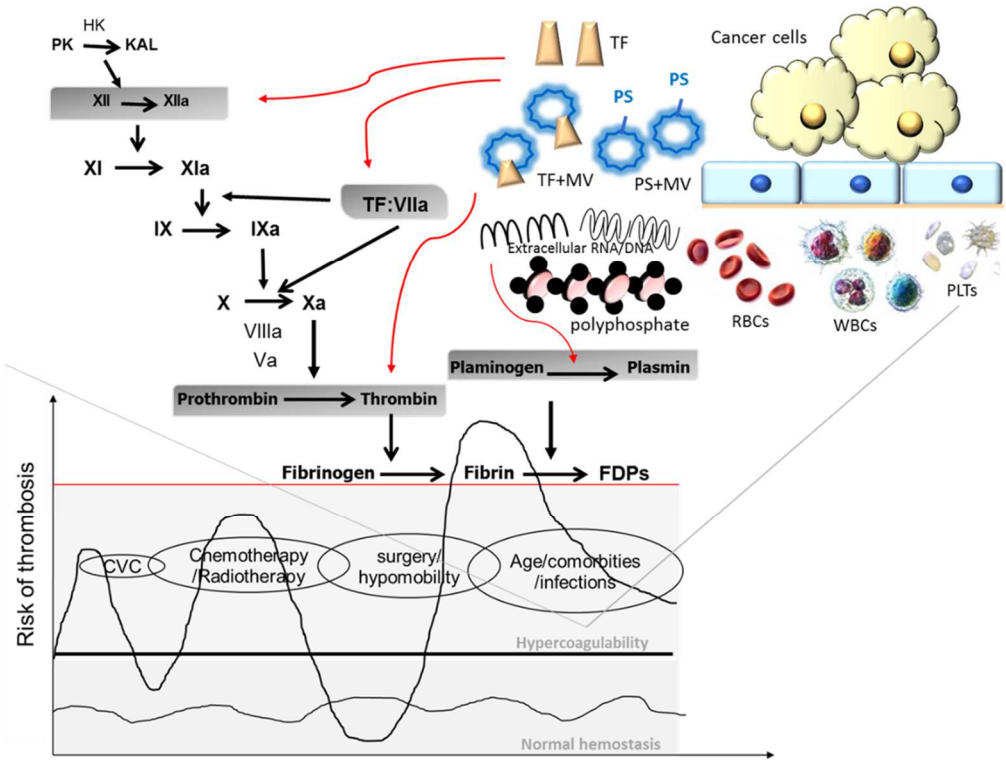
In addition to the induction of fibrin clots, cancer can also result in an inhibition of fibrinolysis. Tumour cells can express plasminogen activator inhibitor-1 (PAI-1), which is a major inhibitor of plasminogen activation by tissue-type plasminogen activator and therefore is an inhibitor of fibrin clot degradation (110). Elevated levels of PAI-1 were reported in individuals with different cancers, including ovarian cancer and multiple myeloma (111, 112). In addition, PAI-1 mRNA was detected in endothelial cells originating from the tumours in patients with colorectal cancer (113).

1.6.3 Induction of inflammatory responses

Malignant cells can also secrete a variety of cytokines including interleukin (IL)-1β, tumour necrosis factor-α and vascular endothelial growth factor (VEGF) (114). These cytokines can in turn induce TF production by vascular endothelial cells, downregulate thrombomodulin expression, promote PAI-1 synthesis and increase endothelial cell expression of heterocellular adhesion molecules (87). The inflammatory response to cancer or chemotherapy can also result in the formation of neutrophil extracellular traps (NETs), which are scaffolds of chromatin fibres lined with antimicrobial proteins (115). The formation of NETs (i.e. NETosis) was originally described as part of the innate immune system, in which the presence of pathogens can induce neutrophil granulocytes to release these extracellular traps that can capture and kill microbes (116). However, NETs are also found within DVTs and have prothrombotic effects (116, 117). In addition, tumours can secrete granulocyte colony stimulating factor (CGSF), a cytokine that can contribute to cancer-associated thrombosis by systemically priming neutrophils toward NETosis (117). In vitro co-localisation of neutrophils with activated endothelial cells (a proinflammatory and procoagulant state of endothelial cells associated with decreased vascular integrity and increased expression of leukocyte adhesion molecules) results in NETosis, partly through the action of cytokines (118). NETs, in turn, promote fibrin formation

through stimulating platelet adhesion and factor XII activation, as well as inducing endothelial cell death (118-120).

Figure 4: Molecular activators of coagulation in cancer. Coagulation activation in cancer-associated thrombosis may be explained by contributions from both the tissue factor ('extrinsic') and FXII-dependent ('intrinsic') pathways. Tissue factor–bearing microvesicles may be released into the circulation by various tumour types and promote thrombin generation and ultimately thrombosis. FXII may be activated in vivo by a variety of negatively charged molecules. These could include phosphatidylserine (e.g. on microvesicles), glycosaminoglycans, polyphosphate, collagen, nucleic acids, and mis-folded proteins. Activation of the contact system in cancer would exacerbate the generation of thrombin, providing accompanying thrombotic risk.



TF: tissue factor; PS: phosphatidylserine; MV: microvesicles; RBCs: red blood cells; WBCs: white blood cells; PLTs: platelets; PK: pre-kallikrein; KAL: Kallicrein; HK: High molecular weight kininogen.

1.7 Microvesicles (MVs)

Microvesicles (MVs) or microparticles (also referred to as shedding vesicles, ectosomes) can range in size from 0.1 to 1.0 μm and include all structures created through budding and fission directly from the plasma membrane (121, 122). MVs are constitutively released from the surface of cells, and their formation can be upregulated by cellular activation and apoptosis. Physiologically, plasma membranes contain various types of phospholipids. Although uncharged phospholipids are mainly present in the outer leaflet of the membrane bilayer, the inner leaflet contains negatively charged aminophospholipids such as phosphatidylserine (PS). During cells activation or apoptosis, the normal lipid bilayer undergoes an alteration by "flipping" internal PS to the external surface. As a result, PS exposing MVs can be released from cells. The membrane composition of MVs reflects their cellular origins. MVs contain functional cytoadhesion proteins, bioactive phospholipids, cytoplasmic components, and various antigens that are characteristic of the state of the originating cell and the type of stimuli (122-125). Increasing evidences point to elevated levels of different phenotypes of MVs in patients with VTE, suggesting that MVs may play an important role in the pathophysiology of VTE (122, 125-127). The role of MVs in thrombogenesis can be summarized, based on previous studies, in these subsequent ways (128): (i) the exposure of PS and the vehiculation of TF, and (ii) MV-induced intercellular communication by cross-talk between inflammation and coagulation. Undoubtedly, the functional interplay among endothelial cells, platelets, inflammatory cells and MVs plays a vital role in VTE.

1.7.1 Procoagulant properties of MVs: the exposure of PS and the vehiculation of TF

Perhaps the best established property of MVs is their ability to promote coagulation (Figure 5), which is largely linked to their physical characteristics with two specific surface features (122, 124). First, the above mentioned externalization of anionic phospholipids (predominantly PS) promotes the interaction with clotting proteins cationic domains, allowing the subsequent assembly of coagulation factors (Va, VIII, IXa) and ultimately thrombin formation. The externalization of PS is believed to be a property of all types of MVs and is a strong promoter of coagulation, thus the optimal thrombin generation and efficient haemostasis. Interestingly, it has been estimated that a platelet-derived MV

generated ex vivo has a 50- to 100-fold higher procoagulant activity than the same blubbing area on an activated platelet membrane (129), which may explain the potential thrombogenicity of MVs. Secondly, some populations of MVs have been shown to display TF on their surface. TF has a high affinity for FVII/FVIIa, and therefore circulating TF-MVs readily binding FVII/FVIIa, can trigger the initiation of coagulation via the extrinsic pathway (122). The presence of TF on MVs dramatically increases their procoagulant activity, especially since PS boosts the procoagulant activity of TF and contributes to the propagation of the coagulation cascade. It is well known that activated monocytes and tumour cells are the primary sources of TF-bearing MVs in the bloodstream, however TF has been identified also on leukocyte MVs, endothelial MVs and platelet MVs (130-133). Platelets and red blood cells produce PS- and PS+MVs, whereas activated monocytes and tumour cells release highly procoagulant PS+TF+MVs (134). Moreover, since the density of active TF on MVs is higher than that on their parental cells (135), the hypothesis that MVs are effective products made in response to a changing environment and that MVs formation is not an entirely random process has been reinforced.

1.7.2 Procoagulant properties of MVs: new mechanisms

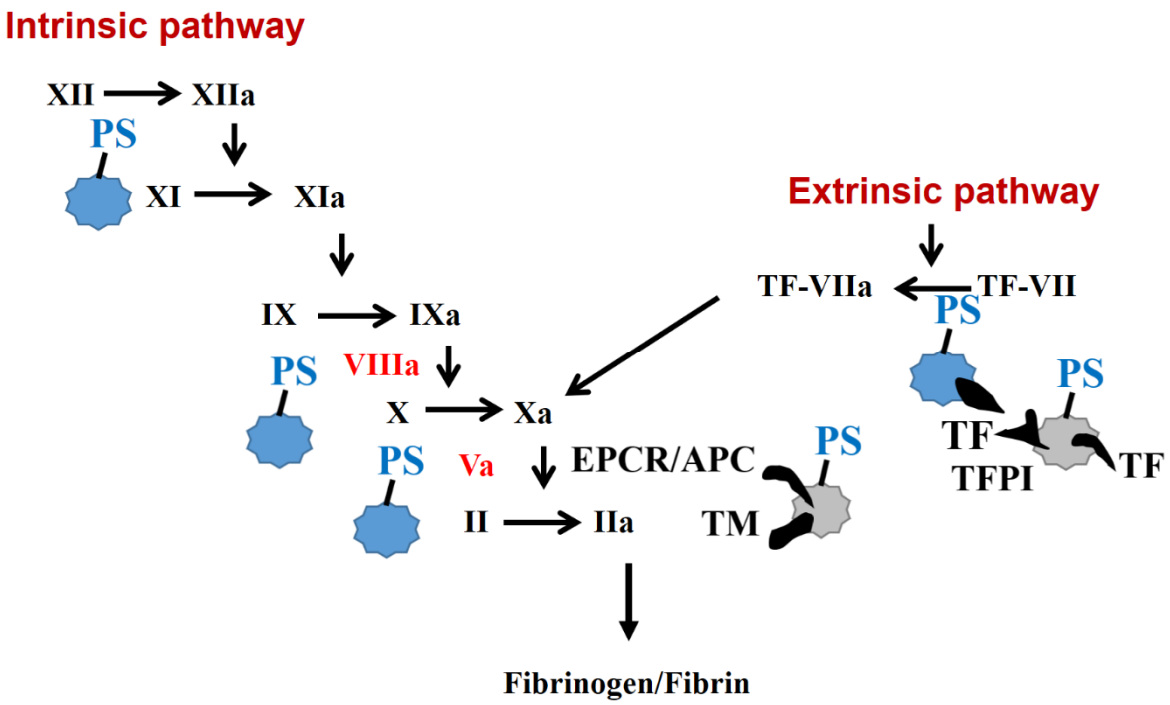
More recent data showed that MVs not only propagate coagulation by exposing PS but also initiate thrombin generation independently of TF and the extrinsic pathway (109, 125). In particular, platelet and erythrocyte-derived MVs have been shown to initiate and support thrombin generation through the intrinsic pathway in a FXII-dependent manner (136). In addition, erythrocyte-derived MVs may be also capable of promoting coagulation in a FXI-dependent manner as shown in sickle cell disease and in blood units (137, 138). These findings shed new light on the procoagulant properties of MVs and their possible hypercoagulable impact.

1.7.3 Procoagulant properties of MVs: regulation of coagulation and fibrinolysis

Apart from their well-known procoagulant activities, evidence exists regarding their ability to regulate coagulation - through anticoagulant antigens - and fibrinolysis. MVs have been proven to harbour functionally active tissue factor pathway inhibitor (TFPI) on their membrane (125, 126), and support

activated protein C and protein S mediated regulation of coagulation (139-141), well-known physiological anticoagulant pathways. Moreover, it has recently been demonstrated that MVs may also expose fibrinolytic properties and support plasmin generation (142, 143). These more recent discoveries open a new scenario where it is likely that the balance between MVs pro- and anticoagulant properties ultimately determines their net effect in haemostasis and thrombosis.

Figure 5: Procoagulant and anticoagulant properties of microvesicles. The best established property of MVs is their ability to promote coagulation, which is largely linked to their physical characteristics: the externalization of anionic phospholipids (predominantly phosphatidylserine [PS]) promotes the interaction with clotting proteins cationic domains, and ultimately thrombin formation. Additionally, some populations of MVs have been shown to display TF on their surface and therefore can trigger the initiation of coagulation via the extrinsic pathway. Moreover, new mechanisms have been recently described. These findings support the evidence that MVs appear also able to activate the intrinsic pathway. Evidence exists also regarding their ability to regulate coagulation through anticoagulant antigens. MVs have been proven to harbor functionally active tissue factor pathway inhibitor (TFPI) on their membrane, and support activated protein C mediated regulation of coagulation.



1.7.4 MV-induced intercellular communication by cross-talk between inflammation and coagulation

Recent studies suggest that MVs are significant mediators of intercellular communication under physiologic and pathologic conditions (121, 144). MVs contain antigens from their cell of origin and can transfer these surface molecules to other cell types and organs. The binding of MV surface antigens to their specific counter receptor may activate intracellular signalling pathways. Inflammation and haemostasis share an interactive relationship because they are linked through common activation pathways and feedback regulation systems. Emerging evidences support the idea that MVs may play a role in cross-talk between inflammation and coagulation because MVs, both from endothelial cells and platelets, have been shown to function as vectors for many inflammatory mediators (145). In vitro experiments have demonstrated that endothelium-derived MVs promote and stabilize platelet aggregates by bearing ultra large von Willebrand factor and that oxidized phospholipids in endothelium-derived MVs may be particularly active in mediating both monocytes adherence to endothelial cells and the activation of neutrophils. These findings suggest that the events surrounding the release of endothelium-derived MVs and its subsequent binding to monocytes might be involved in thrombogenesis (146). Moreover, MVs derived from leukocytes bear P-selectin glycoprotein ligand-1 (PSGL-1), which interacts with P-selectin, a well-known endothelial cell receptor. The interaction between PSGL-1 and P-selectin, and specifically the binding of leukocyte-derived MVs to the activated endothelium, is involved in thrombogenesis, as recently demonstrated (147). Leukocyte-derived MVs also contribute to the development of thrombi through the recruitment of platelets and the accumulation of TF (124, 148). Finally, a series of in vitro studies demonstrated that MVs released by aggregating platelets may facilitate platelets and endothelial cells activation via the transcellular delivery of arachidonic acid or other mediators (145). It has also been suggested that MVs shed by leukocytes stimulate cytokines release and the induction of TF in endothelial cells by activating a signalling pathway which may lead to an increased proinflammatory and procoagulant activity. Other mechanisms contributing to the regulation of MV procoagulant properties rely on the balance between TNF-α and anti-inflammatory cytokines, such as interleukin (IL)-10. Indeed,

endogenous IL-10 was recently reported to downregulate TF vehiculation in monocytes and TF-bearing MVs release, impeding thrombin generation (128, 144, 148).

Table 9. Overall microvesicles potential functions in coagulation, fibrinolysis and inflammation.

Function	Mechanism involved	MV subtype
Procoagulant	- Phosphatidylserine (PS) exposure	Platelet MVs – Monocyte MVs – Erythrocyte MVs – Endothelial MVs
	- Tissue factor (TF) exposure	Platelet MVs – Monocyte MVs – Endothelial MVs – tumour MVs
	- Intrinsic pathway activation (FXII and/or FXI)	Platelet MVs – Erythrocyte MVs
Anticoagulant	- TF pathway inhibitor (TFPI)	Monocyte MVs – Endothelial MVs – tumour MVs
	- Protein C pathway (TM and EPCR)	Monocyte MVs – Endothelial MVs
Fibrinolytic	- tPA	Endothelial MVs
	- uPA/u-PAR	Monocyte MVs – Endothelial MVs – tumour MVs
Anti-fibrinolytic	- PAI-1	Platelet MVs – Monocyte MVs – Endothelial MVs
Pro-inflammatory	- ultra large vWF and oxidized phospholipids	Endothelial MVs
	- P-Selectin glycoprotein ligand 1	Leukocyte MVs
	- TF	Leukocyte MVs
	- arachidonic acid	Platelet MVs
	- cytokines	Leukocyte MVs

TM, thrombomodulin; EPCR, endothelial protein C receptor; t-PA, tissue plasminogen activator; u-PA(R), urokinase (receptor); PAI-1, plasminogen activator inhibitor type 1; vWF, von Willebrand factor.

1.8 Microvesiscles detection

Different methods and combinations of methods have been developed to detect and analyze MVs (Table 10). Appropriate sampling conditions, processing, and sample storage are essential (149). MVs can be directly quantified in platelet-free plasma obtained by serial centrifugation of citrated whole blood. Alternatively, washed MVs can be isolated from platelet-free plasma by ultracentrifugation before re-suspension and analysis. Flow cytometry is the most widely used method because it enables to analyze both quantitative and qualitative characteristics of MVs (150-152). Flow cytometry allows to determine size by assessment of the forward light scatter of each MV. Further accuracy is provided by using calibration beads of a specific diameter for comparison. Additionally, platelet-free plasma or MV suspensions are labeled with fluorescently conjugated monoclonal antibodies against cell-specific

surface antigens. Double staining of MVs allows to determine the origin/cellular source of the MVs. Annexin V or lactadherin binding is used to confirm the phospholipid properties of MVs, although some MVs do not bear these phospholipids antigens, and a variety of cell-specific antibodies have been employed to determine MVs subtypes (153).

On the other hand, functional assays measure the procoagulant activity of isolated MVs. The advantages of functional assays include their high sensitivity, simplicity, and the use of well-defined reagents (124). Solid-phase capture assay consists of measuring the PS content of MVs. Briefly, PS+MVs are captured on an ELISA plate coated with annexin V-streptavidin and incubated with FV, FX, and prothrombin to form the prothrombinase complex that then promotes the cleavage of prothrombin to thrombin. The generated thrombin is detected using a chromogenic substrate (124, 150). Another assay, measures the phospholipid-dependent procoagulant activity of MVs using phospholipid depleted substrate plasma. Factor Xa (and calcium) triggers the coagulation cascade and a shortening clotting time of the sample indicates an increased concentration of procoagulant phospholipid (154). In addition, TF dependent procoagulant activity can be assessed using a specific blocking antibody to TF (155-157). In particular, there are two assays to measure TF activity of MVs isolated from plasma by centrifugation or captured by a monoclonal antibody. The first has been used by a number of different laboratories, the second is quite time-consuming and has not been widely adopted. Finally, new techniques such as laser-induced nanotracking (158), atomic force microscopy (159), and dynamic light scattering have been developed (160). These advances represent exciting possibilities for MV analysis.

1.8.1 Limitations of currently available detection methods

The clinical research on MVs is hampered by the limitations of the currently available detection methods (Table 10). Firstly, pre-analytical conditions highly influence the isolation of MVs (Table 11). For instance, isolation of MVs from blood is affected by vein puncture, tube transportation, time between blood collection and handling, the anticoagulant, centrifugation and washing procedures, the presence of lipoprotein particles and small platelets within the size range of MVs, storage, freezing and thawing procedures (149, 161). The only multicenter study performed by the International

Society on Thrombosis and Haemostasis (ISTH) Vascular Biology Standardization Subcommittee showed that a common pre-analytical protocol can reduce the inter-laboratory variability of flow cytometric enumeration of platelet-derived MVs in healthy individuals. However, the significant variability even when a common protocol is correctly applied remains unacceptably high for the clinical use of MVs (149, 162, 163). Second, it is well known that flow cytometry is the most common method used to determine the size and number of MVs. However this method does have many intrinsic drawbacks, that have been recently well reviewed (160, 161). The major points are: i) only a small fraction of MVs can be detected by commercial flow cytometers for polystyrene beads because of their detection limit (300–500 nm) and because they can resolve only particles that differ by approximately ≥ 280 nm in size (161); ii) quantitative size information are imperfect because they are obtained by comparing the scattering intensity of MVs with that of beads of known size. The scattering intensity, however, depends not only on size but also on shape, refractive index, and absorption (161). Thus, it has been postulated that flow cytometry underestimates the number of MVs in a sample, by up to 100–1000 times (159); iii) fluorescence threshold is also imperfect because no panspecific marker for total MPs exists. Annexin V should not be used to define MVs, as only a minority of circulating MVs expose PS and annexin V binding is highly dependent on Ca2+ concentrations and pre-analytical conditions (164). Indeed, there is no consensus on this point yet; iv) if very small MVs cannot be accurately detected, false-positive signals can arise from other non-cell-derived particles and/or background noise (164). As far as the limitations of functional assays are concerned, these assays do not provide any information on MVs size, cellular source or their physical properties; moreover they does not take into account the purity of the sample. Optimized protocols (Table 11) including a combination of both quantitative and qualitative methods should be used to best characterize MVs in clinical studies (124, 127). However, no consensus on the best method has been reached so far.

Table 10. Methods of MVs quantification (from Burger et al. 2013 (165))

Protocol	Quantification method	Advantages	Limitations
Flow cytometry	Fluorescence and light-scattering properties MVs in suspension	- Available to most research facilities - Rapid - Multiple antigens may be analyzed MVs analyzed on an individual basis	- Quantification of 100–400 nm may be imperfect - Cell origin identification is antibody-dependent
Immunoassays	Immunocapture of MVs and quantification based on the presence of surface antigen	- Available to most research facilities - No size restrictions	- Quantification is done in bulk - Quantifies based on a single antigen - Does not allow for size determination
Functional assays	MVs procoagulant or prothrombinase activity	- Available to most research facilities - Provides an indication of biological activity	- Quantification is done in bulk - Measures only a single biological activity - Does not allow for size determination
Atomic force microscopy	Cantilever is used to scan the MVs surface and tip displacement is related to surface properties	- Allows for very accurate MVs sizing - Allows for 3D view of MV structure - May be used for quantification	- Determination of cell origin requires development of specialized antibody-coated surfaces - Not conducive to large sample numbers
Nanoparticle tracking analysis	MVs are visualized by light microscopy and light scattering is observed; Brownian motion of individual particles is tracked by video	- Clear idea of MV size - Allows for quantification	- Utility of assay for quantification is unclear - Non-universal technology - May be time-consuming

Table 11. Overview of major challenges related to flow cytometric analysis of MVs (from Mooberry et al. 2015 (150)).

Pre-Analytical	Analytical/Technical
• Method of blood collection	• Flow cytometer
– Tourniquet use	– Intrinsic resolution capabilities
– Needle diameter	
– Type of anticoagulant	
• Sample processing	• Size gating
– Sample type (whole blood, plasma, isolated MPs)	– Beads vs biologicals
– Time to sample preparation	
– Centrifugation protocol	
– “Micro-clot” formation	

• Sample handling and storage	• Fluorescence gating
– Sample transportation/agitation	– Proper use of isotype controls (ITCs)
– Fresh vs freeze/thaw	– Detection of dimly expressed antigens
	– Titration of antibodies and ITCs
	– Fluorochrome aggregates
	• MP enumeration
	– Use of counting beads
	– "Swarm effect"

1.9 Microvesicles and cancer

Dvorak et al. (166) first proposed a relationship between tumour-derived MVs and thrombosis. The authors stated that shed vesicles carry procoagulant activity that can account for the activation of the clotting system and the fibrin deposition associated with these and many other types of malignancy in animals and humans. Subsequent studies showed that the procoagulant activity of the tumour-derived MVs was a result of the presence of TF (96, 134, 167-169). Many types of cancer cells express TF and release TF+MVs (96). Furthermore, circulating TF+MVs can be detected in patients with a variety of cancers, including pancreatic, lung, gastric, breast, and brain (169-173). Table 12 summarizes studies evaluating the association between circulating MVs and VTE in patients with cancer. It is likely that these TF+MVs are released from the tumour. For instance, one study in patients with pancreatic cancer found that the TF+MVs co-expressed the epithelial tumour antigen MUC-1 and that pancreatectomy dramatically reduced the level of TF+MVs (173). The majority of studies have examined the relationship between TF+MVs and VTE in patients with pancreatic, brain, colorectal, or lung cancer because these patients have the highest rates of VTE (96). TF+MVs were detected in all these cancers but patients with pancreatic cancer were found to have the highest levels of MV TF activity (170). One possible explanation for this is that the endocrine function of the pancreas provides an easy route for transporting TF+MVs from the tumour into the blood. A longitudinal study with 11 pancreatic cancer patients found a time-dependent elevation of MV-TF activity that proceeded to VTE in 2 patients (98). Several other prospective studies found an association between MV-TF activity in patients with pancreatic cancer but not in patients with lung, gastric, colorectal, ovarian, or brain cancer (170, 172, 174). There was also a strong association

between MV-TF activity and mortality in patients with pancreatic cancer (170, 174). Overall, these studies suggest that TF+MVs contribute to VTE in pancreatic cancer and may be a useful biomarker for assessing the risk of VTE in these patients. It is likely that TF+MVs contribute to VTE in other types of cancer, but the current studies are too small and the current assays are not sensitive or specific enough to reveal a relationship between TF+MVs and VTE (134). Further development of TF+MV assays is needed before they can be used clinically.

In conclusion, a definitive link between TF+MVs and the development of clinical VTE in cancer has yet to be established and remains somewhat debated in view of conflicting evidence (175). Differences in association could be due to varying levels of TF encryption and participation of TF in non-coagulant activities, such as signalling of the TF-FVIIa complex via protease-activated receptor 2 (PAR2) (176). The exact contribution of TF+MVs to the development of VTE may therefore be highly variable between tumour types, and the association in pancreatic cancer may reflect the high level TF expression relative to other cancer types and/or the late stage of disease presentation (175). Interestingly, while around 80% pancreatic cancer patients show increased TF levels, fewer than 30% of these patients are found to develop thrombosis (177), indicating that elevated TF+MV levels in isolation do not unequivocally trigger thrombosis, and raising the interesting question of why/when the clinically recorded thrombosis does occur.

Table 12. Studies evaluating the role of circulating microvesicles (MVs) as biomarkers for venous thromboembolism (VTE) occurrence in cancer.

Study	Patients	without VTE/with VTE at follow-up	Follow-up	Method	MVs detected	Results [VTE risk]
van Doormaal 2012 [178]	Cancer without VTE at study entry	43/5	6 months	FCM and Functional assay	PS+ PMV TF+MV antigen TF+MV activity	- - - ↑
Thaler 2011 [179]	Solid and haematological cancer	728/53	2 years	Functional assay	PS+	-
Campello 2011 [169]	Solid cancer with and without VTE	30/30	n/a	FCM	PS+ PMV EMV TF+MV	↑ ↑ ↑ ↑
Zwicker 2009 [171]	Cancer without VTE	60/5	1 year	Impedance FCM	TF+MV	↑ OR 3.72 [95% CI 1.18–11.76]
Khorana 2008 [172]	Locally advanced or metastatic pancreatic cancer	11/2	Every 4 weeks for 20 weeks	Functional assay	TF+MV TF antigen (ELISA)	↑ ↑
Bharthuar 2010 [174]	Pancreatic-biliary cancer	117/52 (retrospective)	-	Functional assay	TF+MV activity	↑ OR 1.4 [95% CI 1.1-1.6]
Sartori 2013 [180]	Glioblastoma multiforme	61/11	7 months	FCM	TF+MV	↑ RR 4.17 [95% CI 1.57–11.03]
Auwerda 2011 [181]	Multiple myeloma before chemotherapy	122/15	not specified	Functional assay	TF+MV activity	-
Thaler 2012 [170]	Cancer without VTE at study entry	299 (48 pancreatic)/49 (12 pancreatic)	2 years	Functional assay	TF+MV activity chromogenic and kinetic assay	- HR 1.5 [95% CI 1.0–2.4]*

*Association between VTE and TF+MV activity in pancreatic cancer using chromogenic endpoint assay.

PS: phosphatidylserine, PMV: platelet-derived MVs, TF: tissue factor, FCM: flow-cytometry.

2. Aim of the study

Given the multifactorial genesis of hypercoagulability, the need to find clinically meaningful markers able to describe the thrombotic profile and the lack of studies assessing the longitudinal fluctuations of hypercoagulability-associated VTE biomarkers in cancer patients, we conducted a longitudinal cohort study to evaluate the trend of several coagulation parameters in patients with gastro-intestinal cancer with particular focus on tumour-derived MVs and TF+MVs.

Our primary outcome was the description of coagulation fluctuations over a 6-month period following cancer diagnosis and the secondary outcomes were the association between coagulation parameters and clinical outcomes.

Clinical outcomes considered were: surgical radicality (complete-incomplete), disease severity (localized-advanced), and VTE occurrence.

3. Patients and Methods

This was a longitudinal cohort study conducted between September 2015 and September 2017 at the Thrombotic and Haemorrhagic Diseases Unit and General Surgery of the Padua University Hospital. The study was approved by the Institutional Review Board (ref. 4022/AO/16) of the Padua University Hospital and all the participants signed an institutional review board-approved consent form.

3.1 Patients

Patients with a new diagnosis of gastro-intestinal cancer who underwent surgery at the General Surgery Department of Padua University Hospital were consecutively enrolled from September 2015. The enrolment of patients was completed on January 2017.

Inclusion criteria:

- Newly diagnosed esophageal, gastric, colon or pancreatic cancer with surgical indications
- Age > 18 years
- Signed informed consent

Exclusion criteria:

- progression of previous diagnosed cancer after complete or partial remission
- severe liver or renal failure
- low life expectancy (i.e. Karnofsky Performance Status <60%)(182)
- venous or arterial thromboembolism within the past 3 months
- pregnancy/puerperium
- overt/recent bacterial or viral infection (within the past 2 months)

Baseline demographic and clinical data regarding age, sex, body mass index [BMI], blood group, past medical and surgical history, history of VTE, recent anticoagulant/antithrombotic therapy, antithrombotic prophylactic therapy, and preoperative chemo-radiotherapy were collected.

General laboratory determinations, routinely assessed pre-operatively, including haemogram, C-reactive protein (CRP), traditional coagulation tests (prothrombin time (PT) and activated partial

thromboplastin time (aPTT) and levels of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were recorded.

Moreover, clinical variables and cancer-specific variables were prospectively collected.

In particular, we recorded:

- peri/post-operative surgical complications
- possible transfusion therapy
- surgical radicality
- histology (including pathological tumour-node-methastasis [pTNM] and cancer stage (183))
- possible adjuvant chemo-radiotherapy programme

The Khorana Risk Score (66) was calculated for each patient, and the data for the calculation correspond to clinical data obtained within 1 month of recruitment, including blood counts.

All patients received antithrombotic prophylaxis with graduated elastic stockings and low-molecular weight heparin for 30 days after surgery.

3.2 Blood sampling for coagulation tests and pre-analytical conditions

Venous fasted blood samples (12 mL) were collected into 3.2% sodium citrate with a light tourniquet, using a butterfly device with 21-gauge needle without venostasis (the first few ml discarded to avoid the contact phase activation). Platelet-free plasma (PFP) was prepared within 2 hours following the drawing by double centrifugation (2 x 15 min at 2,500 g) at room temperature. Aliquots of plasma were immediately frozen and then stored at -80°C until use.

Blood samples for coagulation tests were collected:

- baseline (after the enrollment) the day before surgery
- 7 days post-surgery
- 1 month post-surgery
- 6 months post-surgery

The baseline blood sample was collected before the start of thromboprophylaxis with low-molecular weight. The 7 days and 1 month post-surgery blood sample were collected before low-molecular

heparin administration (at least 24 hours wash-out), in order to avoid the interference of heparin with coagulation parameters measurements.

Longitudinal samples were collected during follow-up visits at the Ambulatory of the Thrombotic and Haemorrhagic Diseases Unit, and tumour-related clinical history and clinical outcomes were also re-evaluated. For pre-analytical and analytical analysis, we followed the recent standardization protocol by the Scientific Collaborative Workshop of the International Society of Thrombosis and Haemostasis (ISTH) (163).

3.3 Coagulation parameters

3.3.1 Whole blood Thromboelastometry

Viscoelastic clotting measures were performed on whole blood by ROTEM® (Tem International GmbH, Munich, Germany) test according to the manufacturer's protocol (184). In particular, blood was incubated at 37 °C in a heated cup. Within the cup is suspended a pin connected to an optical detector system. The cup and pin are oscillated relative to each other through an angle of 4°45". As fibrin forms between the cup and pin, the transmitted impedance of the rotation of the pin is detected at the pin and a trace generated (Figure 7). All analyses were performed within 2 hours after blood collection and, once initiated, the blood coagulation was allowed to run 60 min. Extrinsic coagulation cascade was studied with EXTEM test (ex-TEM®; Tem International GmbH) and intrinsic coagulation cascade was studied with INTEM test (in-TEM®; Tem International GmbH). The influence of fibrinogen on clot firmness was estimated with the platelet-inactivating FIBTEM test (fib-TEM®; Tem International GmbH). The following ROTEM parameters were analysed (Table 13):

i) Clotting time (**CT, sec**), corresponding to the time from the beginning of the coagulation analysis until an increase in amplitude of 2mm. The CT reflects the initiation phase of the clotting process;

ii) Clot Formation Time (**CFT, sec**), the time between an increase in amplitude of thromboelastogram from 2 to 20 mm. The CFT measures of the propagation phase of clot formation;

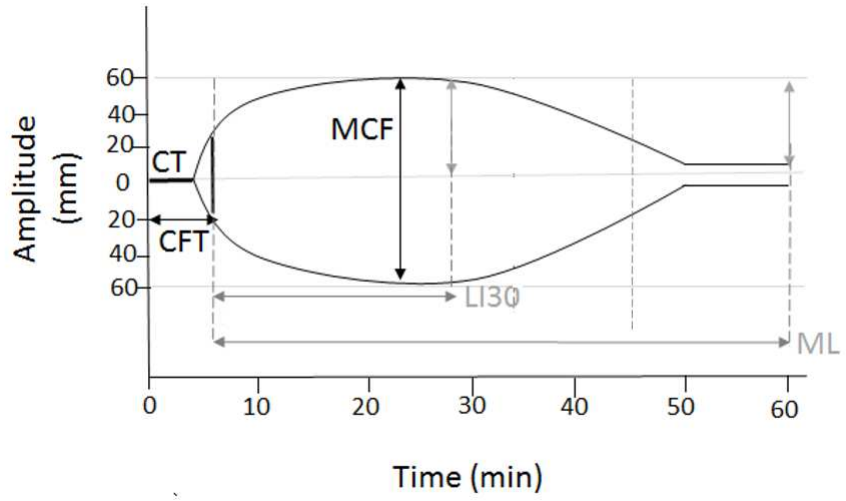
iii) Maximum Clot Firmness (**MCF, mm**), the maximum amplitude in mm reached in thromboelastogram. It correlates with the platelet count and function as well as with the concentration of fibrinogen. The MCF quantifies the strength of the established whole blood coagulum;

iv) Maximum Lysis (**ML, %**), the percentage of lost clot stability (relative to MCF, in %) when the test has been stopped.

Table 13. Summary of the thromboelastometry variables measured.

Parameters	**Description**	**Meaning**	**Correlation to conventional tests**
Clotting time, CT (sec)	Time from start to 2mm above baseline	- Initiation of thrombin generation and clot polymerization. - Expression of coagulation factors activity	PT – aPTT
Clotting formation time, CFT (sec)	Time from start of clotting to amplitude of 20 mm	- Rate of clot formation - Fibrin polymerization and cross-linking with platelet interaction	Fibrinogen concentration, platelet count
Maximum Clot Firmness, MCF (sec)	Maximum strength	Stabilization of clot by platelets and FXIII	Fibrinogen concentration, platelet count
Maximum Lysis, ML (%)	Decrease of clot firmness after MCF	Degree of fibrinolysis after a given amount of time	

Figure 6. Graphical representation of thromboelastometry coagulation and lysis parameters considered in the study. CT: clotting time (sec); CFT: clotting formation time (sec); MCF: maximum clot firmness (mm); ML: maximum lysis (%).



3.3.2 Procoagulant factors and fibrinolysis

Factor VIII and *fibrinogen* activity were measured using fresh unfrozen plasma on a BCS-XP Analyser (Siemens Healthcare Diagnostics, Marburg, Germany).

D-dimer levels were measured with a quantitative latex immunoassay (HemosIL HS D-dimer 500; Diagnostica Stago, Asniers, France) on an ACL TOP® Family Sistems (Diagnostica Stago).

A commercially available enzyme-linked immunosorbent assays (ELISA) was used to measure Plasminogen activator inhibitor-1 antigen (*PAI-1:Ag*) (ZYMUTEST PAI-1 Antigen, HYPHEN BioMed, Neuville-sur-Oise, France).

3.3.3 Hereditary Thrombophilia

Genomic DNA was extracted from whole blood using an automated method (MagCore Extracted System H16, RBC Bioscience, Mew Taipei City, Taiwan). Detection and genotyping for factor V Leiden R506Q (mutation rs6025) and prothrombin G20210A (mutation rs1799963) was performed using commercially available kits for Real-Time PCR (polymerase chain reaction). Real Quality RS-Factor V Leiden (AB Analitica, Padova, Italy) was used to detect factor V Leiden and Real Quality RS Fator II G20210A (AB Analitica, Padova, Italy) to detect prothrombin mutation.

3.3.4 Contact activation pathway

A home-made capture ELISA was used to quantify contact system proteins in order to evaluate the role of the contact system activation in cancer (185). ELISAs for enzyme-inhibitor complexes may serve as biomarkers for recent and/or ongoing contact system activation due to their reduced circulating half-life (185, 186). The levels of FXIa, FXIIa and kallikrein in complex with C1-esterase inhibitor (C1INH) were measured in plasma with ELISAs. Briefly, plates were coated overnight at room temperature with 100 µL of 10 µg/mL MoAb KOK-12, which binds complexed and inactivated C1-inhibitor. The plates were then incubated with plasma samples diluted in PBS-milk 2%. FXIIa-, FXIa- or kallikrein-C1-inhibitor complexes were prepared by incubating 10 µmol/L C1-inhibitor (Behringwerke AG, Marburg, Germany) overnight at 37°C with 1.4 µmol/L FXIIa, 2.7 ·mol/L FXIa, or 2.3 µmol/L kallikrein, respectively. FXIIa-C1INH complexes were detected by incubation for 1

hour of the plates with the biotinylated MoAb F3, FXIa-C1INH by incubation with biotinylated MoAb XI-5 and kallikrein-C1-inhibitor complexes by incubation with biotinylated MoAb K15, each diluted in PBS containing milk 3% and normal mouse serum 1%. Next, the plates were incubated for 30 minutes with a 1:10,000 dilution of polymerized horseradish peroxidase bound to streptavidin (poly-HRP; CLB) in PBS-milk 2%. Absorbance was read at 450 nm on a microplate reader. Kaolin-activated plasma was used as reference (187).

3.4 Circulating microvesicles

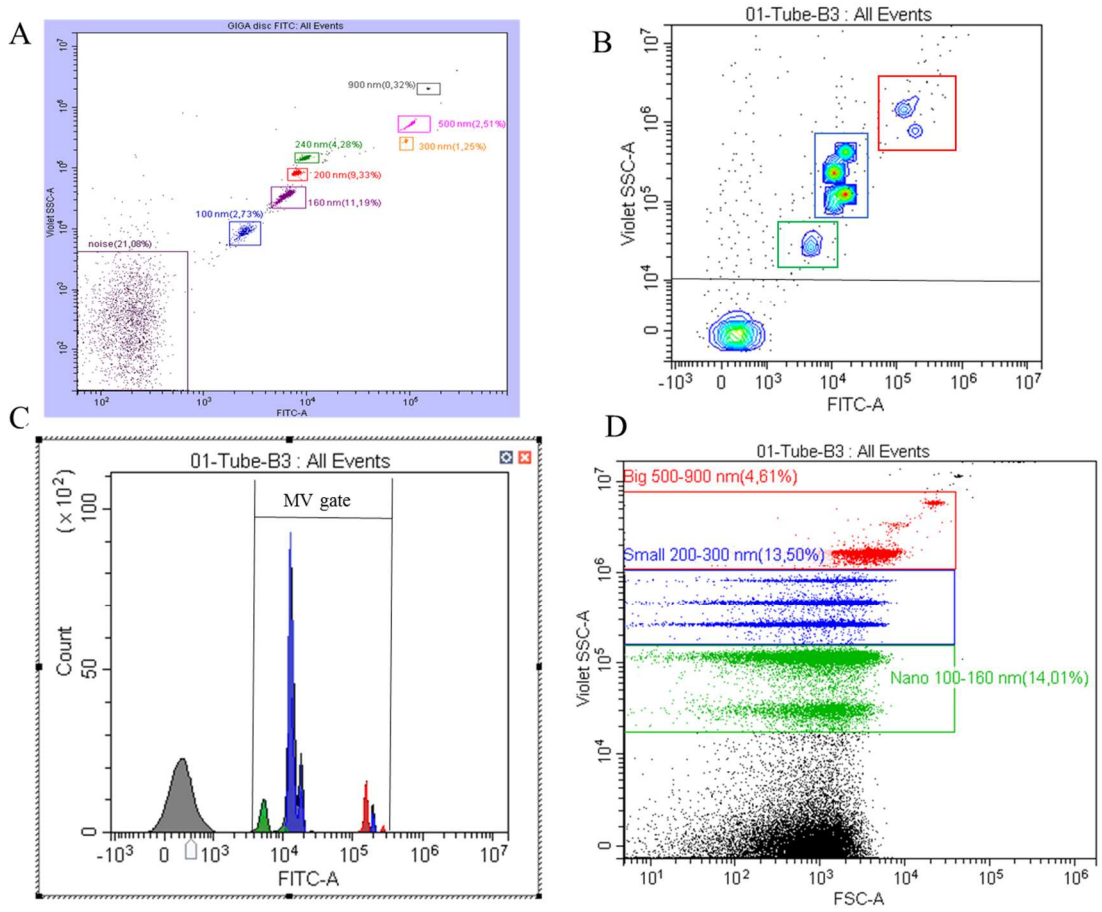
3.4.1 MV-TF activity

For the MV-associated TF activity measurement, MVs were pelleted from 200 µl of PFP by centrifugation at 20,000 g for 30 min at 4 °C, washed twice with HBSA (120mM NaCl, 20mM HEPES, 1mg/mL BSA, pH 7.4), and re-suspended in 200 µl of HBSA. 50 µl aliquots were then added to duplicate wells of a 96-well plate and samples were incubated with either a neutralizing antibody to human TF (hTF1; 4 µg/ml) (1 µl) or an isotype-matched murine monoclonal IgG antibody (4 µg/ml) (1 µl) for 15 min at room temperature. Next, 50 µl of HBSA containing 2 nM FVIIa, 300 nM FX and 10 mMCaCl2 were added to each sample and the mixture incubated for 2 h at 37°C. FXa generation was stopped by 25 µl of 25 mM EDTA buffer and 25 µL of the chromogenic substrate S2765 (1.2 mM) (Chromogenix S-2765) was added. Absorbance at 405 nm was measured using a microplate reader for 30 min every 30 sec. TF activity was calculated by reference to a standard curve generated using Innovin™, a re-lipidated recombinant human TF. The TF-dependent FXa generation (optical density [OD]) was determined by subtracting the amount of FXa generated in the presence of hTF1 from the amount of FXa generated in the presence of the control antibody, and converted to TF concentration (pg/mL) by reference to the standard curve (172, 174, 188).

3.4.2 Flow Cytometric Analysis of Microvesicles

Flow cytometry was performed using a CytoFLEX flow cytometer (Beckman Coulter). The CytoFLEX is equipped with a more sensitive Side Scatter (SSC) resulting in higher particle resolution compared to the Forward Scatter (FSC). The CytoFLEX has a three laser pathway: red laser (638 nm) – blue laser (488 nm) – violet laser (405 nm). This means that normally the blue laser serves as the triggering laser, the red laser gets a positive and the violet laser a negative time delay. To standardize the instrument to detect MVs, standard filter configuration was changed so that the SSC from the 405 nm violet laser (VSSC) was used as trigger signal to discriminate the noise instead of the normally used 488 nm FSC. When the VSSC was used as a trigger signal the noise was significant lower in comparison to the 488 nm SSC when beads were used as standardization reference (189) (Figure 8). For MV size calibration of the flow cytometer fluorescent polystyrene beads (Megamix FSC & SSC Plus, BioCytex, Marseille, France) were used in sizes of 0.1, 0.16, 0.2, 0.24, 0.3, 0.5, and 0.9 μm. VSSC and FL1 channel gain were set to visualize the beads (Figure 8, panel A and B). Megamix bead solution was gated excluding the background noise (due to the solution itself). After turning the set in VSSC and FSC, a rectangular gate was set between the 0.1 mm and 0.9 μm bead populations and defined as MV gate (Figure 8, panel C). According to the diameter of beads three gates were created, in order to detect *big MVs* (0.5-0.9 μm), *small MVs* (0.2-0.3 μm), and *nano MVs* (0.1-0.2 μm) (Figure 8, panel D). A suggested by Wisgrill et al. (189) the run was performed with a maximum event rate up to 2,000 events/sec at the lowest flow rate (10 mL/min).

Figure 8. Microvesicles gate creation with fluorescent polystyrene beads in sizes of 0.1, 0.16, 0.2, 0.24, 0.3, 0.5, and 0.9 µm. (A-D) Fluorescence polystyrene beads of different sizes were used to determine the MV gate between the 0.1 µm and 0.9 µm bead peaks. 0.1 µm fluorescence beads were detectable using the 405 nm side scatter (VSSC).



3.4.3 Microvesicles detection

PFP was thawed in a waterbath for 5 min at 37°C and immediately processed for immunolabeling. PFP was analyzed only after a single freeze-thaw cycle. Prior to the staining, the antibody mixture was centrifuged at 20,000g for 30 minutes to remove fluorescent particles (190).

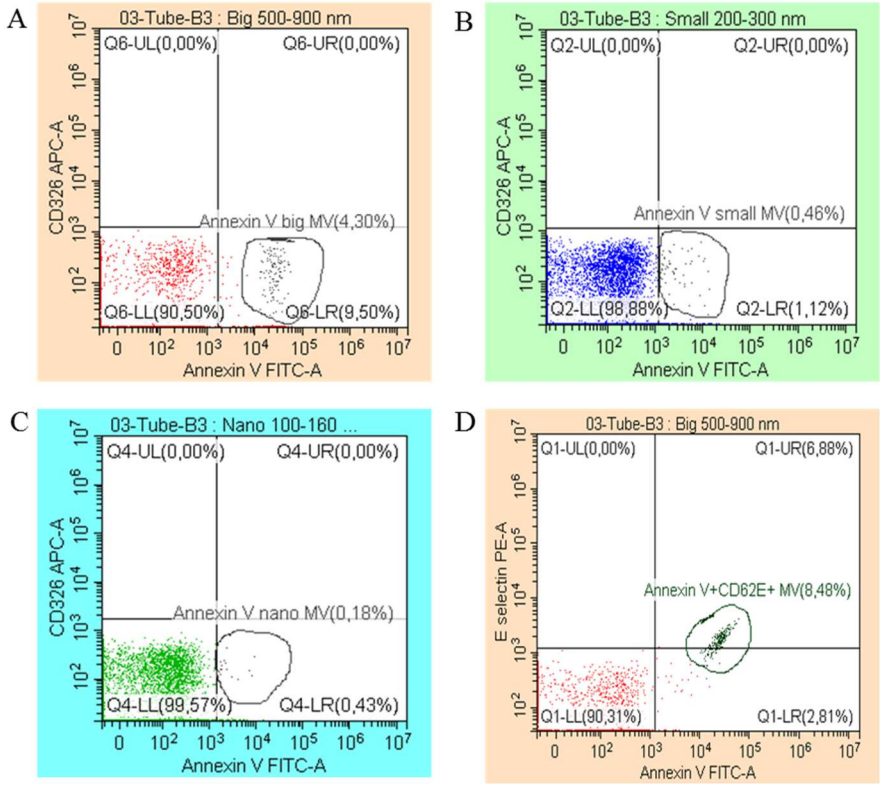
Twenty µL of PFP was stained with 2 µL of FITC-labeled annexin V (eBiosceince, Bender MedSystems GmbH, Vienna, Austria) + 2 µL of PE-labeled anti-CD62E antibody (BioLegend, San Diego, CA) + APC-labeled anti-227 antibody (MUC-1 antigen for pancreatic and gastro-esophageal cancers) (BioLegend, San Diego, CA) or APC-labeled anti-326 antibody (EpCAM antigen for colon cancer) (BioLegend, San Diego, CA) for 15 minutes at 37°C. Parallel incubation was performed with isotype-matched control antibody. True MV events were defined as double positive-stained for

annexin and anti-CD62E and double-stained for annexin and anti-tumour antigen events (Figure 9, panel D). Stained PFP was then diluted 1:400 with annexin binding buffer (Bender MedSystems) containing recombinant Hirudin (Sigma Aldrich, St. Louis, MO) to prevent clot formation. Diluted binding buffer was sterile filtered through a 0.2 µm mesh to reduce background noise. MVs were further characterized by surface staining for following subpopulations:

- Total annexin-positive (PS+)
- Total annexin-negative (PS-)
- Annexin+CD62E+ (endothalial-derived MVs)
- Annexin+CD227+ or Annexin+CD326+ (tumour-derived MVs)

Fluorescence measured with the respective isotype negative control antibody were subtracted in order to avoid unspecific signal. MVs were expressed as events/µL with the volume measurement of the CytoFLEX. Files were exported and data were evaluated by CytExpert (Software Version 1.2, Beckman Coulter).

Figure 9. Microvesicles detection. Using the defined MV gate, all events positive for annexin V were defined as big, small or nano-MV events (Panel A-C). MV were further analysed for surface marker staining; double positive-stained for annexin and anti-CD62E and double-stained for annexin and anti-tumour antigen events were considered (Panel D).



3.5 Outcomes

The primary outcome was the evaluation of coagulative parameters trend over a 6-month period following the diagnosis. Clinical outcomes recorded during the follow-up were: i) surgical radicality (i.e. complete or incomplete); ii) cancer severity (i.e. localized or advanced cancer); iii) any thrombotic event including superficial vein thrombosis (SVT), symptomatic or asymptomatic VTE (DVT and/or PE) or thrombosis in unusual sites. The secondary outcome was the association between the coagulative profile at the different time-points and the clinical outcomes.

DVT/SVT was confirmed by compression ultrasound, and PE was confirmed by pulmonary computed tomography (CT). We considered calf vein thrombosis as distal and thrombosis in popliteal vein or above as proximal. Portal vein thrombosis was diagnosed by abdominal CT or splanchnic venous ultrasound.

Disease progression was evaluated by total body CT, nuclear magnetic resonance (NMR) and/or PET-CT, and tumour markers trend (CEA, CA 19-9), according to the oncologic follow-up of the patient.

Each patient was followed for at least 6 months and for a maximum of 12 months. Clinical follow-up duration was calculated from enrolment to the last contact with patient (phone call or official medical reports).

3.6 Statistical analysis

The qualitative and quantitative variables were expressed as frequencies and median with interquartile range, respectively. The coagulative parameters at the different time-points were compared through Friedman test for non-parametric paired values with Dunn's correction for multiple comparisons. Non-parametric Mann–Whitney or Kruskal-Wallis tests were used to compare median coagulative parameters between two or more non-paired groups, respectively. Spearman correlation test was used to correlate laboratory parameters. The clinical outcomes were described by cumulative incidence and incidence rate (with 95% confidential interval [CI]). Multivariable logistic regression analysis was performed in order to evaluate the possible prediction role of coagulative parameters for surgical radicality and severity of cancer. The association between the odds of VTE and coagulative

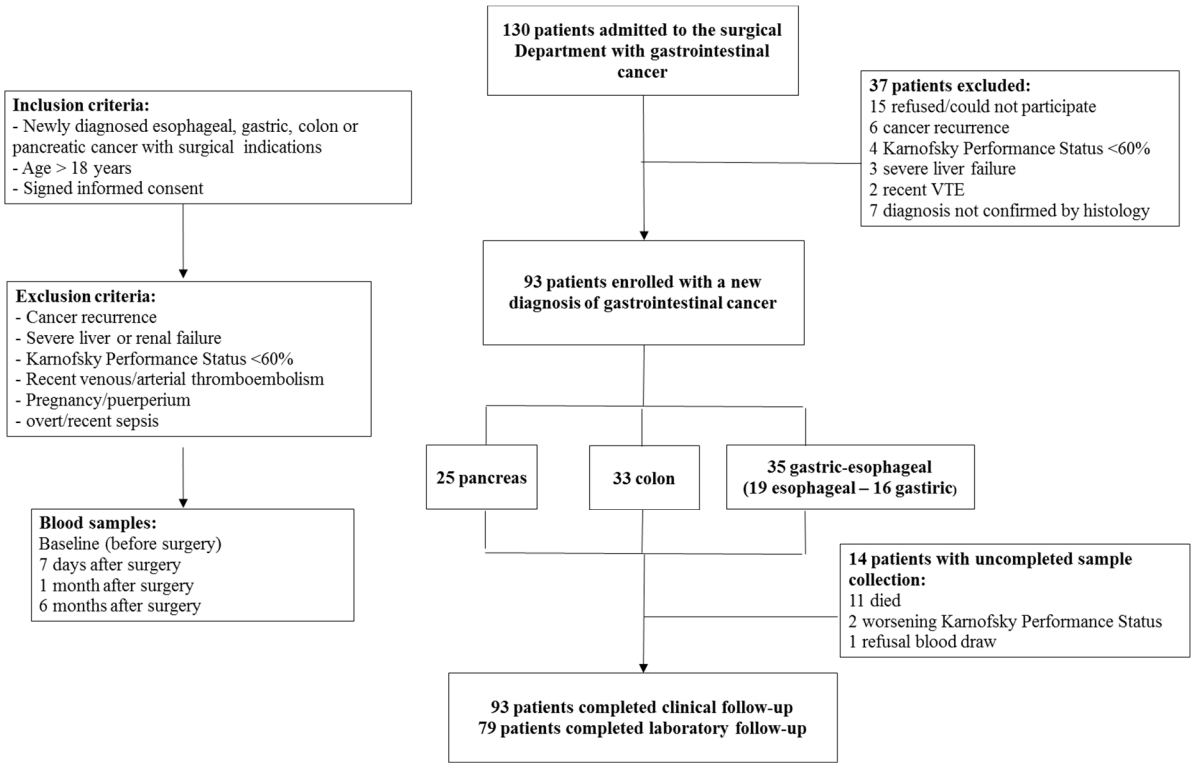
parameters were evaluated. For this purpose, Cox regression multivariable analysis was performed to evaluate which parameters were significantly associated with VTE occurrence. Receiver operating characteristic (ROC) curves for cut-off levels of the parameters detected by Cox were calculated by considering VTE patients cases and non-VTE patients controls. Levels with sensitivity and specificity >80% were used as cut-off points to discriminate between high and low levels. Kaplan-Meier analyses were applied to compare thrombosis-free survival among groups defined by risk scores and log-rank tests were used to test whether differences among groups were statistically significant. Hazard ratios (HR) for VTE were calculated by multivariable Cox regression analyses. All the tests were two-tailed, and differences were considered significant at $p<0.05$. All the analyses were performed using IBM-SPSS 19.0.

4. Results

4.1 Characteristics of the study population

Out of 130 cancer patients consecutively admitted to the General Surgery Department of the Padua University Hospital with a diagnosis of gastrointestinal or pancreatic cancer during the study period, 37 were excluded for the following reasons: i) 5 patients refused to participate; ii) 10 patients lived outside the Veneto Region and the follow-up was not possible; iii) 6 patients underwent surgery for cancer recurrence; iv) 4 patients had a Karnofsky Performance Scale <60%; v) 3 patients had severe liver failure; vi) 2 patients had VTE one month before the surgery; vii) 7 patients were excluded after the first sample because the histology did not confirm the neoplasm or was compatible with different peculiar neoplasms (e.g. neuroendocrine cancer, metastasis from a different site, sarcoma). Thus, 93 patients (25 with pancreatic cancer, 33 with colon cancer and 35 with esophagus or stomach cancer) were enrolled in the study. Out of 35 patients with esophageal-gastric cancer, 19 (54%) had cancer of esophagus or gastric cardia and 16 (46%) had gastric cancer. Figure 10 shows the flow-chart of the study.

Figure 10. Study flow-chart:



The baseline characteristics of the population are reported in Table 14 categorized by cancer type. In particular, median age of the enrolled patients was 69, 72 and 69 years for pancreatic, colon and gastroesophageal cancer, respectively. There was a higher proportion of male, especially in the third group (p ANOVA <0.05). The BMI was similar in all three groups, with median levels of 24.2, 26 and 24.3 Kg/m^2, respectively. The non-0 blood group was the most prevalent in the population. As far as surgery was concerned, surgical radicality was obtained in 48% of pancreatic, 67% of colon and 83% of gastric-esophageal cancer (p ANOVA <0.001). Pancreatic cancer was associated with a higher rate of surgical complications (52% vs 21% and 26% for colon and gastric cancers, respectively, p ANOVA <0.01) and received more blood transfusions (32%) compared to the other cancer subtypes (21 and 11%, respectively, p ANOVA <0.01). Surgical complications included wound dehiscence, anastomotic fistula, post-operative bleeding, parastomal phlegmon, and intra-abdominal fluid collections. The histology from the surgical biopsy showed that 16% and 60% of pancreatic cancer had localized (stage I-IIa) and locally advanced (stage IIb-III) cancer, respectively. The same with respect to colon cancer was 33% and 46%, respectively. Among gastric-esophageal cancer, 40% had localized and 40% locally advanced disease. The proportion of metastatic cancer (stage IV) was similar among groups (around 20%). 60% of pancreatic and colon cancer received adjuvant chemotherapy after surgery vs 46% of gastric cancer. Finally, there was a higher proportion of patients with a high thrombotic risk, according to the Khorana score (≥3), in the pancreatic group (48%) vs gastric group (6%, p<0.001). None of the patients with colon cancer showed a high thrombotic risk profile, with 70% of them having a low risk score (0).

Table 14. Baseline characteristics of the study population.

	Pancreatic cancer n. 25	**Colon cancer n. 33**	**Gastric-esophageal cancer n. 35**
Age – years	69 [62-76]	72 [58.5-77.5]	69 [61-75]
Male – n(%)	13 (52)	20 (61)	26 (74)
BMI – Kg/m^2	24.2 [21.2-25.4]	26 [22.3-28.8]	24.3 [22.2-26.6]
Blood group – n(%)			
- 0 blood group	9 (36)	12 (36.3)	14 (40)
- non 0-blood group	16 (64)	21 (63.6)	21 (60)

Karnofsky Performance Status - %	80 [80-90]	90 [80-90]	90 [80-90]
Neo-adjuvant therapy – n(%)	2 (8)	6 (18)	9 (26)
Surgical radicality – n(%)	12 (48)	22 (67)	29 (83)
Surgical complications – n(%)	13 (52)	7 (21)	9 (26)
Transfusions – n(%)	8 (32)	7 (21)	4 (11)
Stage – n(%)			
- Localized (I-IIa)	4 (16)	11 (33)	14 (40)
- Locally advanced (IIb-III)	15 (60)	15 (46)	14 (40)
- Distant metastasis (IV)	6 (24)	7 (21)	7 (20)
Histology – n(%)			
- Adenocarcinoma	22 (88)	31 (94)	27 (77)
- Squamous carcinoma	-	-	8 (23)
- Other	3 (12)	2 (6)	-
Adjuvant therapy – n(%)	15 (60)	19 (57.6)	16 (46)
- platin protocols	3 (20)	14 (74)	10 (62.5)
- Other	12 (80)	5 (26)	6 (37.5)
Khorana score – n(%)			
- High ≥ 3	12 (48)	-	2 (6)
- Intermediate 1-2	12 (48)	10 (30)	18 (51)
- Low 0	1 (4)	23 (70)	15 (43)

Variables are expressed as median and range interquartile or frequency.

4.2 Clinical outcomes during the follow-up

The median duration of the clinical follow-up was 6 months [6-8.5] for pancreatic, 8 months [7-11] for colon, and 6.5 months for gastric cancer [6-9.25] (Table 15).

Within the pancreatic cancer group, the cumulative incidence of VTE was 20% [95%CI 8.8-39.13] over the 6-month follow-up period (Table 15). The incidence rate of VTE, considering the different follow-up duration for each patient (total person-time 652 months), was 9.21 [95%CI 3.37-20.4] per 100 person-years. As for colon cancer, the cumulative VTE incidence was 12.1% [95%CI 4.8-27.3] over the 8-month follow-up period. Moreover, the VTE incidence rate (total person-time 717 months) was 6.69 [95%CI 2.13-16.2] per 100 person-years. Finally, in the subgroup of esophageal cancer patients, VTE occurred in 17.1% [95%CI 8.1-32.7] over the 6.5-month of follow-up period, whereas the VTE incidence rate (total person-time 688 months) was 10.4 [95%CI 4.24-21.7] per 100 person-years. The median time of VTE occurrence was 2 months after surgery for pancreatic cancer and 3 months after surgery for colon cancer. On the contrary, most of the VTE events (4 out of 6) in the gastric-esophageal group were post-operative, occurring in the first 15 days after surgery.

Disease progression was recorded in 40% of pancreatic cancer [95%CI 23.4-59.3], occurring at a median of 3.5 months after surgery. The proportion of disease progression among colon cancer patients was 21.2% [95%CI 10.7-37.7], occurring at a median of 4 months after surgery. The same figure for gastroesophageal cancer was 22.8% [95%CI 12.07-39.02], being diagnosed at a median of 3 months after surgery.

Five pancreatic cancer patients (20% [95%CI 8.8-39.13]) died during the 6-month follow-up period, and they died at a median of 2 months after enrollment. As for colon cancer, 2 patients died (6.06% [95%CI 1.02-18.6]) during the 8-month follow-up period, at a median of 5.5 months after the enrollment. Finally, 4 patients with esophageal-gastric patients died (11.4% [95%CI 4.5-25.9]) over the 6.5-month follow-up period, at a median of 2.5 months after surgery. Clinical follow-up was complete for the whole study population (100%) through medical/oncologic records, phone calls to patients and treating physicians.

Table 15. Main outcomes during the follow-up

	Pancreatic cancer n. 25	**Colon cancer n. 33**	**Gastric-esophageal cancer n. 35**
Total observation time – months	6 [6-8.5]	8 [7-11]	6.5 [6-9.25]
Thromboembolic events – n(%)	5 (20)	4 (12)	6 (17)
- Superficial vein thrombosis	-	1 (8.3)	-
- Proximal deep vein thrombosis	1 (20)	1 (25)	3 (50)
- Distal deep vein thrombosis	-	1 (25)	1 (16.6)
- Pulmonary embolism	2 (40)	-	1 (16.6)
- Portal vein thrombosis	2 (40)	1 (25)	-
- Upper extremity deep vein thrombosis	-	-	1 (16.6)
Time of VTE occurrence from enrollment– months	2 [1.125-6.5]	3.25 [0.5-6]	0.5 [0.5-1]
Disease progression – n(%)	10 (40)	7 (21)	8 (23)
Time of disease progression from enrollment- months	3.5 [3-5.25]	4 [3-9]	3 [3-7]
Death – n(%)	5 (20)	2 (6)	4 (11.4)
Time of death from enrollment - months	2 [1-3]	5.5 [5-6]	2.5 [1.25-8.25]

Variables are expressed as median and range interquartile or frequency.

4.3 Baseline laboratory and coagulative parameters

Regarding blood samples, 93 patients out of 93 reached the 7 days blood draw; 90 out of 93 reached the 1 month follow-up visit and blood draw (one patient died of pulmonary embolism, two patients died of surgical complications), 80 patients reached the 6-month follow-up visit and blood draw (8 patients died during the follow-up, 2 had a worsening of Karnofsky Performance Status and 1 refused blood draw). Thus, complete coagulation follow-up (6 months after diagnosis) was only available for 79 out of 93 patients (85%) (Figure 10).

Table 16 shows the laboratory and coagulation parameters measured in the study population at baseline categorized by cancer type. Generally speaking, all cancer patients showed white blood cell and platelet counts, PT, and aPTT within the normal range and were slightly anemic. Also, C-reactive protein was slightly increased in all three cancer groups. Levels of factor VIII in pancreatic cancer were significantly increased compared to the normal range and significantly higher compared with the other cancer types (p ANOVA <0.001). As far as the fibrinolytic system was concerned, both D-Dimer and PAI-1 antigen were increased in all cancer patients, with a greater increase within the pancreatic cancer group. Finally, 6 patients out of the overall population (6.4%) patients were carriers of hereditary thrombophilia: namely one factor V Leiden carrier in the pancreatic group, one factor V Leiden and one prothrombin mutation in the colon group, and one factor V Leiden and two prothrombin mutation in the gastric subgroup.

Table 16. Baseline laboratory and coagulation parameters in the study population.

	Pancreatic cancer n. 25	**Colon cancer n. 33**	**Gastric-esophageal cancer n. 35**
White blood cells – $x10^9$/L *(r.v. 4.40-11)*	7.55 [6.32-8.79]	7.00 [5.48-8.25]	7.57 [5.79-9.91]
Hemoglobin – g/dL *(r.v. 13-17)*	115 [108-124]	114 [105-123]	115 [108-131]
Platelets - $x10^9$/L *(r.v. 150-450)*	315 [236-425]	261 [215-367]	313 [249-403]
C-reactive protein –mg/dL *(r.v. 0-6)*	44 [21-58]	49 [28.2-63.5]	55 [35-80]
PT - % *(r.v. 75-11)*	88.5 [66.7-99]	85 [70.5-102]	85 [75-95]

aPTT – sec *(r.v. 22-32)*	25 [24-26]	25 [24-28]	25 [23.2-27]
Factor VIII - % *(r.v. 60-160)*	**189 [207-185]**	146 [104-199]	126 [103-156]
Fibrinogen – mg/dL *(r.v. 150-450)*	339 [294-468]	389 [309-544]	326 [280-421]
D-dimer – ng/mL *(r.v. <500)*	**1001 [395-2274]**	498 [349-1971]	763 [327-1215]
PAI-1 ag – ng/mL *(r.v. 1-10)*	14.2 [8.9-50]	12.8 [9.5-23.3]	13.2 [6.4-19.1]
Thrombophilia – n(%)	1 (4)	2 (6)	3 (8.5)
Factor V Leiden	1(4)	1 (3)	1 (2.8)
Prothrombin mutation	-	1 (3)	2 (5.7)

Data are expressed as median and range interquartile or frequency.

r.v.: reference values; PT: prothrombin time; aPTT: activated partial thromboplastin time; PAI-1: plasminogen activator inhibitor 1.

4.4 Baseline thromboelastometry

Table 17 shows the baseline main thromboelastometric parameters measured in the study population categorized by cancer type. All the ROTEM® parameters were within the normal reference values (Table 17). Patients with pancreatic cancer showed baseline values of MCF in FIBTEM at the upper limit of the standard (23 mm [19-30]) and slightly increased compared with the other cancer patients. They also showed a mild reduction of lysis (ML= 4% [3-8]) compared to both the colon and the gastric subgroups.

Table 17. Baseline thromboelastometric parameters in the study population.

	Pancreatic cancer	**Colon cancer**	**Gastric-esophageal cancer**
INTEM			
CT – sec *(r.v. 100-240)*	176 [170-184]	185 [171-200]	181 [162-200]
CFT – sec *(r.v. 30-110)*	60 [45-77]	58 [44-77]	68.5 [52-79]
MCF- mm *(r.v. 50-72)*	67 [70-72]	67 [73-64]	65 [62-70]
EXTEM			
CT – sec *(r.v. 38-79)*	66 [55-73]	67 [59-75]	65 [59.5-71]
CFT – sec	60 [48-71]	60 [44-77]	69.5 [53-83]

(r.v. 34-159)			
MCF- mm	69 [76-64]	69 [63-76]	66 [63-71]
(r.v. 50-72)			
ML - %	**4 [3-8]**	6 [4-8.75]	6 [3-8.5]
(r.v. 0-15)			
FIBTEM MCF – mm	**23 [19-30]**	20 [16-32]	18.5 [15-26.5]
(r.v. 9-25)			

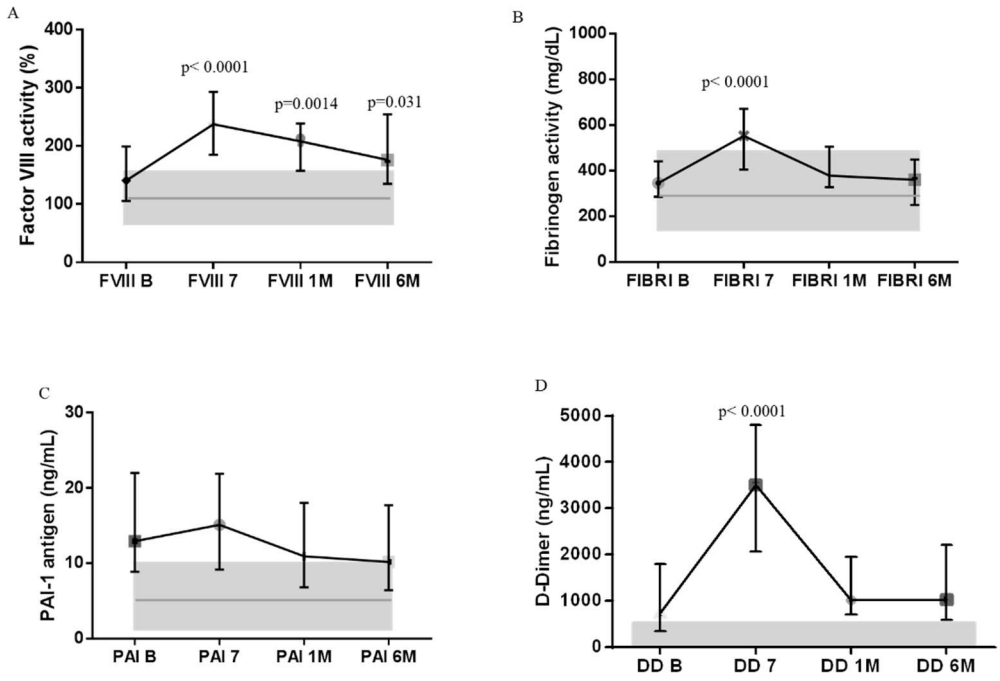
Data are expressed as median and range interquartile.

r.v.: reference values; CT: clotting time; CFT: clotting formation time; MCF: maximum clot firmness; ML: maximum lysis.

4.4.1 Trend of coagulative and thromboelastometry parameters during the follow-up

The analysis for parameters trend was calculated in the 79 patients who completed the sample longitudinal follow-up. Overall, cancer patients showed a significant longitudinal increase of factor VIII activity 7 days (median [IQR] 249 [1214-300] %), 1 month (208 [157-238] %) and 6 months (176 [135-254] %) after surgery, compared to the baseline (140 [105-199] %, $p<0.001$, 0.0014, 0.031, respectively) (Figure 11). They also showed significantly prompt increases of fibrinogen and D-Dimer levels 7 days after surgery with a decrease to pre-surgery levels 1 and 6 months later (Figure 11). Levels of PAI-1 antigen did not significantly change over the follow-up. Interestingly, factor VIII remained significantly higher than the baseline up to 6 months after surgery ($p=0.031$ compared to baseline).

Figure 11. Longitudinal trend of factor VIII activity (A), fibrinogen (B), PAI-1 (C) and D-Dimer (D) in the overall cancer population.

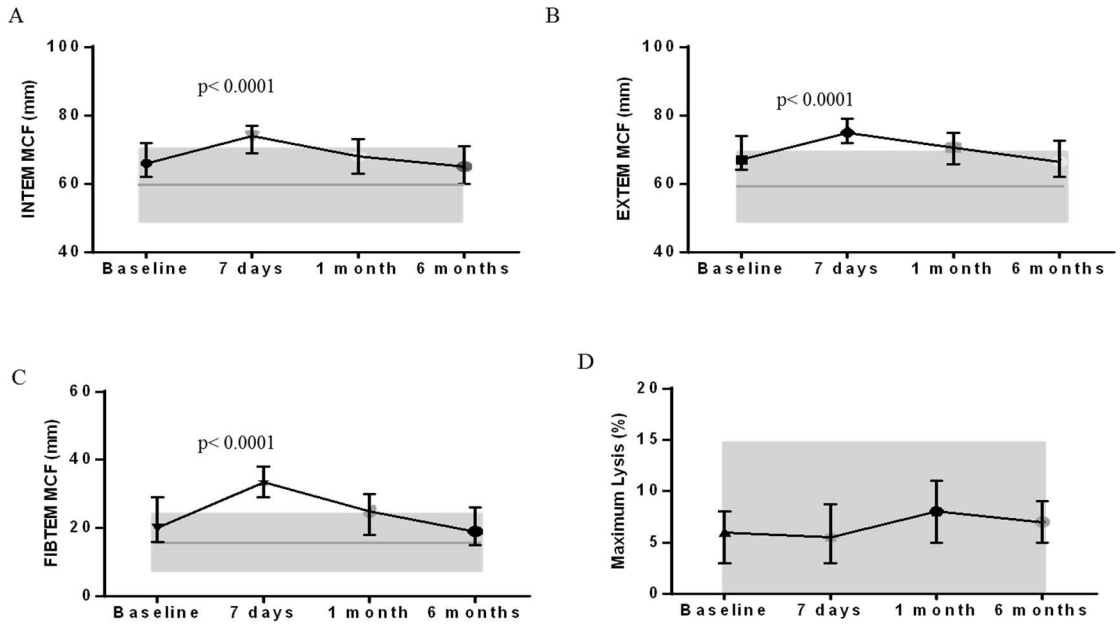


Data are expressed as median and interquartile range.
P values are calculated versus baseline levels. Grey bars indicate normal ranges.
Data are calculated in the 79 patients who completed the overall follow-up.

FVIII: factor VIII; FIBRI: Fibrinogen; PAI: Plasminogen activator inhibitor-1; DD: D-Dimer; B: Baseline; 7: 7 days; 1M: 1 month; 6M: 6 months.

Regarding ROTEM® parameters, there was a significant increase of MCF in INTEM, EXTEM and FIBTEM 7 days after surgery ($p< 0.001$) in all three tests, with a slight decrease towards pre-surgery levels 1 month and 6 months after surgery (Figure 12). The lysis parameter (ML) did not change significantly during the follow-up period (Figure 12).

Figure 12. Longitudinal trend of ROTEM parameters (MCF and ML) in the overall cancer population.



Data are expressed as median and interquartile range.
P values are calculated versus baseline levels. Grey bars indicate normal ranges.
Data are calculated in the 79 patients who completed the overall follow-up.

MCF: Maximum Clot Firmness.

4.5 Baseline contact activation system

As previously mentioned, contact system activation was measured through the presence of complexes made of each of the three contact system factors (FXIIa – FXIa –Kallikrein) and their main inhibitor (C1INH - C1 esterase inhibitor). ELISAs for enzyme-inhibitor complexes may serve as biomarkers for recent and/or ongoing contact system activation. We found increased levels of all three complexes in cancer patients compared to indicative reference values calculated in a group of 10 healthy subjects (half males and half females) (Table 18). Particularly, we detected the highest increase in contact system activation in pancreatic and gastroesophageal cancer compared with colon cancer.

Table 18. Baseline contact system parameters in the study population.

	Pancreatic cancer	**Colon cancer**	**Gastric-esophageal cancer**
FXIIa-C1INH – nM *(r.v. 0.176-0.32)*	2.51 [1.91-4.86]	2.21 [1.02-3.40]	2.55 [1.80-4.22]

FXIa-C1INH – nM *(r.v. 0.131-0.206)*	0.36 [0.14-0.67]	0.21 [0.083-0.56]	0.39 [0.13-0.66]
Kallikrein-C1INH – nM *(r.v. 0.133-0.314)*	1.81 [0.90-3.55]	1.70 [1.20-3.24]	2.31 [1.15-5.24]

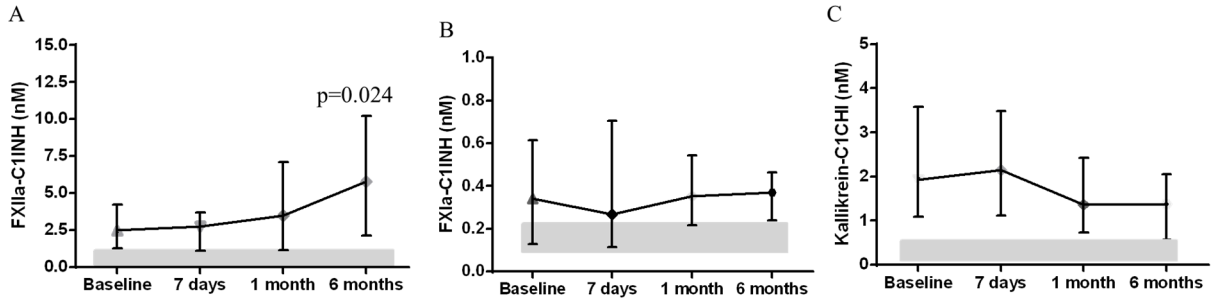
Data are expressed as median and range interquartile.

r.v.: reference values; FXIIa: activated Factor XII; FXIa: activated Factor XI; C1INH: C1 esterase inhibitor.
Indicative reference values were calculated in a group of 10 healthy volunteers (half male).

4.5.1 Trend of contact system parameters over the follow-up

The trend of contact system activation showed a slight increase over time of FXIIa-C1INH complex which became significantly higher than baseline 6 months after surgery (p=0.046). FXIa-C1INH slightly increased over time without reaching statistical significance compared to baseline. Finally, we observed a non-significant decrease of Kallikrein-C1INH complex during the follow-up period (Figure 13). One possible explanation for the increase of FXIIa-C1INH complex 6 months after surgery could be related to the chemotherapy. In fact, we compared levels of contact system complexes in patients undergoing chemotherapy versus patients who did not at the 6-month time-point. Interestingly, cancer patients in chemotherapy showed significantly higher levels of Kallikrein-C1INH (1.668 nM [0.69-4.072]) than patients without chemotherapy (1.248 nM [0.722-2.11], p=0.034) (Figure 14, panel C). They also showed a trend of higher levels of FXIIa-C1INH (7.363 nM [2.73-10.81]) and FXIa-C1INH (0.38 nM [0.22-0.50]) than patients without chemotherapy (5.7 nM [5.70-1.89] and 0.351 nM [0.237-0.51], respectively). However, the differences were not statistically significant (Figure 14).

Figure 13. Longitudinal trend of FXIIa-C1INH (A), FXIa-C1INH (B) and Kallikrein-C1INH (C) in the overall cancer population.

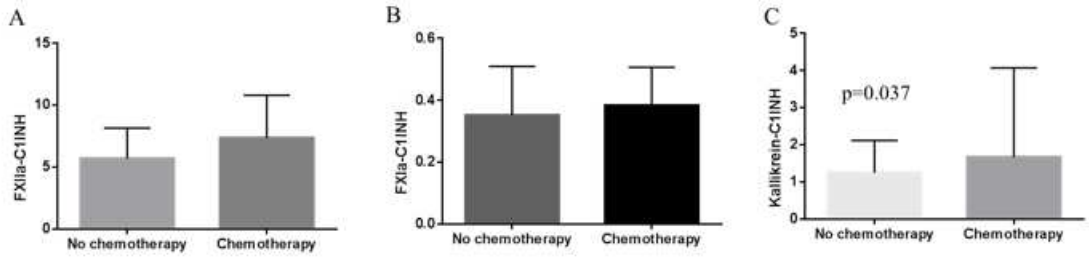


Data are expressed as median and interquartile range.
P values are calculated versus baseline levels. Grey bars indicate levels measured in a reference population.

Data are calculated in the 79 patients who completed the overall follow-up.

FXIIa: activated Factor XII; FXIa: activated Factor XI; C1INH: C1 esterase inhibitor.

Figure 14. Levels of FXIIa-C1INH (A), FXIa-C1INH (B) and Kallikrein-C1INH (C) in patients undergoing or not undergoing chemotherapy at 6-month time point.



4.6 MV-TF activity: baseline and trend

Table 19 shows median levels of MV-TF activity in the study population. Particularly, we once again detected the highest increase of MV-TF dependent procoagulant activity in pancreatic cancer compared to the other cancer subgroups.

Table 19. Baseline MV-TF activity in the study population.

	Pancreatic cancer	Colon cancer	Gastric-esophageal cancer
MV-TF activity – pg/mL *(r.v. 0.01-0.06)*	**0.1 [0.028-0.30]**	0.072 [0.027-0.18]	0.064 [0.027-0.17]

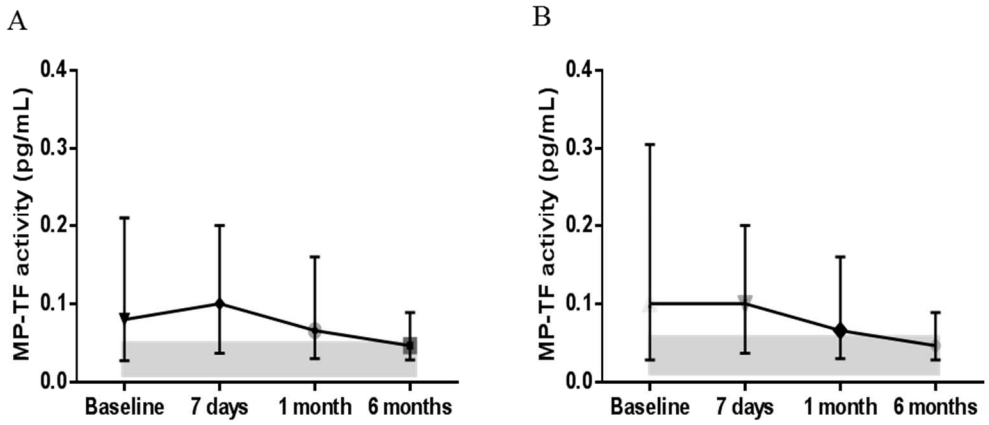
Data are expressed as median and range interquartile.

r.v.: reference values; TF: tissue factor.
Indicative reference values were calculated in a group of 10 healthy volunteers (half male).

The trend of MV-TF activity in the overall population showed a slight increase 7 days post-surgery and a progressive decrease during follow-up reaching 6-month time point levels slightly reduced compared to baseline. However, differences during the follow-up were not statistically significant (Figure 15, panel A). Considering solely the subgroup of pancreatic cancer, patients presented a progressive decrease of MV-TF activity over the course of the disease though we did not detect any statistically significant differences (Figure 15, panel B).

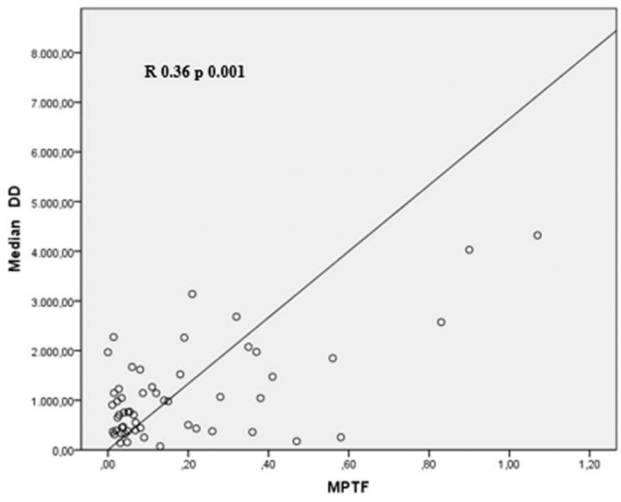
Interestingly enough, levels of MV-TF activity positively correlated with D-dimer (R 0.36 p 0.001) (Figure 16). On the contrary, MV-TF activity did not correlate with the contact activation pathway (FXIIa-C1INH, FXIa-C1INH and KAL-C1INH) neither in the overall population nor the pancreatic cancer subpopulation (data not shown).

Figure 15. Longitudinal trend of MV-TF activity in the overall population (A) and in pancreatic cancer (B).



Data are expressed as median and interquartile range. Grey bars indicate levels measured in a reference population.
P ANOVA for multiple comparisons: 0.04 (A) and 0.036 (B). p values calculated versus baseline levels were not statistically significant.
Data are calculated in the 79 patients who completed the overall follow-up.

Figure 16. Correlation between MV-TF activity and D-Dimer.



4.7 Baseline levels of microvesicles

4.7.1 Total MVs

Table 20 shows median levels of total MVs in the study population categorized by cancer type. Considering the overall phosphatidylserine-positive events (PS+), we once again showed that pancreatic cancer had the highest levels compared with colon and gastric cancers (ANOVA p <0.001) (see also Figure 17). Moreover, even considering the subgroups of PS+MVs according to their size (big, small and nano), pancreatic cancer showed the highest profile (Table 20 and Figure 17). Interestingly enough, the majority of MVs recorded were PS-. However, PS-MVs recorded did not differ according to neoplasm type either considering total PS-MVs or PS-MVs subtypes (Figure 17).

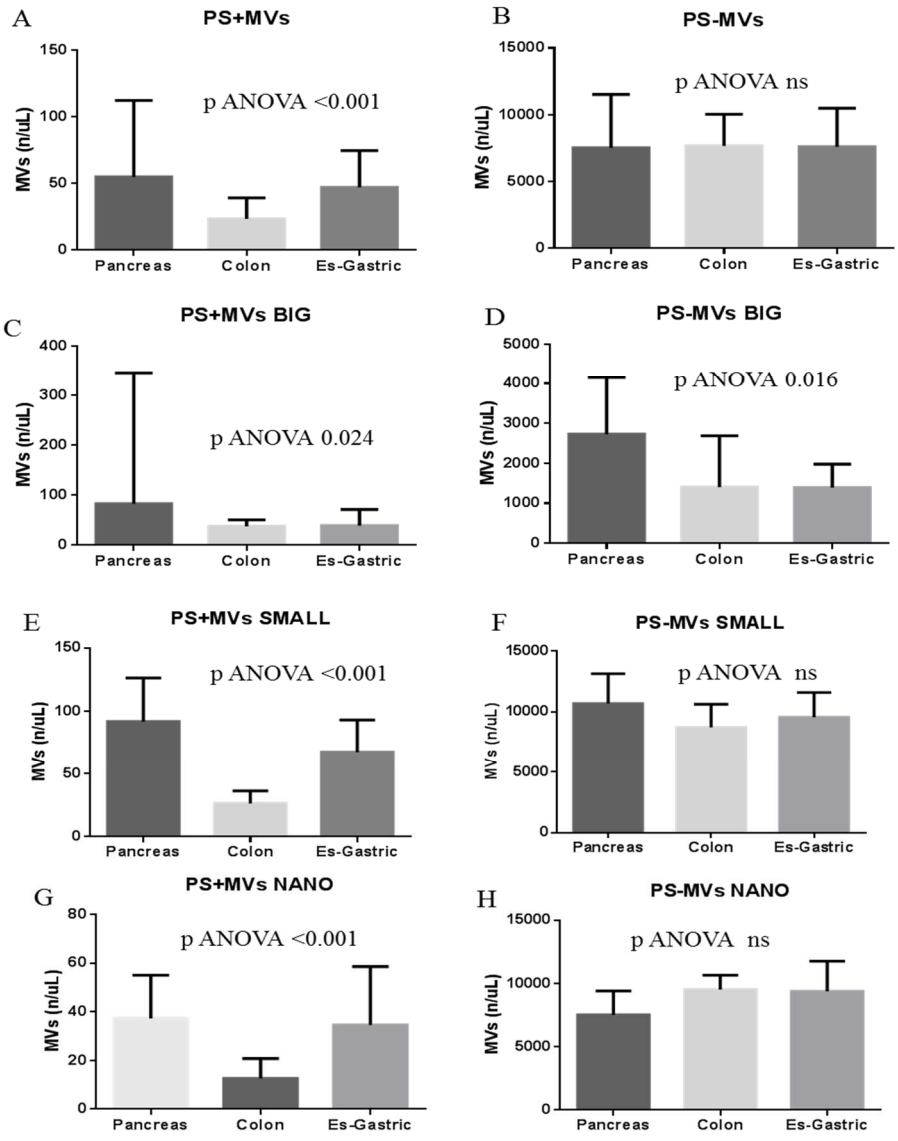
Table 20. Baseline levels of total microvesicles (MVs) in the study population.

	Pancreatic cancer	**Colon cancer**	**Gastric-esophageal cancer**
Total MVs – n/µL			
PS+	**55 [29-112]**	24 [13-39]	47 [23-75]
PS-	7522 [3196-11594]	7671 [2434-10047]	7607 [1960-10498]
Total BIG MVs – n/µL			
PS+	**83 [32-346]**	38 [20-50]	39 [21-71]
PS-	2733 [1734-4157]	1408 [1038-2690]	1395 [1395-871]
Total SMALL MVs - n/µL			
PS+	**92 [43-126]**	26 [18-36]	67 [45-93]
PS-	10702 [7897-13154]	8747 [7646-10642]	9564 [7893-11602]
Total NANO MVs - n/µL			
PS+	37 [16-55]	13 [6-21]	35 [18-59]
PS-	9520 [7671-10661]	7522 [5539-9405]	9376 [7151-11772]

Data are expressed as median and range interquartile.

MVs: microvesicles; PS: phosphatidylserine.

Figure 17. Levels of total (PS+ and PS-) microvesicles (MVs). (A and B) total PS+ and total PS- MVs; (C and D) Total BIG PS+ and total BIG PS- MVs; (E and F) total PS SMALL and total PS- SMALL MVs; (G and H) total PS+ NANO and total PS- NANO MVs.



Data are expressed as median and range interquartile. MVs: microvesicles; PS: phosphatidylserine; Es-Gastric: esophageal-gastric.

4.7.2 Endothelial MVs

Pancreatic and esophageal-gastric cancer patients also showed the highest number of endothelial-MVs (Table 21 and Figure 18). Interestingly, the majority of endothelial events were small.

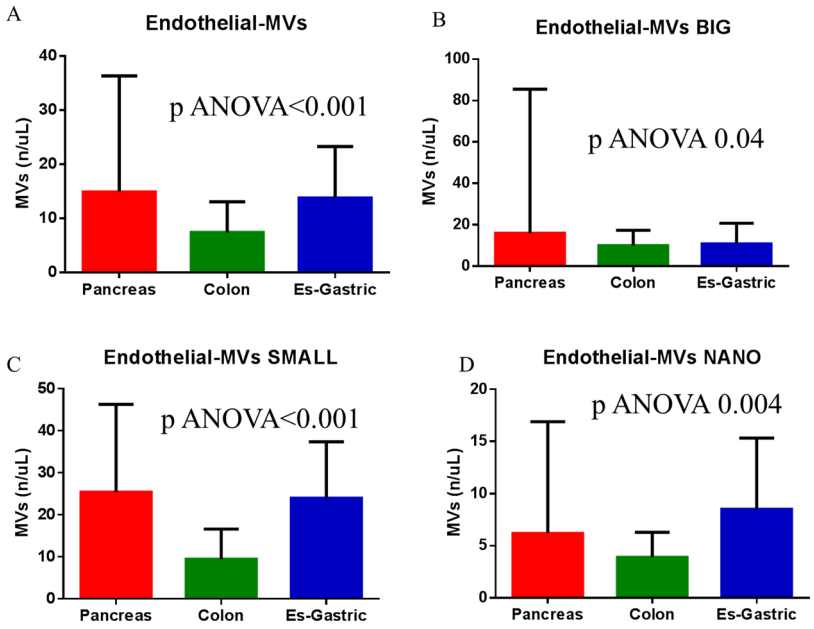
Table 21. Baseline levels of endothelial microvesicles (MVs) in the study population.

	Pancreatic cancer	Colon cancer	Gastric-esophageal cancer
Endothelial MVs – n/µL	**15 [6-36]**	7 [4-13]	**14 [6-23]**
Endothelial BIG – n/µL	16 [8-85]	10 [6-17]	11 [6-21]
Endothelial SMALL - n/µL	**25 [10-46]**	9 [6-17]	**24 [15-37]**
Endothelial NANO - n/µL	6 [2-17]	4 [2-6]	8 [5-15]

Data are expressed as median and range interquartile.

MVs: microvesicles; PS: phosphatidylserine.

Figure 18. Levels of total endothelial-MVs (panel A) and endothelial-MVs classified by size (panel B and C).



Data are expressed as median and interquartile range.

4.7.3 Tumour MVs

Table 22 and Figure 19 show levels of tumour MVs measured in the cancer population. Particularly, we detected tumour MUC-1+ MVs in pancreatic and gastric cancers and tumour EPCAM+MPVs in

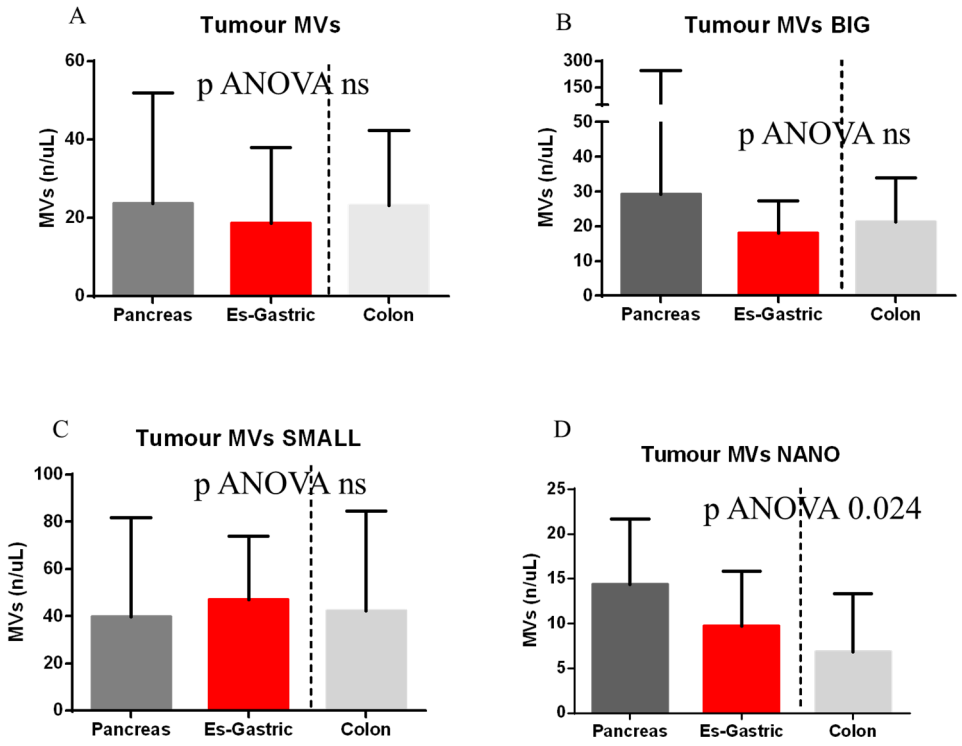
colon cancer. Levels of tumour MVs did not vary significantly with the cancer subpopulation. The majority of tumour MVs were SMALL.

Table 22. Baseline levels of tumour microvesicles (MVs) in the study population.

	Pancreatic cancer (MUC-1+)	Colon cancer (EpCAM+)	Gastric-esophageal cancer (MUC-1+)
Tumour MVs – n/µL	24 [9-52]	23 [13-42]	19 [9-38]
Tumour BIG – n/µL	29 [10-246]	21 [9-34]	18 [9-27]
Tumour SMALL - n/µL	**40 [28-82]**	**42 [34-85]**	**47 [34-74]**
Tumour NANO – n/µL	14 [6-22]	7 [2-13]	10 [5-16]

Data are expressed as median and range interquartile.

Figure 19. Levels of tumour MVs (panel A) and tumour MVs classified by size (panel B-D).



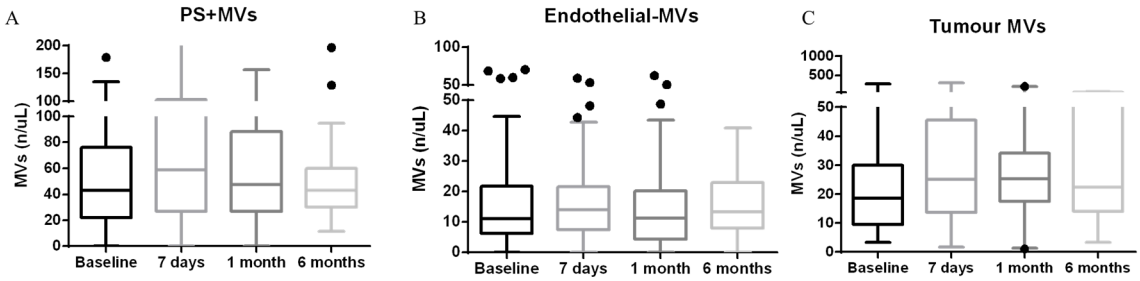
Data are expressed as median and interquartile range.

4.7.4 Trend of MVs during the follow-up

Figure 20 shows the trend of PS+MVs, endothelial and tumour MVs during the follow-up period in the overall cancer population. We observed a slight increase in PS+ and tumour MVs levels 7 days

and 1 month after surgery. However, the differences compared to baseline levels were not statistically significant. On the contrary, endothelial MVs did not change during the follow-up.

Figure 20. Trend of tumour MVs during the follow-up. A) PS+, B) endothelial MVs, C) tumour MVs.



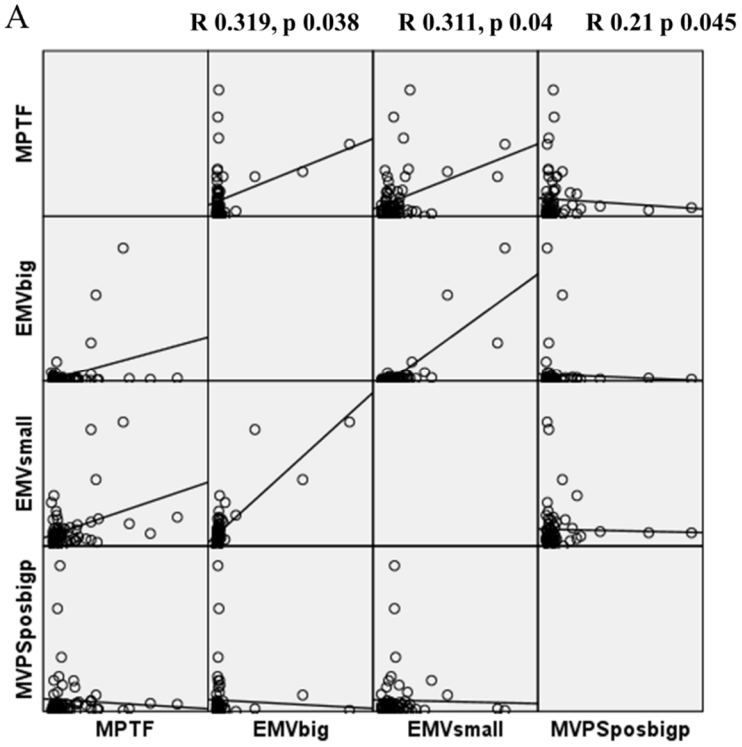
Data are expressed as median and interquartile range. MVs: microvesicles; PS: phosphatidylserine.

4.8 MVs correlations with MV-TF activity and coagulation parameters.

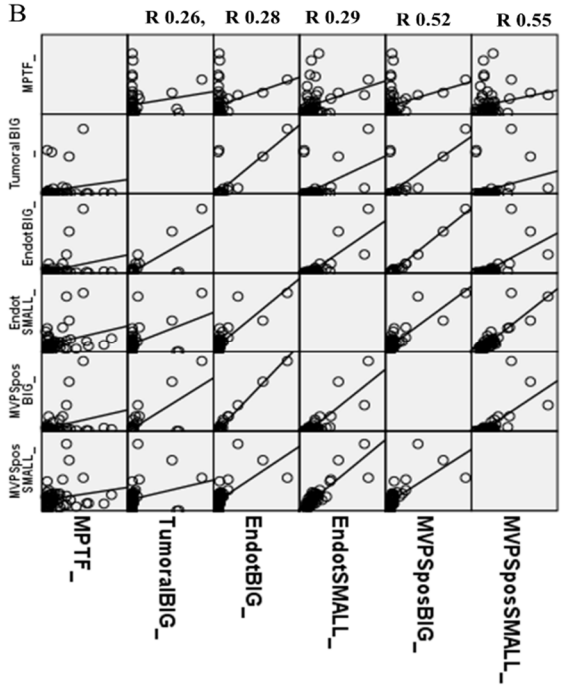
In order to evaluate whether circulating MVs carriers a TF-dependent coagulant activity, we performed a correlation test between each subtype of MVs considered and MV-TF activity detected. Considering the overall cancer population, we showed that MV-TF activity positively correlated with big and small endothelial MVs (R 0.319, p 0.038 and R 0.311, p 0.04, respectively), and weakly correlated with PS+MVs BIG (R 0.21 p 0.045) (Figure 21, A). When we focused the analysis solely on pancreatic cancer (which showed the highest values of MV-TF activity) we observed that (Figure 21, B):

- MV-TF activity positively correlated with PS+MVs (R 0.559 p 0.0001), both BIG (R 0.527 p 0.0001) and SMALL (R 0.556 p 0.007), but not with NANO;
- MV-TF activity positively correlated with endothelial MVs BIG (R 0.28 p 0.028) and SMALL (R 0.29 p 0.022);
- MV-TF activity positively correlated with BIG tumour MVs (R 0.26 p 0.03).
- MV-TF activity did not correlate neither with NANO MVs nor with PS-MVs.

Figure 21. Correlation between circulating MVs and MV-TF activity. A) between MV-TF activity and endothelial MV (big and small) and PS+MV BIG in the overall population. B) between MV-TF activity and tumour MV, endothelial MVs (big and small) and PS+MVs (big and small) in pancreatic cancer.



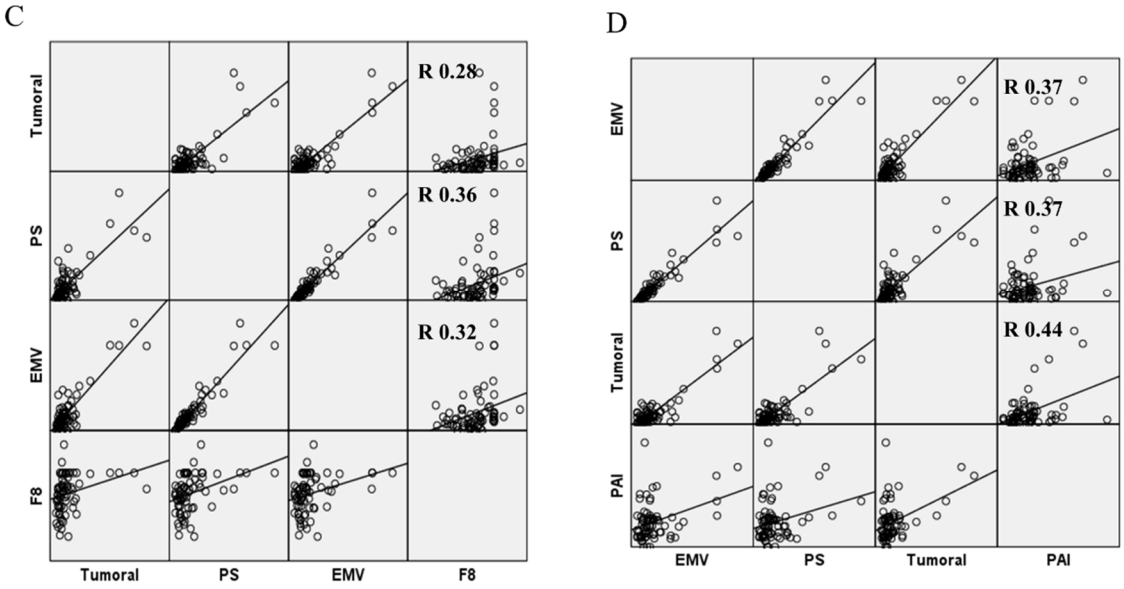
MPTF: MV-TF activity; EMV: endothelial MVs; MVPSpos: PS+MVs.



MPTF: MV-TF activity; Endot: endothelial MVs; MVPSpos: PS+MVs.

We also checked for possible correlations between coagulation parameters and MVs. The only positive correlation found was between MVs and FVIII levels both at baseline and 7 days post-surgery. In particular, PS+MV (R 0.36 p 0.002), endothelial MVs (R 0.32 p 0.006) and tumour MVs (R 0.28 p 0.015) correlated with FVIII levels (Figure 21, C). Also, MVs levels did not correlate with D-dimer and the contact system pathway neither at baseline nor 7 days post-surgery. Finally, we observed a positive correlation between all subtypes of MVs and PAI-1 antigen 7 days post-surgery (see Figure 21, panel D). Interestingly, the correlation between tumour MVs and PAI-1 antigen was highly significant (R 0.44 p <0.0001).

Figure 21. Correlation between circulating MVs with factor VIII and PAI-1 antigen. C) between all MVs subtypes and factor VIII levels at baseline; D) between all MVs subtypes and PAI-1 antigen 7 days after surgery.



Tumour: tumour MVs, PS: PS+MV; EMV: endothelial MVs; F8: factor VIII levels; PAI: PAI-1 antigen.

4.9 Circulating MVs as biomarkers for surgical radicality

The second aim of our study was the possible use of MVs as early biomarkers for cancer clinical outcomes. Firstly, we evaluated their possible association with surgical radicality. We analysed the trend of MV-TF activity and circulating MVs according to surgical radicality and we observed that:

- MV-TF activity was significantly higher in patients without surgical radicality at baseline (p 0.005), 7 months after surgery (0.012) and 1 month after surgery (p 0.029) (Figure 22 panel A-D).

- circulating MVs (endothelial MVs, PS+MVs and tumour MVs) were significantly higher in patients without surgical radicality at baseline, 7 days and 1 month after surgery (Figure 23 panel A-D).

Figure 22. Levels of MV-TF activity in patients with and without surgical radicality.

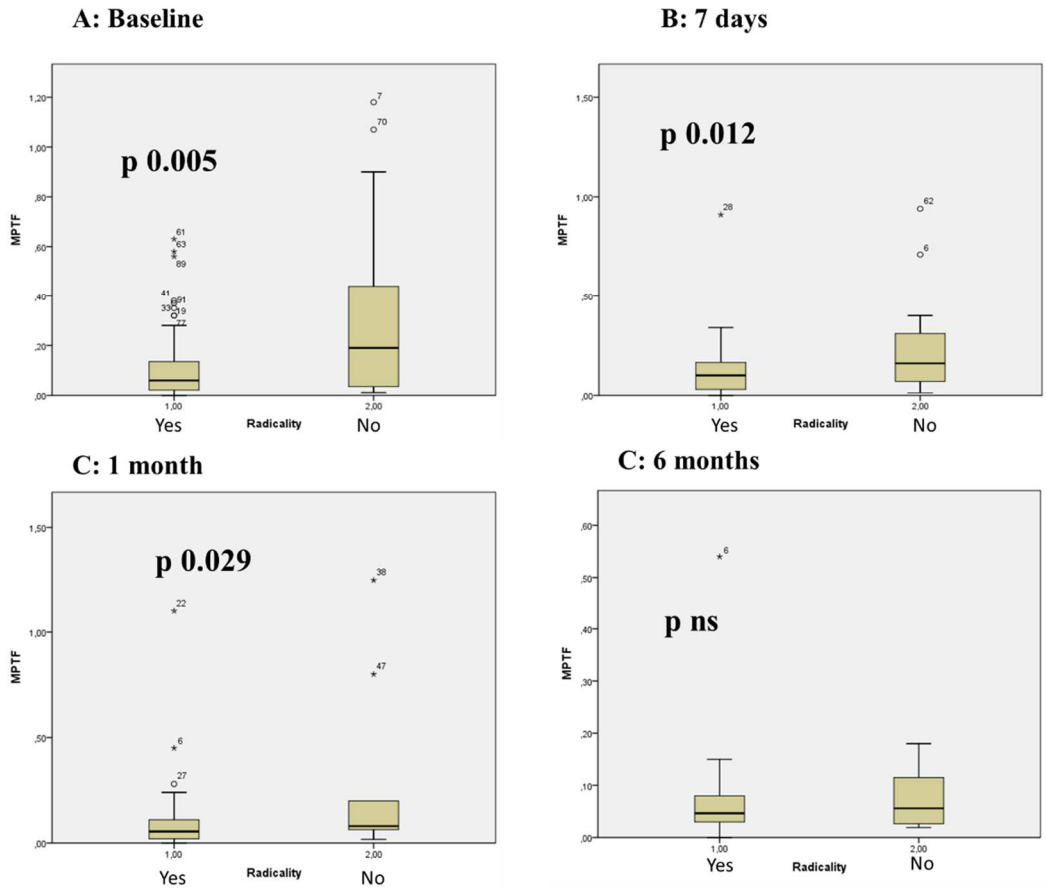
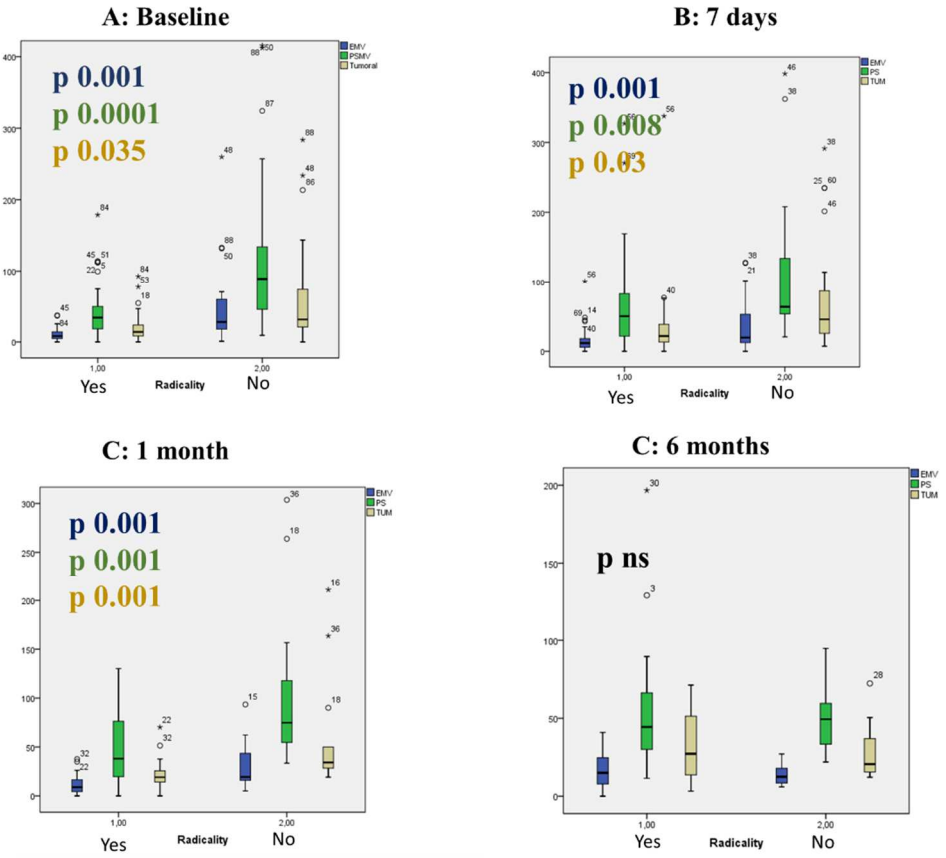


Figure 23. Circulating MVs in patients with and without surgical radicality.



Blue indicates endothelial MVs (EMV), green PS+MVs, and yellow tumour MVs (TUM). P value are calculated between patients with and without radicality at the different time points.

Using a Cox proportional Hazard model, we showed that high levels of MV-TF activity, endothelial MVs and tumour MVs were associated with a significant risk of incomplete surgical eradication. Particularly, MV-TF activity showed a strong association with incomplete surgical radicality (OR 2.25 [95%CI 1.25-7.0]. The OR was significant even after adjustment for age, Karnofsky Performance Status and type of cancer with the presence of esophageal cancer emerging as highly predictive of surgical radicality (see Table 23).

Table 23. Risk factors for incomplete surgical radicality.

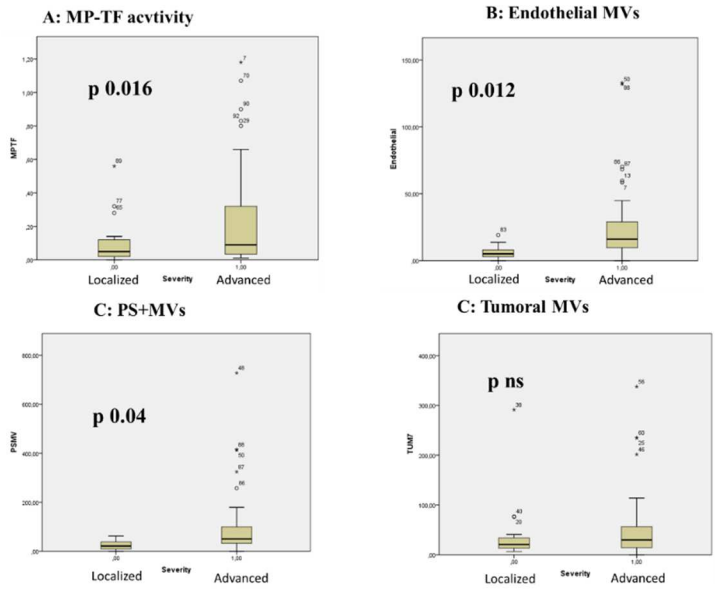
	β coefficient	OR [95%CI]	p
MV TF-activity	0.81	**2.25 [1.25-7.0]**	0.006
Endothelial MVs	0.176	**1.19 [1.04-1.36]**	0.011
Tumour MVs	0.043	1.04 [1.02-1.08]	0.38
Es-Gastric cancer	- 3.1	0.45 [0.30-0.72]	0.28

The model was adjusted for age, Karnofsky Performance Status and cancer type (pancreatic, colon and esophageal-gastric).

4.10 Circulating MVs as biomarkers for cancer severity

When cancer patients were considered according to disease severity (localized: stage I-II or advanced: stage III-IV), we observed that patients with advanced neoplasm had significantly higher levels of MV-TF activity, endothelial MVs and PS+MVs (Figure 24, panel A-C). On the contrary, tumour MVs showed a trend towards higher levels in advanced cancer, but the difference was not statistically significant. This figure was confirmed also at the 7-day time point when advanced cancer had significantly higher levels of MV-TF activity, endothelial MVs and PS+MVs, but not tumour MVs.

Figure 24. Levels of MV-TF activity and MVs in cancer patients with localized or advanced disease.



Using a Cox proportional Hazard model, we showed that high levels of MV-TF activity and endothelial MVs at baseline were associated with a significant risk of advanced neoplasm (see Table 24).

Table 24. Factors associated with advanced cancer.

	β coefficient	**OR [95% CI]**	**P**
MV-TF activity	0.655	**1.87 [1.20-3.8]**	0.008
Endothelial MVs	0.265	**1.30 [1.05-1.6]**	0.013

The model was adjusted for age, Karnofsky Performance Status and cancer type (pancreatic, colon and esophageal-gastric).

4.11 Predictors of venous thromboembolism

Overall and as mentioned previously in paragraph 4.2, 15 (16.1%) patients developed VTE over follow-up. The median time of the event was 2 months for pancreatic, 3.2 months for colon and 0.5 month for gastric cancer after the enrollment. A univariate Cox regression analysis was performed in order to evaluate coagulation biomarkers potentially predictive of VTE. According to this univariate model, variables significantly associated with VTE development were: baseline MV-TF activity, baseline FXIa, 7-day endothelial MVs and PS+MVs, baseline fibrinogen and D-dimer. All remaining parameters did not show any association with VTE development. After adjustment for possible confounders (age, Karnofsky Performance Status, BMI, cancer subtype and severity of disease), the variables that still remained significantly associated with VTE occurrence were baseline MV-TF activity (HR 2.38 [1.81-4.11]) and baseline FXIa (HR 1.66 [1.02-2.9]) (Table 25 and Figure 26). Levels of circulating MVs did not show a significant association with VTE after adjustment for confounders.

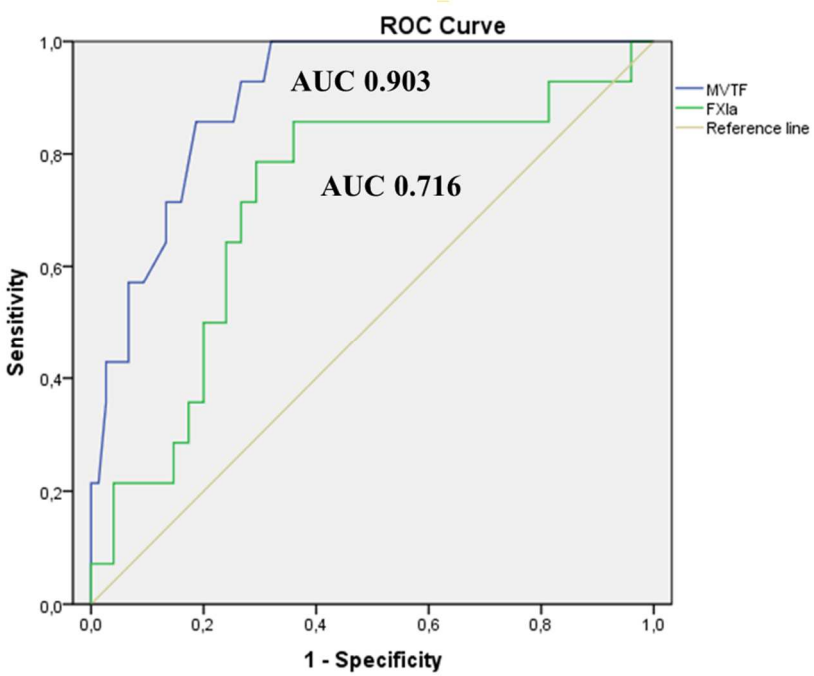
Table 25. Coagulation factors associated with VTE occurrence.

	Univariate		Multivariate	
	β coefficient	**HR [95%CI]**	**β coefficient**	**HR [95%CI]**
Baseline MV-TF	3.04	20 [5.8-74]	0.867	**2.38 [1.81-4.11]**
Baseline FXIa	0.359	1.43 [1.05-2.03]	0.508	**1.66 [1.02-2.9]**
7-day endothelial MVs	0.231	1.22 [1.04-2.11]	0.01	1.01 [1.01-10.9]
7-day PS+MVs	0.183	1.19 [1.06-2.09]	0.075	ns
Baseline fibrinogen	0.09	1.1 [1.03-1.8]	0.088	ns
Baseline D-dimer	0.09	1.09 [1.02-1.9]	0.1	ns

Data were adjusted for age, sex, BMI, Karnofsky Performance Scale, cancer type, surgical radicality and severity of disease.

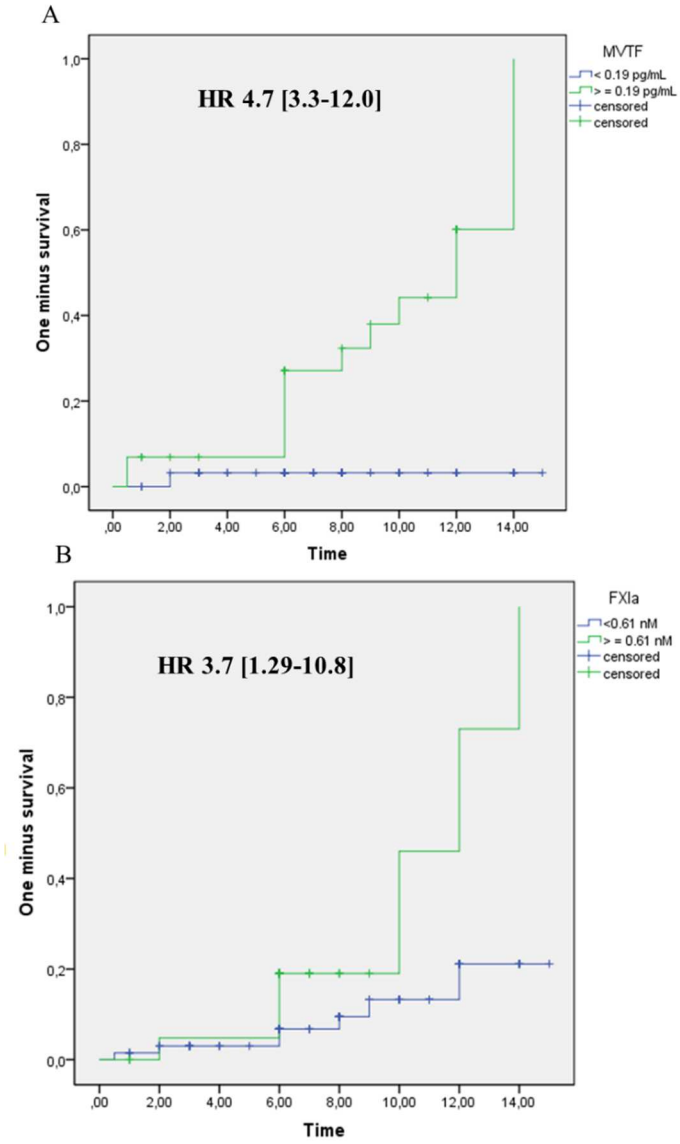
After performing a ROC analysis with MV-TF activity and FXIa levels, we observed that the AUC for discriminating between cases (VTE) and non-cases (non-VTE) was highly discriminant for the former (AUC 0.9) and a little less discriminant for the latter (AUC 0.71) (figure 25).

Figure 25. ROC curve evaluating VTE diagnostic ability of MV-TF activity and FXIa.



The ROC analysis showed that the levels with the highest sensitivity and specificity for VTE were 0.19 pg/mL (sensitivity 85%, specificity 82%), whereas for FXIa levels of 0.61 nM were associated with a sensitivity of 85%, but with lower specificity (70%). A Kaplan-Meier analysis evaluating VTE occurrence dividing the cancer population according to MV-TF activity (≥ 0.19 pg/mL) and FXIa (≥ 0.61 nM) showed a significant risk to develop VTE in patients with levels higher than these calculated cut-off (HR 4.7 [95%CI 3.3-12.0]) for MV-TF activity and 3.7 [95%CI 1.29-10.8] for FXIa) (Figure 26 A and B). HR reported were adjusted for age, sex, BMI, cancer type, severity, and surgical radicality.

Figure 26. Kaplan-Meier analysis showing the high risk to develop VTE for patients with high levels of MV-TF activity (A) and FXIa (B).



5. Discussion

Rationale for the study

It is fully recognized that cancer patients are at significant risk of developing thrombotic events, spanning from asymptomatic deep vein thrombosis and catheter-related thrombosis to recurrent deep vein thrombosis and massive pulmonary embolism, as well as thrombosis in unusual sites (3, 6). The prevention of such complications is of the utmost importance from a clinical standpoint, seeing as they play a considerable part in the morbidity and mortality of these patients (79). The main issue is that the pathogenesis of the cancer-associated coagulopathy is complex and multifactorial. Even without thrombosis, cancer induces different degrees of coagulation activation that can lead to a systemic hypercoagulable state owing to a complex interplay among cancer cells, host cells and the coagulation system. This interplay involves not only direct activation of coagulation – and ultimately thrombin generation – but also the fibrinolytic system, platelets, as well as inflammatory cells and their mediators (32, 79, 90). It is also important to remind that several compounding risk factors for VTE usually coexist in cancer patients, thus raising the baseline prothrombotic state up to the threshold for clinically overt thrombosis. A deep understanding of the mechanisms involved in the pathophysiology of hypercoagulability and cancer-associated thrombosis is essential to control the "prothrombotic-silent-phase" and properly prevent the event occurrence. Additionally, given the multitude of pathways and mediators involved in these pathomechanisms, it is entirely possible that each cancer type may be characterized by its own prothrombotic profile depending on site, differentiation grade, aggressiveness and host immunological response. The individual characteristics of each patient as well as the treatments received over the course of the disease may influence their prothrombotic profile. A lot of work has been done so far in an effort to get a better understanding of the cancer-induced mechanisms of clotting activation or in the interplay between coagulation and inflammation, as well as in the essential role of platelets as cancer mediators in different neoplasms (114, 115, 134, 191). Clinically speaking, however, the only current laboratory biomarkers that have been shown to have some role in predicting the thrombotic risk of patients are white blood cell and platelet counts (33). Several studies have reported that leucocytosis is associated with a greater risk of

VTE in cancer patients and leukocytes have also been shown to contribute to VTE in mouse models (134). However, leucocytosis was most frequently observed in patients with lung or colorectal tumours, while neutrophilia and monocytosis in soft tissue sarcomas (134). Therefore, the clinical significance and use of this marker need further clarification. As for platelet count, thrombocytosis has often been observed in patients with cancer, especially gastrointestinal, lung, breast, and ovarian malignancy. The role of platelets in thrombosis has also been studied in mouse cancer models. Taken together, these clinical and basic studies confirm a role for platelets in the pathogenesis of VTE and suggest that anti-platelet drugs may be useful in preventing VTE in some cancer patients (68, 70, 134, 192, 193). Following these observations, the currently available scoring systems use site of cancer, leukocyte and platelet counts to identify patients with all types of cancer who are at high risk of VTE during chemotherapy (66). However, current scores are heavily weighted on type of cancer, and they have been shown to perform poorly in predicting venous thromboembolism in cancer patients (194, 195). Moreover, leucocytosis and thrombocytosis do not feature in every cancer and sometimes may not correlate with leukocytes or platelets activation. Thus, the identification of novel biomarkers associated with the grade of hypercoagulability in individual malignancies is required to drive the development of cancer-type specific scoring systems with improved predictive value.

The aim of our study was to assess the trend of several coagulation parameters focusing on patients with gastro-intestinal cancer and to determine which parameter may have a role in predicting VTE in this subset of malignancy. Particularly, we focused on gastro-intestinal cancer firstly because pancreatic and gastric cancers have been shown to be highly associated with VTE complications in adult population (incidence rate 14.6 [12.9–16.5] per 100 person-years and 10.8 [9.5–12.3] per 100 person-years, respectively) (12). As for colon cancer, though associated with an intermediate risk of VTE (6.7 [6.3–7.2] x 100 person-years (12)), it is the third most common cancer among men and the second among women; the fourth leading cause of cancer-related mortality worldwide; and an estimated 5-6% of the Western population will suffer from colon cancer during their lifetime (196). Secondly, we decided to homogeneously include only the most common histologic types, such as adenocarcinoma and epithelial cancer, excluding histologic types (such as sarcomas, neuroendocrine tumours, melanomas, etc) that are less prevalent and can have peculiar clinical behaviours. Regarding

the choice of coagulation markers, we focused on coagulation factors associated with both clotting activation and acute-phase inflammation, such as factor VIII and fibrinogen. As cancer-induced hypercoagulability depends demonstrably on fibrinolytic inhibition, we measured PAI-1 antigen levels and D-dimer. Considering the conflicting results yielded by previous studies evaluating the impact of factor V Leiden and prothrombin mutation on the risk of thrombosis in cancer, we opted to perform the genetic research of the most prevalent hereditary thrombophilia in the present study. Additionally, we focused on thromboelastrometric parameters: Rotation ThromboElastoMetry analyser (ROTEM®, Tem International, Munich, Germany) is a point-of-care coagulation monitoring device which assesses the viscoelastic properties of whole blood in order to study the simultaneous and integrated effects of different components (i.e. plasmatic factors, platelets, leukocytes, and red blood cells) involved in the dynamic process of clot formation and lysis (184). Thromboelastometry has shown tremendous potential in detecting a variety of coagulopathies, including states of hypo- as well as hypercoagulability (184). Recent evidence showed that thromboelastometry might be a useful tool in the prediction of cancer-associated thrombosis in cholangiocarcinoma (197). In particular, inasmuch as the point-of care-nature of the test, it may be a very useful tool in the monitoring of post-operative cancer-associated hypercoagulability, namely the risk of post-surgical VTE. We also focused on the contact activation pathway. Increasing interest in the contact pathway of coagulation in the past decade has focused on a possible role in the pathogenesis of thrombosis (198). Moreover, preliminary observations have noted the presence of activation of the contact system in gastrointestinal, lung, breast and prostate cancers (109, 199). The activation of the contact system in cancer might be further enhanced by indwelling ‘foreign’ surfaces (intravenous catheter, port-a-cath), and perhaps chemotherapy, concurrent infection and packed red blood cell transfusions as well. Finally, we addressed our interest in circulating MVs by measuring either their number in plasma or by assessing their antigenic property via flow-cytometry. Given the central role of the TF in the cancer-associated hypercoagulability, we further evaluated MV-TF procoagulant activity (96).

Primary outcome – fluctuations of coagulation parameters: factors, fibrinolysis and thromboelastometry

Our findings showed increased levels of factor VIII at baseline (189% [207-185]) within the subgroup of pancreatic cancer, higher than the reference range and compared to other cancer subtypes. On the contrary, fibrinogen levels were within the normal range in all cancers considered. As for coagulation factors trend, we detected a significant increase both in factor VIII and in fibrinogen 7 days after surgery with a slight decrease 1 and 6 months after surgery. Interestingly, levels of factor VIII were still higher than baseline 6 months after surgery in the overall cancer population. This result is confirmed by evidence in Literature that shows that levels of factor VIII are significantly higher among cancer patients. Moreover, in prospective trials, a high factor VIII level is predictive of cancer-associated thrombosis (200). Factor VIII is an acute-phase protein and it is involved in the interplay between coagulation and inflammation. It is important to point out that, besides this increase observed in cancer which leads to a chronic persistent low-grade inflammation, interventions like surgery can also cause a surge in factor VIII levels that can persist for up to 6 months after the intervention. Therefore, strategies to prevent cancer-associated thrombosis may be improved by also considering anti-inflammatory agents in high-risk situations; for example, aspirin use was associated with a borderline reduction in VTE in patients with ovarian cancer (193). Aspirin may control both inflammatory and platelets-mediated hypercoagulability.

Our findings also showed that pancreatic cancer is associated with higher levels of PAI-1 antigen and D-dimer compared to the other subtypes. D-dimer markedly increased soon after surgery, but then rapidly decreased 1 month after. PAI-1 levels did not change much after surgery and during the follow-up. There are few studies evaluating the fibrinolytic system in cancer associated-thrombosis. A study conducted on patients with pancreatic cancer suggested that elevated levels of PAI-1 antigen and activity may predispose patients to VTE (201). Another study found higher levels of PAI-1 in glioma patients compared with healthy controls (202). Oncogene activation, and perhaps hypoxia, have been shown to inhibit fibrinolysis by up-regulating PAI-1 in cancer (203). Moreover, as previously mentioned, PAI-1 mRNA was detected in endothelial cells originating from the tumours in patients with colorectal cancer, thus directly bestowing cancer cells with the capability to

inhibit fibrinolysis (113). According to our results, surgery did not appear to worsen this hypofibrinolytic tendency of cancer, being the traumatic activation of coagulation and fibrinolysis the most important pathway involved (as evidenced by the marked increase of D-dimer soon after surgery). Interestingly, administration of the anti-VEGF drug bevacizumab increased thrombosis in two mouse models. Bevacizumab increased PAI-1 expression in tumours and in plasma leading to enhanced thrombosis that was reduced by a PAI-1 inhibitor (134).

A few words regarding our findings on thromboelastometry in cancer. Baseline ROTEM® parameters were within the normal range. Baseline FIBTEM MCF (the strength of the coagulum – a functional test for fibrinogen) was higher in pancreatic cancer compared to the other subtypes. Moreover, after surgery there was a prompt increase in the MCF in the three tests considered (INTEM-EXTEM and FIBTEM). These findings mirror the post-surgical phase of coagulation activation. In a recent study involving 27 patients with cholangiocarcinoma, MCF showed a non-significant trend of increased levels in the 6 patients who developed post-operative VTE (197). We can speculate that the ROTEM® parameter MCF may be a useful tool to monitor global coagulation changes after surgery, in order to potentially early detect those cases with particularly high post-surgical acute hypercoagulability that may benefit from "potentiated" thromboprophylaxis or thromboprophylaxis combined with compounds targeting different pathways.

Primary outcome – fluctuations of coagulation parameters: contact system activation

Interestingly enough, by measuring plasma complexes between contact system factors and their main inhibitor, we uncovered increased baseline levels compared to levels measured in a reference healthy population. Pancreatic and gastric cancers showed the highest activation. This means that the intrinsic pathway is also part of the coagulation activation in cancer. More interestingly, surgery did not seem to affect contact system, in fact complexes levels did not change 7 days after surgery. On the contrary, FXIIa-C1INH and FXIa-C1INH complexes slightly increased 1 month and 6 months after surgery. In particular, patients receiving chemotherapy at the 6-month time point showed significantly higher levels of FXIIa-C1INH and kallikrein-C1INH complexes compared to patients without chemotherapy.

This result is of great interest, because it highlights the possible role of chemotherapy in worsening cancer prothrombotic profile by enhancing the intrinsic pathway. Given also that most patients undergoing chemotherapy have external devices, like central vein catheters or port-a-cath, the presence of these surfaces may also potentiate this contact system activation ultimately leading to the thrombotic event. Thrombotic events in cases like this may stem from increased thrombin-generation driven by the intrinsic pathway rather than by the TF pathway. Our findings confirm the results of a past study presented in 1990 (199). Contact system activation was evaluated in 69 patients with gastrointestinal cancer (12 with gastric, 15 with pancreatic and 42 with colon cancer), and in 118 healthy controls. The authors found reduced levels of factor XII, pre-kallikrein and high molecular weight kininogen in cancer compared with controls, whereas C1INH antigen and activity were significantly increased in cancer compared with controls. The reduced levels of non-activated factors and the increased levels of the inhibitor were interpreted as an indication of recent activation of the contact system. Furthermore, patients with metastatic cancer showed a contact system "more activated" than non-metastatic cancer patients. It is important to note that although several observations have documented the activation of the contact system in different cancers, the underlying mechanism(s) remain(s) elusive. Recently, Nickel et al. (109) demonstrated that prostate cancer cells secrete exosomes (prostasomes) that induce lethal pulmonary embolism in mice and can trigger thrombin generation in vitro in a dose-dependent manner. The addition of a recombinant FXIIa inhibitor significantly reduced peak and total thrombin generated by prostasomes; the combined application of FXIIa and TF inhibitors completely stunted thrombin generation. Thus, both the intrinsic and extrinsic pathways seemed to contribute to thrombosis in this model. Moreover, polyphosphate P (PolyP) was found on the surface of prostasomes. Treatment of prostasomes with specific inhibitors of PolyP or with PolyP degrading enzymes abrogated prostasome-induced FXIIa generation in vitro and protected mice from prostasome-induced lethal pulmonary embolism. In aggregate, these findings showed a role for the prostasome – FXII axis mediated by PolyP in the increased procoagulant activity observed in prostate cancer. The confirmation of these findings may open up a new clinical scenario where compounds targeting contact system activation may be used to prevent thrombosis when the intrinsic pathway is more involved, for example - according to our

results - during chemotherapy or catheter-related thrombosis. It is a well-established fact that genetic or pharmacologic inhibition of FXIIa is protective against thrombosis in mouse models of arterial and venous thrombosis without any associated haemorrhagic diathesis (204). Several classes of contact system inhibitors are under development as thrombo-protective and/or anti-inflammatory agents (205).

Primary outcome – fluctuations of coagulation parameters: MV-TF activity

As far as MV-TF activity is concerned, the highest levels were detected in pancreatic cancer. Globally, we saw a slight increase 7 days after surgery and a progressive decrease over follow-up with the 6-month time point levels slightly reduced compared to baseline. Moreover, MV-TF activity significantly correlated with D-Dimer. Several studies have examined the relationship between TF-MVs and VTE in patients with pancreatic, brain, colorectal, or lung cancer. TF-MVs were detected in all these neoplasms but patients with pancreatic cancer showed the highest levels of MV-TF activity (170). One possible explanation for these findings is that the endocrine function of the pancreas provides an easy route for transporting TF-MVs from the tumour into the bloodstream (134). We confirmed the presence of MV-TF activity in pancreatic cancer and observed that while levels of MP-TF activity did not appear to be affected by surgery or chemotherapy, it was a marker related of the "tumour activity" itself. Indeed, it slowly decreased during the follow-up period with some patients going into remission, but most importantly, it was significantly associated with surgical radicality and tumour severity. We found that MV-TF activity was already significantly higher at baseline in patients without surgical radicality, 7 months after surgery up to 1 month after surgery. Moreover, at baseline patients with advanced neoplasm had significantly higher levels of MV-TF activity. One important observation is that, in a multivariate model, baseline levels of MV-TF activity were independent predictors of incomplete surgical removal (with a risk of 2.25 [1.25-7.0]), as they were also significantly associated with cancer severity (baseline high MV-TF activity was associated with 1.87 risk to have advanced cancer). The results were confirmed after adjustment for cancer type.

Primary outcome – fluctuations of coagulation parameters: circulating MVs

When focusing on circulating MVs, we observed that pancreatic cancer had higher levels of total MVs (i.e. PS+MVs), higher endothelial MVs and tumour MVs (detected by MUC-1 antigen). Interestingly, with the new-generation flow-cytometer, we are now able to differentiate MVs according to their size. Old flow-cytometric assays were not sensitive enough to detect all sizes of MVs, given that many of these fall below the detection threshold. In particular, small and nano-MVs were missed. We observed that the majority of detected MVs were small (diameter 0.2-0.4 μm). Moreover, we confirmed that PS-MVs are the majority and thus PS is not the perfect marker to detect total number of MVs. However, PS-MVs did not show a specific pattern according to the cancer population and did not correlate with MV-TF activity. It is widely accepted that MVs expose phosphatidylserine which in turn binds annexin V. A number of recent reports have questioned the use of annexin V for MV detection, stating that a subpopulation of MVs do not demonstrate detectable levels of annexin V binding (206). There may be a number of explanations for failure to detect MVs annexin V binding, including inadequate experimental conditions to allow maximal annexin V binding (calcium concentration) or the presence of inhibitors to annexin V binding. Alternatively, decreased or absent annexin V binding may simply result from insufficient PS exposure on the membrane surface to allow detectable annexin V, thereby representing true annexin V negative MVs. Finally, it is possible that these events may not represent MVs at all, but instead platelet-derived exosomes (resulting from platelet activation), small platelets or the remnants of activated platelets, possessing scattering properties of similar magnitude to MVs. It is worth mentioning that Connor et al. (206) showed that in unstimulated platelet-poor plasma, 80% of platelet-derived MVs failed to bind annexin V and the variation of the assay constituents (buffer, calcium and annexin V concentration) did not increase annexin V binding. However, after in vitro platelets stimulation, the percentage of total events that bound annexin V was dependent upon the type of agonist used. Moreover, the expression of platelet activation markers CD62P and CD63 was significantly decreased in annexin V-MVs, compared to annexin V+MVs, suggesting that the latter are associated with a greater degree of platelet activation. Therefore, Annexin+ events seem to reflect the subgroup of MVs

released after cell activation, the most useful from a clinical point of view. Indeed, we showed that only PS+MVs positively correlated with MV-TF activity, confirming that PS+MVs, and not PS-negative, confer the procoagulant activity. Connor et al. (206) also found that the majority of procoagulant activity was associated with the annexin V binding subpopulation using a phospholipid-dependent clotting time. We added the fact that our functional test measured the procoagulant activity due to TF, and we were able to confirm that PS+MVs had procoagulant potential also by expressing TF. Interestingly, MV-TF activity correlated with big and small MVs, and not with the NANO ones. Finally, the procoagulant activity also correlated with endothelial MVs and BIG tumour MVs, confirming the expression of TF in these subgroups of MVs. A number of investigators have since considered alternatives to annexin V as a marker of PS exposure (lactadherin, diannexin). Nonetheless, annexin V binding remains the most frequently-used marker for MV detection at the moment.

As for the trend of MVs, PS+MVs and tumour MVs showed an increased trend after surgery up to 6 months of follow-up, but we did not detect any significant differences compared to baseline levels. Similarly to MVs-TF activity, it seems that the presence of MVs in plasma is related to the presence of tumour itself, rather than to other events (e.g. chemotherapy, surgery). Endothelial MVs, as well as MV-TF activity, showed a positive association with surgical radicality and tumour severity. We detected an adjusted significant (albeit low) risk of 1.19 [1.04-1.36] to have incomplete cancer resection and of 1.30 [1.05-1.6] to have advanced cancer disease according to baseline levels of endothelial MVs.

In summary, MV-TF activity, presumably conveyed by endothelial MVs, proved to be an early biomarker of cancer aggressiveness and surgical radicality. MVs levels in cancer patients with incomplete surgical resection remained higher than in patients with complete resection up to 1 month after surgery. Finally, it is worth mentioning that all MVs subtypes were shown to correlate with factor VIII activity, both at baseline and 7 days after surgery. This observation confirmed the role of MVs as, not only procoagulant carriers, but also common mediators between coagulation and inflammation. As we mentioned in the introduction, endothelial MVs have a role in promoting platelet aggregation by bearing von Willebrand factor, this property may be mediated by factor VIII (145).

Although a few reports observed a possible activation of the intrinsic pathway mediated by MVs (109, 136), we found that MVs did not correlate with contact activation complexes. Therefore, MVs do not appear to act as an intrinsic pathway activator in gastro-intestinal cancer.

A brief comment regarding the correlation we found between all subtypes of MVs and PAI-1 antigen 7 days after surgery. This observation confirms the possible role of MVs as regulator of the fibrinolytic system. It is plausible that in a situation characterized by enhanced fibrinolytic activity (e.g. post-operative period), MVs may act as fibrinolytic brake (expressing PAI antigen) in order to avoid hyperfibrinolysis (126, 143).

Secondary outcomes – association between coagulation parameters and clinical outcomes

The second aim of our study was to evaluate the possibility to detect a coagulation biomarker able to early predict which patient will suffer thrombotic complications. We found that baseline MV-TF activity and baseline FXIa were independently associated with VTE occurrence. Levels of MV-TF activity ≥0.19 pg/mL conveyed a 2.38 [1.81-4.11] HR for VTE. Furthermore, baseline levels of FXIa >0.61 nM conveyed a 1.66 [1.02-2.9] HR to develop VTE over a median follow-up period of 6 months after diagnosis. This predictive model was adjusted for age, sex, BMI, cancer type, severity, and surgical radicality. Importantly, in the univariate model we singularly included all coagulative parameters individually, as well as the presence of hereditary thrombophilia. Only MV-TF activity, FXIa, fibrinogen, D-dimer, as well as 7-days PS+MVs and 7-days endothelial MVs showed an association with VTE occurrence. After adjustment for confounding factors, only MV-TF activity and FXIa were still significant.

Main findings

In the current scenario where many studies have been conducted or are in progress with the main purpose of identifying the perfect biomarker or the most accurate predictive model for cancer-associated thrombosis, the importance of our results lies in the confirmation of MV-TF activity – a functional test to detect plasma procoagulant activity associated with TF-bearing MVs – as an

independent predictive biomarker of cancer aggressiveness and VTE occurrence, not only in pancreatic but also in colon, stomach and esophagus cancer. Additionally, our findings highlight the importance of the intrinsic pathway in the pathophysiology of cancer-associated thrombosis. The mechanisms of FXI activation requires further elucidation; there is the possibility of direct activation of FXIa by cancer (or cancer-released mediators), or FXI activation could be the result of the enhanced thrombin generation via the TF pathway. More studies are required to understand the contribution of all possible candidates to FXI activation. As TF is upregulated in cancer, it seems reasonable to hypothesize that concomitant activation of both the intrinsic and extrinsic pathways may act synergistically to produce a highly prothrombotic state in cancer. It is tempting to speculate that while TF is the primary initiator, contact system activation may contribute to the amplification of thrombin generation in cancer-associated thrombosis. Further studies are required in order to better understand FXI activation in various cancer types, stages and phases of the disease.

Limitations

Our study does have a few limitations. Firstly, the sample size is too small to adequately design a predictive model for cancer-associated thrombosis. Nevertheless, we collected a very homogenous population that was followed very tightly from a clinical and laboratory point of view. In particular, pre-analytic conditions for sample collection and processing were precisely observed (149, 162). Secondly, flow-cytometry analysis and contact system measurement are not standardized methods at the moment. For the implementation of the former, we strictly followed the current recommendations from the International Society of Thrombosis and Haemostasis for flow-cytometry setting and MVs analysis (163). For the latter, home-made ELISAs were performed following the best method described in Literature at the moment (185-187). Thirdly, we did not evaluate the presence of platelet or leukocyte-derived MVs, well-known mediators of cancer-associated hypercoagulability. The measurement of these MVs subtypes would certainly add information regarding MVs TF-mediated hypercoagulability.

Conclusions

In conclusion, we showed that hypercoagulability in gastro-intestinal cancer is mainly mediated by high levels of factor VIII, increased levels of complexes derived from the activation of the contact system, high MV-TF activity and increased levels of PS+MVs, endothelial and tumour MVs. These prothrombotic factors remained altered up to 6 months after surgical resection of the neoplasm even in patients with surgical radicality, indicating that cancer-associated hypercoagulability persists for months after tumour removal. Increased MV-TF activity and endothelial MVs are independent predictors of advanced disease and incomplete surgical resection. Finally, increased baseline levels of MV-TF activity and FXIa were independent predictors of VTE occurrence over the 6 months following cancer diagnosis.

Future research should focus on the standardization of methods to measure MVs and contact system activation in plasma. Studies with wider populations are then needed to confirm the predictive role of these biomarkers in a wide range of neoplasms. Once the pathways involved are clarified, therapeutic options targeting these pathways could be tested and finally tailored to each specific cancer case.

Bibliography:

1.Trousseau A. Phlegmasia alba dolens. Clin Med Hotel-dieu Paris 1865; 3: 654–712.

2.Varki A. Trousseau's syndrome: Multiple definitions and multiple mechanisms. Blood 2007; 110: 1723-9.

3.Elyamany G, Alzahrani AM, Bukhary E. Cancer-associated thrombosis: an overview. Clin Med Insights Oncol 2014; 8: 129-37.

4.Walker AJ, Card TR, West J, et al. Incidence of venous thromboembolism in patients with cancer - a cohort study using linked United Kingdom databases. Eur J Cancer 2013; 49: 1404-13.

5.Heit JA, O'Fallon WM, Petterson TM, et al. Relative impact of risk factors for deep vein thrombosis and pulmonary embolism: a population-based study. Arch Intern Med 2002; 162: 1245-8.

6.Timp JF, Braekkan SK, Versteeg HH, et al. Epidemiology of cancer-associated venous thrombosis. Blood 2013; 122: 1712-23.

7.Bouillaud S. De l'Obliteration des veines et de son influence sur la formation des hydropisies partielles: consideration sur la hydropisies passive et general. Arch Gen Med 1823; 1: 188-204.

8.Gussoni G, Frasson S, La Regina M, et al. Three-month mortality rate and clinical predictors in patients with venous thromboembolism and cancer. Findings from the RIETE registry. Thromb Res 2013; 131: 24-30.

9.Sallah S, Wan JY, Nguyen NP. Venous thrombosis in patients with solid tumors: determination of frequency and characteristics. Thromb Haemost 2002; 87: 575-9.

10.Stein PD, Beemath A, Meyers FA, et al. Incidence of venous thromboembolism in patients hospitalized with cancer. Am J Med 2006; 119: 60-8.

11.Cronin-Fenton DP, Sondergaard F, Pedersen LA, et al. Hospitalisation for venous thromboembolism in cancer patients and the general population: a population-based cohort study in Denmark, 1997-2006. Br J Cancer 2010; 103: 947-53.

12.Cohen AT, Katholing A, Rietbrock S, et al. Epidemiology of first and recurrent venous thromboembolism in patients with active cancer. A population-based cohort study. Thromb Haemost 2017; 117: 57-65.

13.Blom JW, Doggen CJ, Osanto S, et al. Malignancies, prothrombotic mutations, and the risk of venous thrombosis. JAMA 2005; 293: 715-22.

14.Riedl J, Kaider A, Reitter EM, et al. Association of mean platelet volume with risk of venous thromboembolism and mortality in patients with cancer. Results from the Vienna Cancer and Thrombosis Study (CATS). Thromb Haemost 2014; 111: 670-8.

15.Chew HK, Wun T, Harvey D, et al. Incidence of venous thromboembolism and its effect on survival among patients with common cancers. Arch Intern Med 2006; 166: 458-64.

16.Chew HK, Wun T, Harvey DJ, et al. Incidence of venous thromboembolism and the impact on survival in breast cancer patients. J Clin Oncol 2007; 25: 70-6.

17.Sorensen HT, Mellemkjaer L, Olsen JH, et al. Prognosis of cancers associated with venous thromboembolism. N Engl J Med 2000; 343: 1846-50.
18.Kourlaba G, Relakis J, Mylonas C, et al. The humanistic and economic burden of venous thromboembolism in cancer patients: a systematic review. Blood Coagul Fibrinolysis 2015; 26: 13-31.
19.Lyman GH, Eckert L, Wang Y, et al. Venous thromboembolism risk in patients with cancer receiving chemotherapy: a real-world analysis. Oncologist 2013; 18: 1321-9.
20.Cohoon KP, Ransom JE, Leibson CL, et al. Direct Medical Costs Attributable to Cancer-Associated Venous Thromboembolism: A Population-Based Longitudinal Study. Am J Med 2016; 129: 1000 e15-25.
21.Horsted F, West J, Grainge MJ. Risk of venous thromboembolism in patients with cancer: a systematic review and meta-analysis. PLoS Med 2012; 9: e1001275.
22.Blom JW, Vanderschoot JPM, Oostindier MJ, et al. Incidence of venous thrombosis in a large cohort of 66,329 cancer patients: results of a record linkage study.[see comment]. Journal of Thrombosis & Haemostasis 2006; 4: 529-35.
23.Chew HK, Wun T, Harvey D, et al. Incidence of venous thromboembolism and its effect on survival among patients with common cancers. Archives of Internal Medicine 2006; 166: 458-64.
24.Cronin-Fenton DP, Sondergaard F, Pedersen LA, et al. Hospitalisation for venous thromboembolism in cancer patients and the general population: a population-based cohort study in Denmark, 1997-2006. British Journal of Cancer 2010; 103: 947-53.
25.Otten HM, Mathijssen J, ten Cate H, et al. Symptomatic venous thromboembolism in cancer patients treated with chemotherapy: an underestimated phenomenon. Arch Intern Med 2004; 164: 190-4.
26.Ay C, Vormittag R, Dunkler D, et al. D-dimer and prothrombin fragment 1 + 2 predict venous thromboembolism in patients with cancer: results from the Vienna Cancer and Thrombosis Study. Journal of Clinical Oncology 2009; 27: 4124-9.
27.Hall IE, Andersen MS, Krumholz HM, et al. Predictors of venous thromboembolism in patients with advanced common solid cancers. J Cancer Epidemiol 2009; 2009: 182521.
28.Connolly GC, Khorana AA, Kuderer NM, et al. Leukocytosis, thrombosis and early mortality in cancer patients initiating chemotherapy. Thrombosis Research 2010; 126: 113-8.
29.Abdel-Razeq HN, Hijjawi SB, Jallad SG, et al. Venous thromboembolism risk stratification in medically-ill hospitalized cancer patients. A comprehensive cancer center experience. Journal of Thrombosis & Thrombolysis 2010; 30: 286-93.
30.Di Nisio M, Ferrante N, De Tursi M, et al. Incidental venous thromboembolism in ambulatory cancer patients receiving chemotherapy. Thromb Haemost 2010; 104: 1049-54.
31.Reeves D, Liu CY. Retrospective evaluation of venous thromboembolism prophylaxis in the adult cancer population. J Oncol Pharm Pract 2010; 16: 27-31.

32.Ay C, Pabinger I, Cohen AT. Cancer-associated venous thromboembolism: Burden, mechanisms, and management. Thromb Haemost 2017; 117: 219-30.
33.Khorana AA, Dalal M, Lin J, et al. Incidence and predictors of venous thromboembolism (VTE) among ambulatory high-risk cancer patients undergoing chemotherapy in the United States. Cancer 2013; 119: 648-55.
34.Khorana AA, Francis CW, Culakova E, et al. Frequency, risk factors, and trends for venous thromboembolism among hospitalized cancer patients. Cancer 2007; 110: 2339-46.
35.Dickmann B, Ahlbrecht J, Ay C, et al. Regional lymph node metastases are a strong risk factor for venous thromboembolism: results from the Vienna Cancer and Thrombosis Study. Haematologica 2013; 98: 1309-14.
36.Ahlbrecht J, Dickmann B, Ay C, et al. Tumor grade is associated with venous thromboembolism in patients with cancer: results from the Vienna Cancer and Thrombosis Study. J Clin Oncol 2012; 30: 3870-5.
37.Kroger K, Weiland D, Ose C, et al. Risk factors for venous thromboembolic events in cancer patients. Ann Oncol 2006; 17: 297-303.
38.Lee YG, Lee E, Kim I, et al. Cisplatin-Based Chemotherapy Is a Strong Risk Factor for Thromboembolic Events in Small-Cell Lung Cancer. Cancer Res Treat 2015; 47: 670-5.
39.Moore RA, Adel N, Riedel E, et al. High incidence of thromboembolic events in patients treated with cisplatin-based chemotherapy: a large retrospective analysis. J Clin Oncol 2011; 29: 3466-73.
40.Starling N, Rao S, Cunningham D, et al. Thromboembolism in patients with advanced gastroesophageal cancer treated with anthracycline, platinum, and fluoropyrimidine combination chemotherapy: a report from the UK National Cancer Research Institute Upper Gastrointestinal Clinical Studies Group. J Clin Oncol 2009; 27: 3786-93.
41.Seng S, Liu Z, Chiu SK, et al. Risk of venous thromboembolism in patients with cancer treated with Cisplatin: a systematic review and meta-analysis. J Clin Oncol 2012; 30: 4416-26.
42.Nalluri SR, Chu D, Keresztes R, et al. Risk of venous thromboembolism with the angiogenesis inhibitor bevacizumab in cancer patients: a meta-analysis. JAMA 2008; 300: 2277-85.
43.Pritchard KI, Paterson AH, Paul NA, et al. Increased thromboembolic complications with concurrent tamoxifen and chemotherapy in a randomized trial of adjuvant therapy for women with breast cancer. National Cancer Institute of Canada Clinical Trials Group Breast Cancer Site Group. J Clin Oncol 1996; 14: 2731-7.
44.Bohlius J, Wilson J, Seidenfeld J, et al. Recombinant human erythropoietins and cancer patients: updated meta-analysis of 57 studies including 9353 patients. J Natl Cancer Inst 2006; 98: 708-14.
45.Bennett CL, Silver SM, Djulbegovic B, et al. Venous thromboembolism and mortality associated with recombinant erythropoietin and darbepoetin administration for the treatment of cancer-associated anemia. JAMA 2008; 299: 914-24.

46.White RH, Zhou H, Romano PS. Incidence of symptomatic venous thromboembolism after different elective or urgent surgical procedures. Thromb Haemost 2003; 90: 446-55.
47.Baumann Kreuziger L, Jaffray J, Carrier M. Epidemiology, diagnosis, prevention and treatment of catheter-related thrombosis in children and adults. Thromb Res 2017; 157: 64-71.
48.Kamphuisen PW, Lee AY. Catheter-related thrombosis: lifeline or a pain in the neck? Hematology Am Soc Hematol Educ Program 2012; 2012: 638-44.
49.Saber W, Moua T, Williams EC, et al. Risk factors for catheter-related thrombosis (CRT) in cancer patients: a patient-level data (IPD) meta-analysis of clinical trials and prospective studies. J Thromb Haemost 2011; 9: 312-9.
50.Debourdeau P, Farge D, Beckers M, et al. International clinical practice guidelines for the treatment and prophylaxis of thrombosis associated with central venous catheters in patients with cancer. J Thromb Haemost 2013; 11: 71-80.
51.Chopra V, Anand S, Hickner A, et al. Risk of venous thromboembolism associated with peripherally inserted central catheters: a systematic review and meta-analysis. Lancet 2013; 382: 311-25.
52.Geerts W. Central venous catheter-related thrombosis. Hematology Am Soc Hematol Educ Program 2014; 2014: 306-11.
53.Dentali F, Gianni M, Agnelli G, et al. Association between inherited thrombophilic abnormalities and central venous catheter thrombosis in patients with cancer: a meta-analysis. J Thromb Haemost 2008; 6: 70-5.
54.Rajasekhar A, Streiff MB. How I treat central venous access device-related upper extremity deep vein thrombosis. Blood 2017; 129: 2727-36.
55.Alcalay A, Wun T, Khatri V, et al. Venous thromboembolism in patients with colorectal cancer: incidence and effect on survival. J Clin Oncol 2006; 24: 1112-8.
56.Mandala M, Barni S, Prins M, et al. Acquired and inherited risk factors for developing venous thromboembolism in cancer patients receiving adjuvant chemotherapy: a prospective trial. Ann Oncol 2010; 21: 871-6.
57.Konigsbrugge O, Lotsch F, Reitter EM, et al. Presence of varicose veins in cancer patients increases the risk for occurrence of venous thromboembolism. J Thromb Haemost 2013; 11: 1993-2000.
58.Gran OV, Smith EN, Braekkan SK, et al. Joint effects of cancer and variants in the factor 5 gene on the risk of venous thromboembolism. Haematologica 2016; 101: 1046-53.
59.Kovac M, Kovac Z, Tomasevic Z, et al. Factor V Leiden mutation and high FVIII are associated with an increased risk of VTE in women with breast cancer during adjuvant tamoxifen - results from a prospective, single center, case control study. Eur J Intern Med 2015; 26: 63-7.

60.Imberti D, Agnelli G, Ageno W, et al. Clinical characteristics and management of cancer-associated acute venous thromboembolism: findings from the MASTER Registry. Haematologica 2008; 93: 273-8.
61.Martinelli I, De Stefano V. Rare thromboses of cerebral, splanchnic and upper-extremity veins. A narrative review. Thromb Haemost 2010; 103: 1136-44.
62.Prandoni P, Lensing AW, Cogo A, et al. The long-term clinical course of acute deep venous thrombosis. Ann Intern Med 1996; 125: 1-7.
63.Prandoni P, Lensing AW, Piccioli A, et al. Recurrent venous thromboembolism and bleeding complications during anticoagulant treatment in patients with cancer and venous thrombosis. Blood 2002; 100: 3484-8.
64.Trujillo-Santos J, Nieto JA, Tiberio G, et al. Predicting recurrences or major bleeding in cancer patients with venous thromboembolism. Findings from the RIETE Registry. Thromb Haemost 2008; 100: 435-9.
65.Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS 2010; 5: 463-6.
66.Khorana AA, Kuderer NM, Culakova E, et al. Development and validation of a predictive model for chemotherapy-associated thrombosis. Blood 2008; 111: 4902-7.
67.Pabinger I, Posch F. Flamethrowers: blood cells and cancer thrombosis risk. Hematology Am Soc Hematol Educ Program 2014; 2014: 410-7.
68.Simanek R, Vormittag R, Ay C, et al. High platelet count associated with venous thromboembolism in cancer patients: results from the Vienna Cancer and Thrombosis Study (CATS). J Thromb Haemost 2010; 8: 114-20.
69.Ay C, Dunkler D, Simanek R, et al. Prediction of venous thromboembolism in patients with cancer by measuring thrombin generation: results from the Vienna Cancer and Thrombosis Study. J Clin Oncol 2011; 29: 2099-103.
70.Ay C, Simanek R, Vormittag R, et al. High plasma levels of soluble P-selectin are predictive of venous thromboembolism in cancer patients: results from the Vienna Cancer and Thrombosis Study (CATS). Blood 2008; 112: 2703-8.
71.Ay C, Vormittag R, Dunkler D, et al. D-dimer and prothrombin fragment 1 + 2 predict venous thromboembolism in patients with cancer: results from the Vienna Cancer and Thrombosis Study. J Clin Oncol 2009; 27: 4124-9.
72.Chen M, Geng JG. P-selectin mediates adhesion of leukocytes, platelets, and cancer cells in inflammation, thrombosis, and cancer growth and metastasis. Arch Immunol Ther Exp (Warsz) 2006; 54: 75-84.
73.Adam SS, Key NS, Greenberg CS. D-dimer antigen: current concepts and future prospects. Blood 2009; 113: 2878-87.

74.Teitel JM, Bauer KA, Lau HK, et al. Studies of the prothrombin activation pathway utilizing radioimmunoassays for the F2/F1 + 2 fragment and thrombin--antithrombin complex. Blood 1982; 59: 1086-97.
75.Stender MT, Frokjaer JB, Larsen TB, et al. Preoperative plasma D-dimer is a predictor of postoperative deep venous thrombosis in colorectal cancer patients: a clinical, prospective cohort study with one-year follow-up. Dis Colon Rectum 2009; 52: 446-51.
76.Pasceri V, Willerson JT, Yeh ET. Direct proinflammatory effect of C-reactive protein on human endothelial cells. Circulation 2000; 102: 2165-8.
77.Kanz R, Vukovich T, Vormittag R, et al. Thrombosis risk and survival in cancer patients with elevated C-reactive protein. J Thromb Haemost 2011; 9: 57-63.
78.Reitter EM, Kaider A, Ay C, et al. Longitudinal analysis of hemostasis biomarkers in cancer patients during antitumor treatment. J Thromb Haemost 2016; 14: 294-305.
79.Falanga A, Russo L, Milesi V, et al. Mechanisms and risk factors of thrombosis in cancer. Crit Rev Oncol Hematol 2017; 118: 79-83.
80.Ay C, Dunkler D, Marosi C, et al. Prediction of venous thromboembolism in cancer patients. Blood 2010; 116: 5377-82.
81.Mandala M, Clerici M, Corradino I, et al. Incidence, risk factors and clinical implications of venous thromboembolism in cancer patients treated within the context of phase I studies: the 'SENDO experience'. Ann Oncol 2012; 23: 1416-21.
82.Verso M, Agnelli G, Barni S, et al. A modified Khorana risk assessment score for venous thromboembolism in cancer patients receiving chemotherapy: the Protecht score. Intern Emerg Med 2012; 7: 291-2.
83.Palumbo A, Rajkumar SV, Dimopoulos MA, et al. Prevention of thalidomide- and lenalidomide-associated thrombosis in myeloma. Leukemia 2008; 22: 414-23.
84.Louzada ML, Carrier M, Lazo-Langner A, et al. Development of a clinical prediction rule for risk stratification of recurrent venous thromboembolism in patients with cancer-associated venous thromboembolism. Circulation 2012; 126: 448-54.
85.den Exter PL, Kooiman J, Huisman MV. Validation of the Ottawa prognostic score for the prediction of recurrent venous thromboembolism in patients with cancer-associated thrombosis. J Thromb Haemost 2013; 11: 998-1000.
86.Lyman GH, Bohlke K, Khorana AA, et al. Venous thromboembolism prophylaxis and treatment in patients with cancer: american society of clinical oncology clinical practice guideline update 2014. J Clin Oncol 2015; 33: 654-6.
87.Karimi M, Cohan N. Cancer-associated thrombosis. Open Cardiovasc Med J 2010; 4: 78-82.
88.Bick RL. Cancer-associated thrombosis. N Engl J Med 2003; 349: 109-11.
89.Rickles FR. Mechanisms of cancer-induced thrombosis in cancer. Pathophysiol Haemost Thromb 2006; 35: 103-10.

90.Falanga A, Marchetti M, Russo L. The mechanisms of cancer-associated thrombosis. Thromb Res 2015; 135 Suppl 1: S8-S11.
91.Falanga A, Marchetti M, Vignoli A. Coagulation and cancer: biological and clinical aspects. J Thromb Haemost 2013; 11: 223-33.
92.Falanga A, Alessio MG, Donati MB, et al. A new procoagulant in acute leukemia. Blood 1988; 71: 870-5.
93.Kazmierczak M, Lewandowski K, Wojtukiewicz MZ, et al. Cancer procoagulant in patients with adenocarcinomas. Blood Coagul Fibrinolysis 2005; 16: 543-7.
94.Mackman N. The many faces of tissue factor. J Thromb Haemost 2009; 7 Suppl 1: 136-9.
95.Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler Thromb Vasc Biol 2007; 27: 1687-93.
96.Geddings JE, Mackman N. Tumor-derived tissue factor-positive microparticles and venous thrombosis in cancer patients. Blood 2013; 122: 1873-80.
97.Callander NS, Varki N, Rao LV. Immunohistochemical identification of tissue factor in solid tumors. Cancer 1992; 70: 1194-201.
98.Khorana AA, Ahrendt SA, Ryan CK, et al. Tissue factor expression, angiogenesis, and thrombosis in pancreatic cancer. Clin Cancer Res 2007; 13: 2870-5.
99.Davila M, Amirkhosravi A, Coll E, et al. Tissue factor-bearing microparticles derived from tumor cells: impact on coagulation activation. J Thromb Haemost 2008; 6: 1517-24.
100.Kakkar AK, Lemoine NR, Scully MF, et al. Tissue factor expression correlates with histological grade in human pancreatic cancer. Br J Surg 1995; 82: 1101-4.
101.Thaler J, Preusser M, Ay C, et al. Intratumoral tissue factor expression and risk of venous thromboembolism in brain tumor patients. Thromb Res 2013; 131: 162-5.
102.Ueno T, Toi M, Koike M, et al. Tissue factor expression in breast cancer tissues: its correlation with prognosis and plasma concentration. Br J Cancer 2000; 83: 164-70.
103.Uno K, Homma S, Satoh T, et al. Tissue factor expression as a possible determinant of thromboembolism in ovarian cancer. Br J Cancer 2007; 96: 290-5.
104.Kasthuri RS, Taubman MB, Mackman N. Role of tissue factor in cancer. J Clin Oncol 2009; 27: 4834-8.
105.van den Berg YW, Osanto S, Reitsma PH, et al. The relationship between tissue factor and cancer progression: insights from bench and bedside. Blood 2012; 119: 924-32.
106.Magnus N, D'Asti E, Meehan B, et al. Oncogenes and the coagulation system--forces that modulate dormant and aggressive states in cancer. Thromb Res 2014; 133 Suppl 2: S1-9.
107.Minnema MC, Fijnheer R, De Groot PG, et al. Extremely high levels of von Willebrand factor antigen and of procoagulant factor VIII found in multiple myeloma patients are associated with activity status but not with thalidomide treatment. J Thromb Haemost 2003; 1: 445-9.

108.Auwerda JJ, Sonneveld P, de Maat MP, et al. Prothrombotic coagulation abnormalities in patients with newly diagnosed multiple myeloma. Haematologica 2007; 92: 279-80.
109.Nickel KF, Ronquist G, Langer F, et al. The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis. Blood 2015; 126: 1379-89.
110.Binder BR, Christ G, Gruber F, et al. Plasminogen activator inhibitor 1: physiological and pathophysiological roles. News Physiol Sci 2002; 17: 56-61.
111.Yagci M, Sucak GT, Haznedar R. Fibrinolytic activity in multiple myeloma. Am J Hematol 2003; 74: 231-7.
112.Casslen B, Bossmar T, Lecander I, et al. Plasminogen activators and plasminogen activator inhibitors in blood and tumour fluids of patients with ovarian cancer. Eur J Cancer 1994; 30A: 1302-9.
113.Pyke C, Kristensen P, Ralfkiaer E, et al. The plasminogen activation system in human colon cancer: messenger RNA for the inhibitor PAI-1 is located in endothelial cells in the tumor stroma. Cancer Res 1991; 51: 4067-71.
114.Seruga B, Zhang H, Bernstein LJ, et al. Cytokines and their relationship to the symptoms and outcome of cancer. Nat Rev Cancer 2008; 8: 887-99.
115.Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. Arterioscler Thromb Vasc Biol 2012; 32: 1777-83.
116.Martinod K, Wagner DD. Thrombosis: tangled up in NETs. Blood 2014; 123: 2768-76.
117.Demers M, Krause DS, Schatzberg D, et al. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. Proc Natl Acad Sci U S A 2012; 109: 13076-81.
118.Gupta AK, Joshi MB, Philippova M, et al. Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. FEBS Lett 2010; 584: 3193-7.
119.Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A 2010; 107: 15880-5.
120.von Bruhl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 2012; 209: 819-35.
121.Gyorgy B, Szabo TG, Pasztoi M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci 2011; 68: 2667-88.
122.Campello E, Spiezia L, Radu CM, et al. Microparticles as biomarkers of venous thromboembolic events. Biomark Med 2016; 10: 743-55.
123.Gardiner C, Harrison P, Belting M, et al. Extracellular vesicles, tissue factor, cancer and thrombosis - discussion themes of the ISEV 2014 Educational Day. J Extracell Vesicles 2015; 4: 26901.
124.Owens AP, 3rd, Mackman N. Microparticles in hemostasis and thrombosis. Circ Res 2011; 108: 1284-97.

125.Mooberry MJ, Bradford R, Hobl EL, et al. Procoagulant microparticles promote coagulation in a factor XI-dependent manner in human endotoxemia. J Thromb Haemost 2016; 14: 1031-42.
126.Lacroix R, Dubois C, Leroyer AS, et al. Revisited role of microparticles in arterial and venous thrombosis. J Thromb Haemost 2013; 11 Suppl 1: 24-35.
127.Rautou PE, Mackman N. Microvesicles as risk markers for venous thrombosis. Expert Rev Hematol 2013; 6: 91-101.
128.Zhou L, Qi XL, Xu MX, et al. Microparticles: new light shed on the understanding of venous thromboembolism. Acta Pharmacol Sin 2014; 35: 1103-10.
129.Sinauridze EI, Kireev DA, Popenko NY, et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thromb Haemost 2007; 97: 425-34.
130.Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood 2003; 102: 2678-83.
131.Biro E, Sturk-Maquelin KN, Vogel GM, et al. Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. J Thromb Haemost 2003; 1: 2561-8.
132.Abid Hussein MN, Meesters EW, Osmanovic N, et al. Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo. J Thromb Haemost 2003; 1: 2434-43.
133.Siddiqui FA, Desai H, Amirkhosravi A, et al. The presence and release of tissue factor from human platelets. Platelets 2002; 13: 247-53.
134.Hisada Y, Mackman N. Cancer-associated pathways and biomarkers of venous thrombosis. Blood 2017; 130: 1499-506.
135.Thomas GM, Panicot-Dubois L, Lacroix R, et al. Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. J Exp Med 2009; 206: 1913-27.
136.Van Der Meijden PE, Van Schilfgaarde M, Van Oerle R, et al. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. J Thromb Haemost 2012; 10: 1355-62.
137.Rubin O, Delobel J, Prudent M, et al. Red blood cell-derived microparticles isolated from blood units initiate and propagate thrombin generation. Transfusion 2013; 53: 1744-54.
138.van Beers EJ, Schaap MC, Berckmans RJ, et al. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Haematologica 2009; 94: 1513-9.
139.Koshiar RL, Somajo S, Norstrom E, et al. Erythrocyte-derived microparticles supporting activated protein C-mediated regulation of blood coagulation. PLoS One 2014; 9: e104200.
140.Somajo S, Koshiar RL, Norstrom E, et al. Protein S and factor V in regulation of coagulation on platelet microparticles by activated protein C. Thromb Res 2014; 134: 144-52.
141.Tans G, Rosing J, Thomassen MC, et al. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. Blood 1991; 77: 2641-8.
142.Lacroix R, Dignat-George F. Microparticles: new protagonists in pericellular and intravascular proteolysis. Semin Thromb Hemost 2013; 39: 33-9.

143.Lacroix R, Plawinski L, Robert S, et al. Leukocyte- and endothelial-derived microparticles: a circulating source for fibrinolysis. Haematologica 2012; 97: 1864-72.
144.Yanez-Mo M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 2015; 4: 27066.
145.Freyssinet JM. Cellular microparticles: what are they bad or good for? J Thromb Haemost 2003; 1: 1655-62.
146.Ye R, Ye C, Huang Y, et al. Circulating tissue factor positive microparticles in patients with acute recurrent deep venous thrombosis. Thromb Res 2012; 130: 253-8.
147.Furie B, Furie BC. Mechanisms of thrombus formation. N Engl J Med 2008; 359: 938-49.
148.Puddu P, Puddu GM, Cravero E, et al. The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. Can J Cardiol 2010; 26: 140-5.
149.Lacroix R, Judicone C, Poncelet P, et al. Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. J Thromb Haemost 2012; 10: 437-46.
150.Mooberry MJ, Key NS. Microparticle analysis in disorders of hemostasis and thrombosis. Cytometry A 2015.
151.Lacroix R, Robert S, Poncelet P, et al. Overcoming limitations of microparticle measurement by flow cytometry. Semin Thromb Hemost 2010; 36: 807-18.
152.Shantsila E, Montoro-Garcia S, Gallego P, et al. Circulating microparticles: challenges and perspectives of flow cytometric assessment. Thromb Haemost 2014; 111: 1009-14.
153.Nomura S, Niki M, Nisizawa T, et al. Microparticles as Biomarkers of Blood Coagulation in Cancer. Biomark Cancer 2015; 7: 51-6.
154.Campello E, Spiezia L, Radu CM, et al. Evaluation of a procoagulant phospholipid functional assay as a routine test for measuring circulating microparticle activity. Blood Coagul Fibrinolysis 2014; 25: 534-7.
155.Osumi K, Ozeki Y, Saito S, et al. Development and assessment of enzyme immunoassay for platelet-derived microparticles. Thromb Haemost 2001; 85: 326-30.
156.Jy W, Horstman LL, Jimenez JJ, et al. Measuring circulating cell-derived microparticles. J Thromb Haemost 2004; 2: 1842-51.
157.Key NS, Mackman N. Tissue factor and its measurement in whole blood, plasma, and microparticles. Semin Thromb Hemost 2010; 36: 865-75.
158.Dragovic RA, Gardiner C, Brooks AS, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. Nanomedicine 2011; 7: 780-8.
159.Yuana Y, Oosterkamp TH, Bahatyrova S, et al. Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles. J Thromb Haemost 2010; 8: 315-23.
160.van der Pol E, Coumans F, Varga Z, et al. Innovation in detection of microparticles and exosomes. J Thromb Haemost 2013; 11 Suppl 1: 36-45.

161.van der Pol E, Hoekstra AG, Sturk A, et al. Optical and non-optical methods for detection and characterization of microparticles and exosomes. J Thromb Haemost 2010; 8: 2596-607.
162.Lacroix R, Judicone C, Mooberry M, et al. Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost 2013.
163.Cointe S, Judicone C, Robert S, et al. Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop. J Thromb Haemost 2017; 15: 187-93.
164.Inglis HC, Danesh A, Shah A, et al. Techniques to improve detection and analysis of extracellular vesicles using flow cytometry. Cytometry A 2015; 87: 1052-63.
165.Burger D, Schock S, Thompson CS, et al. Microparticles: biomarkers and beyond. Clin Sci (Lond) 2013; 124: 423-41.
166.Dvorak HF, Quay SC, Orenstein NS, et al. Tumor shedding and coagulation. Science 1981; 212: 923-4.
167.Dvorak HF, Van DeWater L, Bitzer AM, et al. Procoagulant activity associated with plasma membrane vesicles shed by cultured tumor cells. Cancer Res 1983; 43: 4434-42.
168.Campello E, Radu CM, Spiezia L, et al. Modulating thrombotic diathesis in hereditary thrombophilia and antiphospholipid antibody syndrome: a role for circulating microparticles? Clin Chem Lab Med 2017; 55: 934-43.
169.Campello E, Spiezia L, Radu CM, et al. Endothelial, platelet, and tissue factor-bearing microparticles in cancer patients with and without venous thromboembolism. Thromb Res 2011; 127: 473-7.
170.Thaler J, Ay C, Mackman N, et al. Microparticle-associated tissue factor activity, venous thromboembolism and mortality in pancreatic, gastric, colorectal and brain cancer patients. J Thromb Haemost 2012; 10: 1363-70.
171.Zwicker JI, Liebman HA, Neuberg D, et al. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. Clin Cancer Res 2009; 15: 6830-40.
172.Khorana AA, Francis CW, Menzies KE, et al. Plasma tissue factor may be predictive of venous thromboembolism in pancreatic cancer. J Thromb Haemost 2008; 6: 1983-5.
173.Tesselaar ME, Romijn FP, Van Der Linden IK, et al. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? J Thromb Haemost 2007; 5: 520-7.
174.Bharthuar A, Khorana AA, Hutson A, et al. Circulating microparticle tissue factor, thromboembolism and survival in pancreaticobiliary cancers. Thromb Res 2013; 132: 180-4.
175.Date K, Ettelaie C, Maraveyas A. Tissue factor-bearing microparticles and inflammation: a potential mechanism for the development of venous thromboembolism in cancer. J Thromb Haemost 2017.

176.Ruf W, Disse J, Carneiro-Lobo TC, et al. Tissue factor and cell signalling in cancer progression and thrombosis. J Thromb Haemost 2011; 9 Suppl 1: 306-15.
177.Maraveyas A, Ettelaie C, Echrish H, et al. Weight-adjusted dalteparin for prevention of vascular thromboembolism in advanced pancreatic cancer patients decreases serum tissue factor and serum-mediated induction of cancer cell invasion. Blood Coagul Fibrinolysis 2010; 21: 452-8.
178.van Doormaal F, Kleinjan A, Berckmans RJ, et al. Coagulation activation and microparticle-associated coagulant activity in cancer patients. An exploratory prospective study. Thromb Haemost 2012; 108: 160-5.
179.Thaler J, Ay C, Weinstabl H, et al. Circulating procoagulant microparticles in cancer patients. Ann Hematol 2011; 90: 447-53.
180.Sartori MT, Della Puppa A, Ballin A, et al. Circulating microparticles of glial origin and tissue factor bearing in high-grade glioma: a potential prothrombotic role. Thromb Haemost 2013; 110: 378-85.
181.Auwerda JJ, Yuana Y, Osanto S, et al. Microparticle-associated tissue factor activity and venous thrombosis in multiple myeloma. Thromb Haemost 2011; 105: 14-20.
182.Yates JW, Chalmer B, McKegney FP. Evaluation of patients with advanced cancer using the Karnofsky performance status. Cancer 1980; 45: 2220-4.
183.Brierley J, Gospodarowicz M, O'Sullivan B. The principles of cancer staging. Ecancermedicalscience 2016; 10: ed61.
184.Campello E, Spiezia L, Zabeo E, et al. Hypercoagulability detected by whole blood thromboelastometry (ROTEM(R)) and impedance aggregometry (MULTIPLATE(R)) in obese patients. Thromb Res 2015; 135: 548-53.
185.Konings J, Govers-Riemslag JW, Spronk HM, et al. Activation of the contact system in patients with a first acute myocardial infarction. Thromb Res 2013; 132: 138-42.
186.Govers-Riemslag JW, Smid M, Cooper JA, et al. The plasma kallikrein-kinin system and risk of cardiovascular disease in men. J Thromb Haemost 2007; 5: 1896-903.
187.Minnema MC, Pajkrt D, Wuillemin WA, et al. Activation of clotting factor XI without detectable contact activation in experimental human endotoxemia. Blood 1998; 92: 3294-301.
188.Hisada Y, Alexander W, Kasthuri R, et al. Measurement of microparticle tissue factor activity in clinical samples: A summary of two tissue factor-dependent FXa generation assays. Thromb Res 2016; 139: 90-7.
189.Wisgrill L, Lamm C, Hartmann J, et al. Peripheral blood microvesicles secretion is influenced by storage time, temperature, and anticoagulants. Cytometry A 2016; 89: 663-72.
190.Aass HC, Ovstebo R, Troseid AM, et al. Fluorescent particles in the antibody solution result in false TF- and CD14-positive microparticles in flow cytometric analysis. Cytometry A 2011; 79: 990-9.

191.Mezouar S, Frere C, Darbousset R, et al. Role of platelets in cancer and cancer-associated thrombosis: Experimental and clinical evidences. Thromb Res 2016; 139: 65-76.
192.Larocca A, Cavallo F, Bringhen S, et al. Aspirin or enoxaparin thromboprophylaxis for patients with newly diagnosed multiple myeloma treated with lenalidomide. Blood 2012; 119: 933-9; quiz 1093.
193.Shai A, Rennert HS, Rennert G, et al. Statins, aspirin and risk of thromboembolic events in ovarian cancer patients. Gynecol Oncol 2014; 133: 304-8.
194.Mansfield AS, Tafur AJ, Wang CE, et al. Predictors of active cancer thromboembolic outcomes: validation of the Khorana score among patients with lung cancer. J Thromb Haemost 2016; 14: 1773-8.
195.van Es N, Di Nisio M, Cesarman G, et al. Comparison of risk prediction scores for venous thromboembolism in cancer patients: a prospective cohort study. Haematologica 2017; 102: 1494-501.
196.Hamzehzadeh L, Yousefi M, Ghaffari SH. Colorectal Cancer Screening: A Comprehensive Review to Recent Non-Invasive Methods. Int J Hematol Oncol Stem Cell Res 2017; 11: 250-61.
197.Blasi A, Molina V, Sanchez-Cabus S, et al. Prediction of thromboembolic complications after liver resection for cholangiocarcinoma: is there a place for thromboelastometry? Blood Coagul Fibrinolysis 2017.
198.Long AT, Kenne E, Jung R, et al. Contact system revisited: an interface between inflammation, coagulation, and innate immunity. J Thromb Haemost 2016; 14: 427-37.
199.Roeise O, Sivertsen S, Ruud TE, et al. Studies on components of the contact phase system in patients with advanced gastrointestinal cancer. Cancer 1990; 65: 1355-9.
200.Tafur AJ, Dale G, Cherry M, et al. Prospective evaluation of protein C and factor VIII in prediction of cancer-associated thrombosis. Thromb Res 2015; 136: 1120-5.
201.Andren-Sandberg A, Lecander I, Martinsson G, et al. Peaks in plasma plasminogen activator inhibitor-1 concentration may explain thrombotic events in cases of pancreatic carcinoma. Cancer 1992; 69: 2884-7.
202.Sciacca FL, Ciusani E, Silvani A, et al. Genetic and plasma markers of venous thromboembolism in patients with high grade glioma. Clin Cancer Res 2004; 10: 1312-7.
203.Denko NC, Giaccia AJ. Tumor hypoxia, the physiological link between Trousseau's syndrome (carcinoma-induced coagulopathy) and metastasis. Cancer Res 2001; 61: 795-8.
204.Renne T, Pozgajova M, Gruner S, et al. Defective thrombus formation in mice lacking coagulation factor XII. J Exp Med 2005; 202: 271-81.
205.Fredenburgh JC, Gross PL, Weitz JI. Emerging anticoagulant strategies. Blood 2017; 129: 147-54.

206.Connor DE, Exner T, Ma DD, et al. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. Thromb Haemost 2010; 103: 1044-52.