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1 **Microvesicles and cancer associated thrombosis**

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16  
17 **Running Title:** Microvesicles and cancer associated thrombosis

1 **Abstract**

2 Microvesicles (MVs) are small membrane enclosed structures released into the  
3 extracellular space by virtually all cell types. Their composition varies according to the cell  
4 origin and the stimulus which caused their formation. They harbor functional molecules and  
5 participate in intercellular communication. Endothelium, inflammatory cells, and cancer cells  
6 produce procoagulant MVs which contribute to cancer-associated thrombosis (CAT) in  
7 animal models. The tissue factor (TF) conveyed by these MVs was shown to play a key role  
8 in different animal models of experimental CAT. Alternatively, other molecular mechanisms  
9 involving polyphosphates or phosphatidylethanolamine could also be involved. In clinical  
10 practice, an association between an increase in the number of TF-positive or the procoagulant  
11 activity of these MVs and the occurrence of CAT has indeed been demonstrated in pancreatic-  
12 biliary cancers, suggesting that they could behave as a biomarker predictive for CAT.  
13 However, to date, this association was not confirmed in other types of cancer. Potential causes  
14 explaining this limited association between MVs and CAT are 1) the diversity of mechanisms  
15 associating MVs and different types of cancer; 2) a more complex role of MVs in hemostasis  
16 integrating their anticoagulant and fibrinolytic activity; 3) the lack of sensitivity,  
17 reproducibility and standardization of current methodologies permitting measurement of  
18 MVs. Each of these hypotheses constitutes an interesting exploration path for a future  
19 reassessment of the clinical interest of the MVs in CAT.

20

21 **Key words:** extracellular vesicles, microvesicles, cancer-associated thrombosis

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24

## 1 **Introduction**

2 Extracellular vesicles (EVs) constitute a heterogeneous population of small membrane  
3 enclosed structures released into the extracellular space by virtually all cell types. They can be  
4 divided in subpopulations based on specific characteristics like, biogenesis, size, content and  
5 biological function. EVs have been divided in 3 main categories according to their biogenesis.  
6 Exosomes are generated by fusion of the multivesicular bodies with the membrane.  
7 Ectosomes originate from the budding of cell membranes and are more often referred to as  
8 microparticles or microvesicles (MVs) whereas apoptotic bodies result from the late stages of  
9 apoptosis (1,2). In this review, we will refer mainly to MVs, small membrane vesicles with a  
10 size between 100 and 1000 nm released from activated or apoptotic cells (3). Once released  
11 by the cells, MVs circulate in a variety of body fluids and vectorize information to multiple  
12 target cells in the body (3). MVs are extremely diverse depending on the inducer triggering  
13 their biogenesis and their parent cell. They are considered both as molecular signature of the  
14 cells from which they originate and as a depository of important biological information that  
15 can be exchanged with neighboring cells (4). Diversity of MVs origin is responsible for a  
16 diversity of structures and composition in lipids, proteins and nucleic acid such as mRNA and  
17 miRNA resulting in a diversity of functions. Because of the panel of selected molecules they  
18 bear, MVs have been involved in different pathophysiological processes such as coagulation,  
19 inflammation, angiogenesis or endothelial dysfunction (5,6). This coagulo-inflammatory  
20 profile has logically led over the past decades to the question about their involvement in  
21 thrombosis. To address the question of the role of MVs in cancer associated thrombosis  
22 (CAT), MVs have to be integrated in their complex vascular environment with all partners  
23 contributing to thrombus formation including innate immune cells such as polymorphonuclear  
24 cells (PMNs) and monocytes, endothelial cells, platelets and also tumor cells. Additional  
25 partners resulting from cellular activation also include neutrophil extracellular traps (NETs)

1 and Polyphosphates (PolyPs) (7). Moreover, MVs originating from normal and tumoral cells  
2 contribute to CAT, explaining that both common and cancer specific mechanisms are  
3 involved. After a rapid overview of MVs involvement in coagulation and CAT and their  
4 association with the development of clinical thrombosis, we will address the current causes  
5 potentially explaining why MVs were not always found associated to CAT including: 1) the  
6 diversity of CAT mechanisms, 2) the more complex role of MVs in hemostasis integrating  
7 their anticoagulant and fibrinolytic activity and 3) the lack of sensitivity, reproducibility and  
8 standardization of current methodologies allowing to measure MVs in clinical situations.

9

## 10 **MV involvement in coagulation and CAT**

11 MVs are procoagulant because they provide a catalytic surface for the assembly of  
12 different clotting factor complexes. Their procoagulant properties mainly rely on the exposure  
13 of anionic phospholipids, especially the phosphatidylserine (PS) on the external leaflet of the  
14 membrane after a calcium-dependent cellular activation (6). PS allows the binding of  
15 coagulation factors at the MVs surface by their carboxylglutamic acid-rich (GLA)-domains. A  
16 critical transporter with scramblase activity responsible for the PS exposure was identified as  
17 an anoctamin, the calcium-dependent channel TMEM16F (8). Accordingly, mutated form of  
18 TMEM16F lead to a defective PS membrane exposure and MVs shedding which is associated  
19 with a bleeding disorder in rare Scott Syndrome patients (9).

20 Besides PS, the presence of the coagulation initiator tissue factor (TF) on subsets of  
21 MVs also significantly contributes to their procoagulant activity. TF was initially identified on  
22 MVs after the observation that tumor cells release procoagulant vesicles (10,11). PS and TF  
23 are not independent each other, indeed TF needs anionic phospholipids to be fully activated. It

1 is the concentration of the same restricted surface of both molecules which lead to highly  
2 procoagulant MVs. Incorporation of TF in PS+MVs is tightly regulated (12–16).

3         Once release in the circulation, TF positive MVs (TF+MVs) participate in a transfer of  
4 information between cells at the crossroad between inflammation and thrombosis. Indeed, it  
5 was demonstrated that MVs released by activated endothelial cells induce TF-dependent  
6 pathways on monocytes (17) whereas activation of monocytes generate TF+MVs which  
7 induce a switch of the endothelium phenotype towards a proinflammatory profile. This profile  
8 is associated with the release of inflammatory cytokines, TF expression and release of new  
9 TF+MVs which can in turn transferred TF to platelets or activate monocytes and increase  
10 their TF expression (18). Consequently, TF expression on cells or MVs which initially do not  
11 express TF, is enabled by different mechanisms of membrane fusion (19) or endocytosis  
12 recycling (20).

13         The procoagulant potential of MVs depends on their cellular origin and the stimuli which  
14 triggered their formation. Platelet MVs (PMVs), the main source of MVs present the blood  
15 circulation (21,22) are natively lacking TF-dependent procoagulant activity (23). When  
16 observed, the TF activity on PMVs may result from the fusion of endothelial or monocyte-  
17 derived TF+MVs with activated platelets as described above. Moreover, the PS-rich  
18 environment generated by PMVs may amplify the TF+MV activity from MVs from another  
19 source. It was also showed that PMVs represent an appropriate surface to activate the contact  
20 phase pathway (24). Similarly, erythrocyte-derived MVs (EryMV) trigger the coagulation  
21 independently of TF but in a contact phase-dependent manner (25).

22         Among leucocytes, monocyte-derived MVs (MoMV) represent a major component of  
23 the TF+MV circulating pool (26). As recently reviewed by Halim et al (27), TF on MoMV  
24 can be upregulated by several factors including LPS (lipopolysaccharide) and  
25 proinflammatory cytokines such as TNF $\alpha$  and Interleukin (IL)-1 $\beta$ . Interestingly, it was

1 recently shown that the different human monocyte subsets generated MVs with different  
2 procoagulant potential after stimulation by IL-33 (28). In contrast to MoMVs, granulocyte-  
3 derived MVs (Gran-MVs) generated only a low level of TF activity, if any, while this  
4 property may be observed in pathological situations (29), although MoMVs transfer to  
5 granulocytes cannot be excluded (30).

6 Endothelial-derived MVs (EMVs) harbor PS and TF-dependent procoagulant activity  
7 in response to a variety of stimuli including cytokines, complement, and LPS and therefore  
8 contribute to the circulating pool of TF+MVs in clinical situation where the endothelium is  
9 activated (5). Cancer MVs represent another set of procoagulant MVs. Cultured human tumor  
10 cell lines of different origins express variable levels of TF and release cancer MVs with  
11 various procoagulant potentials in their supernatant (31,32). As illustrated for breast cancer,  
12 according to the cell line, mechanisms involve TF-independent pathways which promote  
13 platelet activation and aggregation and/or TF-dependent thrombin generation (33).

14 Evidence of MVs involvement in thrombus formation *in vivo* is supported by different  
15 studies using endogeneously formed or exogeneously injected MVs and mice mouse models  
16 of thrombosis. The pioneering work by Furies's group in 2003 showed that MoMVs rapidly  
17 accumulate at the site of injury in a laser injury model of small cremaster muscle arteries  
18 (34,35). This result was reproduced in various murine model of arterial and venous  
19 thrombosis with different sources of MVs (36–40).

20

## 21 **MVs association with the development of clinical thrombosis**

22 Cancer patients have an increased risk of thrombosis, especially venous thromboembolism  
23 (VTE), which differs according to the type of cancer (31,41). Although cancer is known as a  
24 hypercoagulable state, the pathogenesis is not totally elucidated. TF bearing MVs has been

1 assessed as a risk factor for cancer related VTE in several studies. The 5 main retrospective  
2 studies (31,42–45) that analyzed the link between TF+MVs and VTE demonstrated increased  
3 levels of circulating TF+MVs(measured by flow cytometry and functional assays) in cancer  
4 patients with VTE compared with cancer patient without VTE, with the highest values found  
5 in patients with pancreatic cancer.

6 The main prospective studies are listed in table 1. The first one supporting the hypothesis that  
7 high levels of TF+MVs activity predicts VTE in pancreatic cancer was performed by Khorana  
8 et al (46). Since this initial work, several other prospective studies, measured TF-MVs to  
9 evaluate the relationship between TF+MVs and future development of VTE. A positive  
10 association was found in studies using reported methodologies (43,47–49) including the most  
11 recent studies (50–52) whereas it was not found in other large studies (53–55). A recent meta-  
12 analysis on the relationship between TF+MVs and cancer patients with VTE concluded that  
13 the increased presence of TF+MVs represents an increased risk for cancer patients VTE with  
14 an overall odds ratio (OR) of 1.76 (56). However, due to the heterogeneity of study design,  
15 methods to measure TF-MVs, cancer origins and causes of death, more well-designed studies  
16 and more comprehensive adjustments for confounders are needed to confirm this association.  
17 Indeed, it is most likely that the results of the aforementioned studies are primarily driven by  
18 pancreaticobiliary cancer.

19

20 From these clinical studies, it seems that MVs have a real potential to predict CAT.  
21 However, most of these studies have limitations including a low number of patients, and the  
22 fact that few of them rely on multicenter prospective designs. More importantly these studies  
23 display a broad methodological heterogeneity and lack of standardization. We will now  
24 address the potential causes explaining that MVs are not always associated to CAT including:  
25 1) the diversity of CAT mechanisms, 2) the more complex role of MVs in hemostasis



1 integrating their anticoagulant and fibrinolytic activity and 3) the lack of sensitivity,  
2 reproducibility and standardization of current methodologies allowing to measure MVs in  
3 clinical situations.

4

## 5 **MVs and the diversity of cancer associated thrombosis mechanisms**

6 Over the past years, a special attention has been given to CAT and the underlying  
7 mechanisms linking MVs and the thrombus formation (Figure 1). Several mouse models of  
8 cancer-associated thrombosis have been used with different mouse strains, tumors, tumor sites  
9 and thrombosis models (40). As a result, it seems that the importance of MVs and the  
10 mechanisms involved may vary according to the cancer origin (57). Consequently, the  
11 relevance of measuring MVs as a biomarker of CAT may similarly vary according to the  
12 cancer origin. Moreover, the exact nature of what should be measured on these MVs may also  
13 not be restricted to TF.

14 Pancreatic cancer was the most studied, due to its high rate of CAT (58). The role of  
15 pancreatic cancer-derived MVs in thrombosis has been first studied with the model of PancO2  
16 pancreatic tumor cell lines. Using the ferric chloride mesentery vessel model and an ectopic  
17 pancreatic cancer mice model, a first study demonstrated that MVs released by PancO2 cell  
18 are detectable in mice peripheral circulation and accumulated in developing thrombus.  
19 Thrombus formation was reduced by inhibition of P selectin suggesting a P-selectin / PSGL-1  
20 interaction may be involved (59). In this model, thrombus growth induced by both  
21 exogeneously infused tumor-MVs and endogenous MVs, was prevented by inhibition of  
22 platelet activation by clopidrogrel and by blocking  $\alpha v\beta 1/\beta 3$  integrins, suggesting that tumor-  
23 MVs bind to the thrombus on activated platelets in an integrin-dependent manner (60).  
24 Injection of the PancO2 derived MVs in the inferior vena cava (IVC) stenosis models

1 significantly increased the number of mice developing a thrombus but also the thrombus size  
2 (61). Interestingly, when MVs with low TF were injected, significantly less mice developed a  
3 thrombus, showing that TF from tumoral MVs is essential to their thrombus promoting  
4 activity. It was also recently shown that infusion of tumor-MVs resulted in the activation of  
5 platelets both *in vitro* and *in vivo* as suggested by the accumulation of platelets in the lung  
6 (62). Thus TF+MVs enhance VTE both by increasing fibrin formation but also by activating  
7 platelets. The direct demonstration of the role of TF on MVs originating from the tumor cell  
8 itself has recently been confirmed in a recent study performed with human pancreatic BxPc-3  
9 grown orthotopically in nude mice (Figure 1A). In the IVC stenosis model, inhibition of  
10 human TF reduced the clot size in tumor-bearing mice but did not affect clot size in control  
11 mice. These results demonstrate that TF+MVs released from pancreatic tumors are  
12 responsible for increased clot size in tumor bearing mice (63). Interestingly, inflammation is a  
13 further putative pathway that could provide the trigger driving the procoagulant response with  
14 a particular relevance for the development of VTE in pancreatic cancer. In this model, high  
15 levels of TF+MVs are present and increased leukocyte infiltration contributes to promotes TF  
16 pathway and proinflammatory effects. Anticoagulant mechanisms are overwhelmed by the  
17 high levels of TF which accumulate in endothelial cells (ECs) and TF is recycled to the EV  
18 surfaces with PS, resulting in apoptosis, junctional weakening and subsequent endothelial  
19 denudation, resulting in activation of the extrinsic pathway via Xa activation TF+MVs in the  
20 context of acute inflammation (64). Beside TF pathway, another alternative novel pathway  
21 was also recently reported, using an IVC flow restricted model and strains of genetically  
22 engineered mice lacking GPIb or expressing reduced levels of TF (65). The mechanisms are  
23 independent of platelets and myeloid cells and involves the synergistic activation of  
24 coagulation by pancreatic MVs and host TF. A major contribution of the procoagulant activity  
25 of pancreatic MVs was provided by phosphatidylethanolamine (PE) externalization which

1 supported robust generation of Xa (Figure 1C). Disrupting PE dependent activation of factor  
2 X suppressed pancreatic MVs induced DVT without causing changes in hemostasis (65). In  
3 terms of therapy, this study confirms the observation that low molecular weight heparins are  
4 effective for prevention and treatment of CAT (66,67) but also suggests that oral factor Xa  
5 inhibitors may also be even more effective and tolerated (68).

6 In other cancers, TF is not necessary the major pathway by which MVs can promote  
7 VTE. For example, in prostate cancer, a recent work using mice knock-out for FXII, FXI and  
8 kininogen showed that these mice were protected against pulmonary embolism by the  
9 infusion of prostate cancer-derived MVs (69). These tumor-MVs also exhibit polyphosphates  
10 which suggests that tumor generated polyphosphate-rich MVs may activate the contact  
11 pathway (Figure 1B), favoring the thrombus formation (70). In brain cancers, platelet  
12 aggregation is a mechanism by which brain tumors may release podoplanin (PDPN) positive  
13 MVs in the circulation, that could activate circulating platelets (Figure 1D), resulting in  
14 increased VTE in patients with brain cancer (71–73). Additionally, mammary carcinoma  
15 derived EVs induce the formation of NETs which may represent another mechanism linking  
16 MVs and thrombosis (74). Thus according to the type of cancer, TF-independent mechanisms  
17 associated with MVs can also be involved in CAT and thus represent a potential cause  
18 explaining why an association between the biomarker TF+MV and CAT was not always  
19 found.

20

### 21 **A more complex role of MVs in hemostasis**

22 Beside their well-documented procoagulant activity, MVs vectorize anticoagulant and  
23 fibrinolytic molecules which may balance the resulting effect of MVs in the hemostasis  
24 equilibrium (Figure 2).

1           Anticoagulant molecules have been described at the surface of MVs originating of  
2 cells which may be activated in the tumoral environment such as platelets, leukocytes and  
3 endothelial cells. Anticoagulant proteins S and C, as others vitamin K-dependent molecules,  
4 can bind to the MVs surface on anionic PS residues and directly inhibit the procoagulant  
5 activity of platelet MVs (75–77). The endothelial protein C receptor (EPCR) (78) and  
6 thrombomodulin (79) have been reported not only on endothelial-derived MVs generated in  
7 vitro but also among circulating MVs in patients (80,81). Regulatory molecules such as TFPI  
8 (TF pathway inhibitor) was detected on leukocytes and endothelial MVs (82). Thus, the  
9 thrombogenicity of MVs depends on a balance between the pro- and anti-coagulant molecules  
10 which may vary among various disease conditions. For instance, the TF/TFPI balance of MVs  
11 has been evaluated in 26 patients with cancer from various origins compared to 92 healthy  
12 control volunteers. As a result, the ratio of TF/TFPI MVs was significantly increased in the  
13 cancer patients due to a significant decrease of the percentage of TFPI+ MVs (83). The same  
14 team also recently showed that this balance is modified by chemotherapy drugs. Indeed, a  
15 significant increase in the circulating MVs TF/TFPI ratio was found after the first 24 hours of  
16 doxorubicin treatment (84). Consistently, tumoral MVs derived from MDA-MB-231 cells  
17 stimulated with high-dose doxorubicin display a strong decrease in the TFPI levels resulting  
18 in a six-fold increase in their procoagulant activity (82). Interestingly, in these studies, the  
19 critical variable was the level of the anticoagulant molecule on the MVs surface. These results  
20 stress/emphasize the importance of taking into account both the procoagulant and the  
21 anticoagulant molecules on the MVs surface to better reflect their global effect. In practice,  
22 the presence of these anticoagulant molecules such as TPFPI may impact in the resulting MVs  
23 procoagulant activity measured by functional assays. Indeed, the inhibition of TFPI increases  
24 their sensitivity to procoagulant MVs (85,86). However, how the balance of TF/TFPI on

1 circulating MVs is modified by the pre-analytical and analytical protocols remains to be  
2 clarified.

3         The role of MVs in hemostasis is further complicated by the presence of fibrinolytic  
4 activity. Initially, proteolytic molecules such as metalloproteinases and the  
5 urokinase(uPA)/uPA receptor have been reported on MVs from tumoral cell lines in vitro  
6 (87–89) and from ascites from patients with ovarian cancers (90–92). Tissue-type  
7 plasminogen activator (t-PA) was also identified on MVs derived from a prostate cancer cell  
8 line (93). This tumoral MV proteolytic activity was first associated with an increased cellular  
9 invasiveness and metastasis (94–96). It was shown that hsp90alpha is secreted in small EV  
10 and interacts with tPA and its receptor annexin II in invasive cancer cells and contributes to  
11 their invasive nature by enhancing the conversion of plasminogen to active plasmin (97).  
12 Finally, the complete panel of molecules allowing MVs to efficiently generate plasmin and to  
13 vectorize a fibrinolytic activity was found to be present at their surface. This was first  
14 demonstrated on MVs from endothelial origin in vitro (98,99) and extended to leucocyte MVs  
15 (100). The plasmin generation capacity involves the binding of plasminogen on a specific  
16 receptor such as alpha-enolase and its activators such as uPA or tPA on receptors specific to  
17 surface of the MVs (101). Interestingly, this MV-dependent activity was found on circulating  
18 MVs of patients including cancer patients with acute promyelocytic leukemia (100,102). The  
19 fibrinolytic potential varies according to the cellular origin and the vesicular subsets.  
20 Comparison of the thrombotic and fibrinolytic activity of exosomes and MVs from several  
21 tumor cell lines showed that exosomes and/or MVs from most cell lines (MCF7, NB4, MDA-  
22 MB-231) with the exception of the A549 cell line, displayed fibrinolytic activity toward a  
23 pure fibrin clot, while only exosomes from MDA-MB-231 cells could degrade a fibrin clot  
24 formed in plasma (103). Interestingly, this fibrinolytic potential could be transferred from cell

1 to cell by exosomes. Indeed, the incubation of MDA-MB-231 cell-derived exosomes with  
2 A549 cells increased plasmin generation by these cells (103).

3 Plasmin generation is a highly regulated process controlled by a balance between  
4 activators and inhibitors. The plasminogen activator inhibitor (PAI-1) has been detected on  
5 tumoral MVs surface. MVs derived from leukemic mast cells inhibit the process of  
6 fibrinolysis by increasing the expression and secretion of PAI-1 (104). It has been also  
7 recently shown that the active oncogene EGFRvIII increased the concentration of TF and  
8 PAI-1 in prostate cancer derived MVs increasing their procoagulant activity (105).

9 Presence of both procoagulant and profibrinolytic activity at the surface of MVs lead  
10 us to propose that the resulting effect on bleeding or thrombotic events will be oriented as a  
11 function of the balance between the MV-dependent procoagulant and fibrinolytic activities  
12 (106,107). This concept of the coagulo-fibrinolytic balance of MVs was recently evaluated in  
13 the context of the fibrinolytic imbalance associated with septic shock. In a preliminary study,  
14 we showed, that the MVs fibrinolytic activity was significantly higher in septic shock patients  
15 who survived compared to those who died and that this MV-dependent activity was inversely  
16 correlated with multiorgan failure scores and ischemic markers (108). However, to what  
17 extent the fibrinolytic side may counterbalance the procoagulant side and play a significant  
18 role in the cancer-associated thrombosis pathophysiology is still largely unknown. Similarly,  
19 whether the coagulo-fibrinolytic balance of MVs is a better predictor of the occurrence of  
20 thrombosis in cancer patients rather their procoagulant activity alone, remains to be evaluated  
21 with suitable methodologies.

22

23 **Methodological considerations**

1           The reported limited performance of MVs as biomarker to accurately predict the risk  
2 of thrombosis in cancer patients, may also originate from methodological issues.

3           Pre-analytics remains an important source of artifacts. Recommendations have been  
4 made but they are not always easy to apply in clinical studies (109,110). Moreover, several  
5 functional assays require MV isolation from the body fluids before performing the test. For  
6 many years, ultracentrifugation has been used. However, the centrifugation step significantly  
7 decreases the reproducibility of the assays and may impede inter-laboratory comparisons.  
8 Immunocapture on magnetic beads may represent a useful alternative (108).

9           The two main approaches which have been applied to evaluate the potential of MVs  
10 biomarker of cancer associated thrombosis are: 1) the antigenic detection of procoagulant  
11 molecules at the surface of MVs (111), or 2) the functional capacity of MVs to generate factor  
12 Xa, thrombin or a clot. Combined strategies have also been developed (112) (Table 2).

13           Among antigenic assays, flow cytometry (FCM) was used for the detection of  
14 procoagulant molecules such as PS with annexin V or lactadherin and TF with specific  
15 antibodies. Over the past few years, significant improvements have been made regarding the  
16 sensitivity of FCM to detect single vesicles with a diameter of < 300 nm which have further  
17 established this methodology as the most promising tool for routine enumeration of EV  
18 subsets (113,114). However, the lack of sensitivity of the current flow cytometers to  
19 characterize small MVs and to detect small amount of molecules at the MVs surface still  
20 impede this evaluation. Moreover, most FCM studies did not take into account the presence of  
21 other active PLs, such as PE or the presence of physiological inhibitors regulating the  
22 procoagulant activity such as TFPI. As an interesting alternative, we measured fibrin+  
23 (positive) MVs by FCM in pancreatic and colorectal cancers. The hypothesis was that as  
24 fibrin is the final product of the coagulation cascade, the level of fibrin+MV s may directly  
25 reflect the activation state of the blood coagulation cascade. As a result, fibrin+MV s were

1 significantly increased in patients with thrombosis (39). Other antigenic methods have been  
2 recently proposed such as the detection of TF+MVs by scanning confocal microscopy (115).

3 Regarding functional tests, a first group of methods focuses on the PS-dependent  
4 procoagulant activity of MV:

5 1. PS contribution can be evaluated measuring a PL-dependent coagulation time after  
6 dilution in a PL-depleted plasma and activation with factor Xa (FXa) and calcium.  
7 Thus, the only PLs present in the reaction mixture are provided by the patient sample.  
8 This test, initially described by Exner et al. (116), is commercially available (XaCT  
9 test, Haematex, Australia; STA-Procoag-PPL, Stago, Paris, France) and can be fully  
10 automated on routine hemostasis instruments.

11 2. Other assays combine solid-phase capture of MVs on annexin V and thrombin  
12 generation (Zymuphen-MP ELISA kit, Hyphen Biomed, Neuville-sur-Oise, France)  
13 (117). The generation of thrombin after adding prothrombin, FXa, FVa and calcium, is  
14 measured via cleavage of a chromogenic thrombin substrate producing absorbance at  
15 405 nm. A standard curve of known MVs concentration is used to convert the  
16 absorbance into nM PS. This test may be adapted to quantify different subsets of MVs  
17 substituting annexin V by various capture antibodies. However, the sensitivity of these  
18 methods is limited by a heterogeneous and passive sedimentation of MVs and a  
19 limited contact surface.

20 3. The Calibrated Automated Thrombogram assay was originally developed by Hemker  
21 et al. for measuring thrombin generation in platelet-rich or platelet-poor plasma in a  
22 variety of clinical settings (Calibrated Automated Thrombogram, Thrombinoscope  
23 BV, Maastricht, NL and Stago) (118). To evaluate the PS contribution of MVs,  
24 thrombin generation is initiated by the addition of a reagent containing tissue factor  
25 and a minimal amount of PLs (platelet poor plasma reagent). Thrombin generation is



1 monitored with a thrombin fluorogenic substrate. The use of a thrombin calibrator for  
2 each individual plasma corrects for donor-to-donor differences in color of plasma,  
3 inner filter effects and substrate consumption. This assay is sensitive to the contact  
4 phase activation. Alternatively, plasmatic MVs can be pelleted, washed and re-  
5 suspended in MV-free plasma. The reproducibility of the measurement will decrease,  
6 but the measurement of thrombin generation will not be affected by soluble factors,  
7 the presence of anticoagulants or a coagulopathy.

8 A second group of assays focuses on the measurement of TF-dependent procoagulant  
9 activity of MVs :

- 10 1. The calibrated automated thrombogram assay can be modified to be more sensitive to  
11 TF. Thrombin generation in platelet free plasma or purified MVs spiked in MV-free  
12 plasma is initiated in presence of a "MP reagent" which contains only PLs and no TF.  
13 This assay is sensitive to the contact phase activation. Even if this test is more  
14 sensitive than the Zymuphen-TF (described below) in a *Neisseria meningitidis*-  
15 stimulated whole blood model (119), high concentrations of TF+MV are necessary to  
16 be detectable probably because of a masking effect due to the high concentration of  
17 PLs added in the test.
- 18 2. Most of the studies evaluating the potential interest of MVs as a biomarker of the  
19 cancer-associated thrombosis have measured procoagulant MVs with FXa generation  
20 assays as a read-out of TF-FVIIa activity (120). These assays have been performed  
21 using either antibody-mediated MVs captured on coated plate or isolation of the MVs  
22 by high-speed centrifugation. In the commercially available assay (Zymuphen-TF,  
23 Hyphen Biomed), MVs are captured using an anti-TF antibody which does not  
24 interfere with TF activity. Unfortunately, no anti-TF inhibitor antibody is incorporated  
25 during the measurement of FXa. It was found that this assay has reduced specificity

1 and sensitivity compared with an “in-house” assay that isolates MVs using  
2 centrifugation (121). Two slightly different "in house" assays have been reported for  
3 the measurement of TF-MVs activity after isolation of MVs by high speed  
4 centrifugation. MVs are washed then resuspended in buffer before measuring FXa.  
5 Both assays use commercially available Dade thromboplastin reagent Innovin as a  
6 standard. FVII is added with synthetic PLs in the "kinetic assays" (42) whereas FVIIa  
7 is used without adding PLs in the endpoint assay (46). A moderate correlation was  
8 found ( $r=0.61$ ,  $P<0.001$ ) between the two assays in 54 pancreatic cancer patients (54).  
9 Several ongoing developments are improving the sensitivity of these assays (122,123).  
10 Alternatively, a more global assay has been proposed monitoring the fibrin generation  
11 (FGT; fibrin generation test) after incubating purified MVs by high speed centrifugation in a  
12 plasma in presence of anti-TF or anti-FXII blocking antibodies (124,125). Clotting is initiated  
13 by addition of calcium and fibrin formation is monitored by kinetic measurement of the  
14 optical density at 405 nm.

15 Data comparing all these methods to measure the procoagulant potential of MVs are  
16 lacking. Moreover, FCM is the only method which benefits from a standardization effort so  
17 far which lead to different tools to reduce the inter-laboratory variability (123,124). This work  
18 is currently ongoing in a collaborative workshop on the standardization of EVs combining the  
19 experts from three international societies: International Society on Extracellular Vesicles  
20 (ISEV), International Society on Advancement of Cytometry (ISAC), and the International  
21 Society on Thrombosis and Haemostasis (ISTH). However, development of international  
22 standards is still needed to compare the results in the field. In a timely manner a new ISTH  
23 workshop, comparing the sensitivity and the specificity of assays to measure TF-MV in  
24 plasma samples will start this year. Results of these studies will certainly provide important

1 information to progress toward the optimal methods to measure MVs and standardization  
2 tools mandatory for robust multicenter studies in the cancer associated thrombosis field.

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## 5 **Conclusions**

6 Up to now, several issues explain why MVs are not systemically found associated with CAT,  
7 including the diversity of mechanisms associating MVs and different types of cancer, the  
8 complex role of MVs in hemostasis integrating their anticoagulant and fibrinolytic activity  
9 and the limited sensitivity, reproducibility and standardization of current methodologies  
10 allowing to measure MVs. Continuation of the technical development for measuring MVs,  
11 standardization of methods in combination with efforts to define the most interesting  
12 antigenic or functional target to be measured on MVs in different clinical settings are issues  
13 that need to be addressed during the coming years. In addition, modulating MVs with  
14 treatment, and the benefits of regulating MVs in the prevention of future cardiovascular  
15 events are to be tested in prospective large randomized trials. Altogether, the rapidly growing  
16 knowledge on the biology of EV combined with the many technological advances set high  
17 expectations for future clinical applications of MVs as a predictive biomarker for CAT.

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Patients	Wo VTE / W VTE	Follow-up	MV phenotype	Method	Reference
Locally advanced or metastatic pancreatic cancer	11/02	Every 4 weeks for 20 weeks	TF + MV	Functional assay	Khorana, JTH, 2008 (46)
Cancer Wo VTE	60/5	1 year	TF + MV	Impedance FMC	Zwicker, Clin. Cancer Res., 2009 (43)
Solid and hematological cancer	728/53	2 years	PS + MV	Functional assay	Thaler, Ann. Hematol., 2011 (53)
Cancer Wo VTE	299 (48 pancreatic) / 49 (12 pancreatic)	2 years	TF + MV	Functional assay (chromogenic end point and kinetic)	Thaler, JTH, 2012 (54)
Cancer Wo VTE at study entry	43/5	6 months	Annexin V + MV	FCM	Van Doormaal, Thromb. Haemost., 2012 (47)
Pancreatic Cancer	252/40	10 months	TF + MV	FCM	Hernandez, Thromb. Haemostat., 2013 (55)
Cancer Wo VTE at study entry	43/5	6 months	TF + MV	Functional assay	Van Doormaal, Thromb. Haemost., 2012 (47)
Glioblastoma multiforme	61/11	7 months	TF + MV	FCM	Sartori, Thromb. Haemost., 2013 (48)
Pancreatobiliary cancer	117/52	Not specified	TF + MV	Functional assay	Bharthuar, Thromb. Res., 2013 (49)
Pancreatic cancer	65/11	6months – 1 year	TF + MV	Functional assay	Woei-A-Jin, B J Cancer, 2016 (50)
Cancer Wo VTE	608 (90 pancreatic) / 40 (10 pancreatic)	180 days	TF + MV	Functional assay (Fibrin generation and FXa generation)	Van Es, Thromb. Res., 2018 (51)
Pancreatic cancer	41/12	1 year	TF + MV	Functional assay	Faille, Oncotarget, 2018 (52)

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9 **Table 1** : Role of MVs as prognostic biomarkers of VTE in cancer patients. FCM: Flow-

10 cytometry; FXa: Activated factor X; MV: Microvesicle; PS: Phosphatidylserine; TF: Tissue

1 factor; VTE: Venous thromboembolism; Wo/W: Without/With. White lines represent studies  
 2 no associations found between cancer and VTE and grey lines represent studies with a  
 3 significant association between cancer and VTE.

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Assays	Strategy	Target	Method	Preanalytics	EV commercial kit	Reference
Flow cytometry	Antigenic	PS, TF, TF/TFPI	Immunofluorimetry	PFP	No	Poncelet et al Thromb Res 2015 (113), Nolan et al Platelets 2017 (114)
Flow cytometry	Antigenic	Fibrin	Immunofluorimetry	PFP	No	Mege et al Oncotarget 2017 (39)
Scanning confocal microscopy	Antigenic	TF	Immunofluorimetry	PFP / Centrifugation	No	Hisada et al Thromb Res 2017 (115)
Calibrated Automated Thrombogram	Functional	PS or TF	Thrombin generation	PFP	Yes	Hemker et al, Pathophysiol. Haemost. Thromb. 2002 (118)
STA-Procoag PPL	Functional	PS	Coagulation time	PFP	Yes	Exner et al, Blood Coagul. Fibrinolysis, 2003 (116)
Zymuphen-MP ELISA Kit	Combined	PS	Thrombin generation	Immunocapture (PS) on PFP	Yes	Laroche et al Platelets 2017 (112)
Zymuphen-MP TF	Combined	TF	FXa generation	Immunocapture (PS) on PFP	Yes	Laroche et al Platelets 2017 (112)
Factor Xa generation assays	Functional	TF	FXa generation	Centrifugation	No	Khorana et al, JTH, 2008 (46), Woei-A-Jin, B J Cancer, 2016 (50)
Fibrin generation assay	Functional	PS + TF	Fibrin generation	Centrifugation	No	Berckmans et al, Blood, 2011(125)

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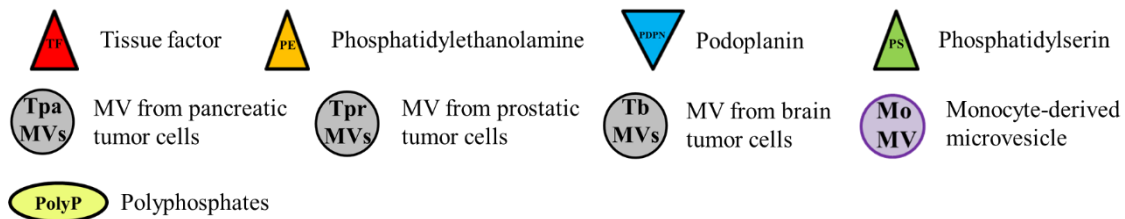
9 **Table 2:** Procoagulant assay. Different strategies were used: antigenic assay using flow  
 10 cytometry, functional assay using coagulation time, factor Xa (FXa) generation assay,  
 11 thrombin generation assay or fibrin generation assay. PFP: Platelet Free Plasma; PS:  
 12 Phosphatidylserin; TF: Tissue factor; TFPI: TF pathway inhibitor.

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**Figure 1: Diversity of mechanisms involving MVs in cancer associated thrombosis.**

MoMVs and MVs originating from the tumor cell itself rapidly accumulate at the site injury, increase fibrin formation and activating platelets(A). Tumor-MVs also exhibit polyphosphates that may activate the contact pathway (B). Pancreatic MVs support robust generation of Xa provided by phosphatidylethanolamine externalization (C). Brain tumors may release Podoplanin (PDPN)-MVVs that activate circulating platelets (D). High levels of TF which accumulate in ECs and is recycled to the ECs surfaces (E).

**Legend**



**Figure 2: Prothrombotic and antithrombotic molecular pattern of MVs.** MVs are initially

described to bear procoagulant molecules like phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), tissue factor (TF) and polyphosphates. Anticoagulant molecules have been more recently described at the surface of MVs like TFPI, thrombomodulin (TM) that activate proteins C, its cofactor protein S and the receptor EPCR. The role of MVs in hemostasis was complicated by the presence of proteins of the fibrinolytic system : the tissue-type plasminogen activator (tPA) and urokinase (uPA) and its receptor uPAR and their principal inhibitor (PAI-1). MVs can also carry directly plasminogen on its receptors such as  $\alpha$ -enolase.

**Bibliography**

- 1 1. van der Pol E, Böing AN, Harrison P, et al. Classification, functions, and clinical relevance of  
2 extracellular vesicles. *Pharmacol Rev* 2012; 64: 676–705.
- 3 2. Boulanger CM, Loyer X, Rautou P-E, et al. Extracellular vesicles in coronary artery disease. *Nat*  
4 *Rev Cardiol* 2017; 14: 259–72.
- 5 3. György B, Szabó TG, Pásztói M, et al. Membrane vesicles, current state-of-the-art: emerging  
6 role of extracellular vesicles. *Cell Mol Life Sci CMLS* 2011; 68: 2667–88.
- 7 4. Ciardiello C, Cavallini L, Spinelli C, et al. Focus on Extracellular Vesicles: New Frontiers of Cell-  
8 to-Cell Communication in Cancer. *Int J Mol Sci* 2016; 17: 175.
- 9 5. Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler*  
10 *Thromb Vasc Biol* 2011; 31: 27–33.
- 11 6. Ridger VC, Boulanger CM, Angelillo-Scherrer A, et al. Microvesicles in vascular homeostasis  
12 and diseases. *Thromb Haemost* 2017; 117: 1296–316.
- 13 7. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat*  
14 *Rev Immunol* 2013; 13: 34–45.
- 15 8. Suzuki J, Umeda M, Sims PJ, et al. Calcium-dependent phospholipid scrambling by TMEM16F.  
16 *Nature* 2010; 468: 834–8.
- 17 9. Lhermusier T, Chap H, Payrastre B. Platelet membrane phospholipid asymmetry: from the  
18 characterization of a scramblase activity to the identification of an essential protein mutated  
19 in Scott syndrome. *J Thromb Haemost JTH* 2011; 9: 1883–91.
- 20 10. Dvorak HF, Quay SC, Orenstein NS, et al. Tumor shedding and coagulation. *Science* 1981; 212:  
21 923–4.
- 22 11. Dvorak HF, Van DeWater L, Bitzer AM, et al. Procoagulant activity associated with plasma  
23 membrane vesicles shed by cultured tumor cells. *Cancer Res* 1983; 43: 4434–42.
- 24 12. Collier MEW, Ettelaie C. Regulation of the incorporation of tissue factor into microparticles by  
25 serine phosphorylation of the cytoplasmic domain of tissue factor. *J Biol Chem* 2011; 286:  
26 11977–84.
- 27 13. Collier MEW, Maraveyas A, Ettelaie C. Filamin-A is required for the incorporation of tissue  
28 factor into cell-derived microvesicles. *Thromb Haemost* 2014; 111: 647–55.
- 29 14. Rothmeier AS, Marchese P, Petrich BG, et al. Caspase-1-mediated pathway promotes  
30 generation of thromboinflammatory microparticles. *J Clin Invest* 2015; 125: 1471–84.
- 31 15. Ettelaie C, Collier MEW, Featherby S, et al. Oligoubiquitination of tissue factor on Lys255  
32 promotes Ser253-dephosphorylation and terminates TF release. *Biochim Biophys Acta* 2016;  
33 1863: 2846–57.
- 34 16. Rothmeier AS, Marchese P, Langer F, et al. Tissue Factor Prothrombotic Activity Is Regulated  
35 by Integrin-arf6 Trafficking. *Arterioscler Thromb Vasc Biol* 2017; 37: 1323–31.
- 36 17. Sabatier F, Roux V, Anfosso F, et al. Interaction of endothelial microparticles with monocytic  
37 cells in vitro induces tissue factor-dependent procoagulant activity. *Blood* 2002; 99: 3962–70.

- 1 18. Meziani F, Delabranche X, Asfar P, et al. Bench-to-bedside review: circulating microparticles--a  
2 new player in sepsis? *Crit Care Lond Engl* 2010; 14: 236.
- 3 19. Del Conde I, Shrimpton CN, Thiagarajan P, et al. Tissue-factor-bearing microvesicles arise from  
4 lipid rafts and fuse with activated platelets to initiate coagulation. *Blood* 2005; 106: 1604–11.
- 5 20. Collier MEW, Mah P-M, Xiao Y, et al. Microparticle-associated tissue factor is recycled by  
6 endothelial cells resulting in enhanced surface tissue factor activity. *Thromb Haemost* 2013;  
7 110: 966–76.
- 8 21. Cointe S, Lacroix R, Dignat-George F. Platelet-Derived Microparticles. *Platelets Thromb Non-  
9 Thromb Disord* 2017.
- 10 22. Mazurov A, Antonova O, Golubeva, N. Procoagulant Properties of Microparticles Produced by  
11 Monocytes, Granulocytes, Platelets and Endothelial Cells. *Res Pr Thromb Haemost Berlin*;  
12 2017; 1:443-444.
- 13 23. Tripisciano C, Weiss R, Eichhorn T, et al. Different Potential of Extracellular Vesicles to Support  
14 Thrombin Generation: Contributions of Phosphatidylserine, Tissue Factor, and Cellular Origin.  
15 *Sci Rep* 2017; 7: 6522.
- 16 24. Van Der Meijden PEJ, Van Schilfgaarde M, Van Oerle R, et al. Platelet- and erythrocyte-derived  
17 microparticles trigger thrombin generation via factor XIIa. *J Thromb Haemost JTH* 2012; 10:  
18 1355–62.
- 19 25. Rubin O, Delobel J, Prudent M, et al. Red blood cell-derived microparticles isolated from blood  
20 units initiate and propagate thrombin generation. *Transfusion (Paris)* 2013; 53: 1744–54.
- 21 26. Angelillo-Scherrer A. Leukocyte-derived microparticles in vascular homeostasis. *Circ Res* 2012;  
22 110: 356–69.
- 23 27. Halim ATA, Ariffin NAFM, Azlan M. Review: the Multiple Roles of Monocytic Microparticles.  
24 *Inflammation* 2016; 39: 1277–84.
- 25 28. Stojkovic S, Thulin Å, Hell L, et al. IL-33 stimulates the release of procoagulant microvesicles  
26 from human monocytes and differentially increases tissue factor in human monocyte subsets.  
27 *Thromb Haemost* 2017; 117: 1379–90.
- 28 29. Kambas K, Chrysanthopoulou A, Vassilopoulos D, et al. Tissue factor expression in neutrophil  
29 extracellular traps and neutrophil derived microparticles in antineutrophil cytoplasmic  
30 antibody associated vasculitis may promote thromboinflammation and the thrombophilic  
31 state associated with the disease. *Ann Rheum Dis* 2014; 73: 1854–63.
- 32 30. Egorina EM, Sovershaev MA, Olsen JO, et al. Granulocytes do not express but acquire  
33 monocyte-derived tissue factor in whole blood: evidence for a direct transfer. *Blood* 2008;  
34 111: 1208–16.
- 35 31. Geddings JE, Mackman N. Tumor-derived tissue factor-positive microparticles and venous  
36 thrombosis in cancer patients. *Blood* 2013; 122: 1873–80.
- 37 32. Ettelaie C, Collier ME, Featherby S, et al. Analysis of the potential of cancer cell lines to release  
38 tissue factor-containing microvesicles: correlation with tissue factor and PAR2 expression.  
39 *Thromb J* 2016; 14: 2.



- 1 33. Gomes FG, Sandim V, Almeida VH, et al. Breast-cancer extracellular vesicles induce platelet  
2 activation and aggregation by tissue factor-independent and -dependent mechanisms.  
3 *Thromb Res* 2017; 159: 24–32.
- 4 34. Falati S, Liu Q, Gross P, et al. Accumulation of tissue factor into developing thrombi in vivo is  
5 dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *J Exp*  
6 *Med* 2003; 197: 1585–98.
- 7 35. Chou J, Mackman N, Merrill-Skoloff G, et al. Hematopoietic cell-derived microparticle tissue  
8 factor contributes to fibrin formation during thrombus propagation. *Blood* 2004; 104: 3190–7.
- 9 36. Owens AP, Mackman N. Microparticles in hemostasis and thrombosis. *Circ Res* 2011; 108:  
10 1284–97.
- 11 37. Zwicker JI, Trenor CC, Furie BC, et al. Tissue factor-bearing microparticles and thrombus  
12 formation. *Arterioscler Thromb Vasc Biol* 2011; 31: 728–33.
- 13 38. Reitsma PH, Versteeg HH, Middeldorp S. Mechanistic view of risk factors for venous  
14 thromboembolism. *Arterioscler Thromb Vasc Biol* 2012; 32: 563–8.
- 15 39. Mege D, Crescence L, Ouaisi M, et al. Fibrin-bearing microparticles: marker of thrombo-  
16 embolic events in pancreatic and colorectal cancers. *Oncotarget* 2017; 8: 97394–406.
- 17 40. Hisada Y, Mackman N. Mouse models of cancer-associated thrombosis. *Thromb Res* 2018; 164  
18 Suppl 1: S48–53.
- 19 41. Sørensen HT, Mellekjaer L, Olsen JH, et al. Prognosis of cancers associated with venous  
20 thromboembolism. *N Engl J Med* 2000; 343: 1846–50.
- 21 42. Tesselaar MET, Romijn FPHTM, Van Der Linden IK, et al. Microparticle-associated tissue factor  
22 activity: a link between cancer and thrombosis? *J Thromb Haemost* 2007; 5: 520–7.
- 23 43. Zwicker JI, Liebman HA, Neuberg D, et al. Tumor-derived tissue factor-bearing microparticles  
24 are associated with venous thromboembolic events in malignancy. *Clin Cancer Res Off J Am*  
25 *Assoc Cancer Res* 2009; 15: 6830–40.
- 26 44. Manly DA, Wang J, Glover SL, et al. Increased microparticle tissue factor activity in cancer  
27 patients with Venous Thromboembolism. *Thromb Res* 2010; 125: 511–2.
- 28 45. Campello E, Spiezia L, Radu CM, et al. Endothelial, platelet, and tissue factor-bearing  
29 microparticles in cancer patients with and without venous thromboembolism. *Thromb Res*  
30 2011; 127: 473–7.
- 31 46. Khorana AA, Francis CW, Menzies KE, et al. Plasma tissue factor may be predictive of venous  
32 thromboembolism in pancreatic cancer. *J Thromb Haemost* 2008; 6: 1983–5.
- 33 47. van Doormaal F, Kleinjan A, Berckmans RJ, et al. Coagulation activation and microparticle-  
34 associated coagulant activity in cancer patients. An exploratory prospective study. *Thromb*  
35 *Haemost* 2012; 108: 160–5.
- 36 48. Sartori MT, Della Puppa A, Ballin A, et al. Circulating microparticles of glial origin and tissue  
37 factor bearing in high-grade glioma: a potential prothrombotic role. *Thromb Haemost* 2013;  
38 110: 378–85.

- 1 49. Bharthuar A, Khorana AA, Hutson A, et al. Circulating microparticle tissue factor,  
2 thromboembolism and survival in pancreaticobiliary cancers. *Thromb Res* 2013; 132: 180–4.
- 3 50. Woei-A-Jin FJSH, Tesselaar MET, Garcia Rodriguez P, et al. Tissue factor-bearing microparticles  
4 and CA19.9: two players in pancreatic cancer-associated thrombosis? *Br J Cancer* 2016; 115:  
5 332–8.
- 6 51. van Es N, Hisada Y, Di Nisio M, et al. Extracellular vesicles exposing tissue factor for the  
7 prediction of venous thromboembolism in patients with cancer: A prospective cohort study.  
8 *Thromb Res* 2018; 166: 54–9.
- 9 52. Faille D, Bourrienne M-C, de Raucourt E, et al. Biomarkers for the risk of thrombosis in  
10 pancreatic adenocarcinoma are related to cancer process. *Oncotarget* 2018; 9: 26453–65.
- 11 53. Thaler J, Ay C, Weinstabl H, et al. Circulating procoagulant microparticles in cancer patients.  
12 *Ann Hematol* 2011; 90: 447–53.
- 13 54. Thaler J, Ay C, Mackman N, et al. Microparticle-associated tissue factor activity, venous  
14 thromboembolism and mortality in pancreatic, gastric, colorectal and brain cancer patients. *J*  
15 *Thromb Haemost* 2012; 10: 1363–70.
- 16 55. Hernández C, Orbe J, Roncal C, et al. Tissue factor expressed by microparticles is associated  
17 with mortality but not with thrombosis in cancer patients. *Thromb Haemost* 2013; 110: 598–  
18 608.
- 19 56. Cui C-J, Wang G-J, Yang S, et al. Tissue Factor-bearing MPs and the risk of venous thrombosis  
20 in cancer patients: A meta-analysis. *Sci Rep* 2018; 8: 1675.
- 21 57. Hisada Y, Mackman N. Cancer-associated pathways and biomarkers of venous thrombosis.  
22 *Blood* 2017; 130: 1499–506.
- 23 58. Mege D, Mezouar S, Dignat-George F, et al. Microparticles and cancer thrombosis in animal  
24 models. *Thromb Res* 2016; 140: 21–6.
- 25 59. Thomas GM, Panicot-Dubois L, Lacroix R, et al. Cancer cell-derived microparticles bearing P-  
26 selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. *J Exp Med* 2009; 206:  
27 1913–27.
- 28 60. Mezouar S, Darbousset R, Dignat-George F, et al. Inhibition of platelet activation prevents the  
29 P-selectin and integrin-dependent accumulation of cancer cell microparticles and reduces  
30 tumor growth and metastasis in vivo. *Int J Cancer* 2015; 136: 462–75.
- 31 61. Thomas GM, Brill A, Mezouar S, et al. Tissue factor expressed by circulating cancer cell-derived  
32 microparticles drastically increases the incidence of deep vein thrombosis in mice. *J Thromb*  
33 *Haemost* 2015; 13: 1310–9.
- 34 62. Geddings JE, Hisada Y, Boulaftali Y, et al. Tissue factor-positive tumor microvesicles activate  
35 platelets and enhance thrombosis in mice. *J Thromb Haemost* 2016; 14: 153–66.
- 36 63. Hisada Y, Ay C, Auriemma AC, et al. Human pancreatic tumors grown in mice release tissue  
37 factor-positive microvesicles that increase venous clot size. *J Thromb Haemost* 2017; 15:  
38 2208–17.

- 1 64. Date K, Ettelaie C, Maraveyas A. Tissue factor-bearing microparticles and inflammation: a  
2 potential mechanism for the development of venous thromboembolism in cancer. *J Thromb*  
3 *Haemost JTH* 2017; 15: 2289–99.
- 4 65. Stark K, Schubert I, Joshi U, et al. Distinct Pathogenesis of Pancreatic Cancer Microvesicle-  
5 Associated Venous Thrombosis Identifies New Antithrombotic Targets In Vivo. *Arterioscler*  
6 *Thromb Vasc Biol* 2018; 38: 772–86.
- 7 66. Lee AYY, Levine MN, Baker RI, et al. Low-molecular-weight heparin versus a coumarin for the  
8 prevention of recurrent venous thromboembolism in patients with cancer. *N Engl J Med* 2003;  
9 349: 146–53.
- 10 67. Lee AYY, Kamphuisen PW, Meyer G, et al. Tinzaparin vs Warfarin for Treatment of Acute  
11 Venous Thromboembolism in Patients With Active Cancer: A Randomized Clinical Trial. *JAMA*  
12 2015; 314: 677–86.
- 13 68. Streiff MB, Milentijevic D, McCrae K, et al. Effectiveness and safety of anticoagulants for the  
14 treatment of venous thromboembolism in patients with cancer. *Am J Hematol* 2018; 93: 664–  
15 71.
- 16 69. Nickel KF, Ronquist G, Langer F, et al. The polyphosphate-factor XII pathway drives  
17 coagulation in prostate cancer-associated thrombosis. *Blood* 2015; 126: 1379–89.
- 18 70. Nickel KF, Labberton L, Long AT, et al. The polyphosphate/factor XII pathway in cancer-  
19 associated thrombosis: novel perspectives for safe anticoagulation in patients with  
20 malignancies. *Thromb Res* 2016; 141 Suppl 2: S4-7.
- 21 71. Suzuki-Inoue K, Kato Y, Inoue O, et al. Involvement of the snake toxin receptor CLEC-2, in  
22 podoplanin-mediated platelet activation, by cancer cells. *J Biol Chem* 2007; 282: 25993–6001.
- 23 72. Thaler J, Koder S, Kornek G, et al. Microparticle-associated tissue factor activity in patients  
24 with metastatic pancreatic cancer and its effect on fibrin clot formation. *Transl Res J Lab Clin*  
25 *Med* 2014; 163: 145–50.
- 26 73. Riedl J, Preusser M, Nazari PMS, et al. Podoplanin expression in primary brain tumors induces  
27 platelet aggregation and increases risk of venous thromboembolism. *Blood* 2017; 129: 1831–  
28 9.
- 29 74. Leal AC, Mizurini DM, Gomes T, et al. Tumor-Derived Exosomes Induce the Formation of  
30 Neutrophil Extracellular Traps: Implications For The Establishment of Cancer-Associated  
31 Thrombosis. *Sci Rep* 2017; 7: 6438.
- 32 75. Tans G, Rosing J, Thomassen MC, et al. Comparison of anticoagulant and procoagulant  
33 activities of stimulated platelets and platelet-derived microparticles. *Blood* 1991; 77: 2641–8.
- 34 76. Dahlbäck B, Wiedmer T, Sims PJ. Binding of anticoagulant vitamin K-dependent protein S to  
35 platelet-derived microparticles. *Biochemistry* 1992; 31: 12769–77.
- 36 77. Stavenuiter F, Davis NF, Duan E, et al. Platelet protein S directly inhibits procoagulant activity  
37 on platelets and microparticles. *Thromb Haemost* 2013; 109: 229–37.
- 38 78. Pérez-Casal M, Downey C, Fukudome K, et al. Activated protein C induces the release of  
39 microparticle-associated endothelial protein C receptor. *Blood* 2005; 105: 1515–22.

- 1 79. Satta N, Freyssinet JM, Toti F. The significance of human monocyte thrombomodulin during  
2 membrane vesiculation and after stimulation by lipopolysaccharide. *Br J Haematol* 1997; 96:  
3 534–42.
- 4 80. Steppich B, Mattisek C, Sobczyk D, et al. Tissue factor pathway inhibitor on circulating  
5 microparticles in acute myocardial infarction. *Thromb Haemost* 2005; 93: 35–9.
- 6 81. Tsimerman G, Roguin A, Bachar A, et al. Involvement of microparticles in diabetic vascular  
7 complications. *Thromb Haemost* 2011; 106: 310–21.
- 8 82. Aharon A, Tamari T, Brenner B. Monocyte-derived microparticles and exosomes induce  
9 procoagulant and apoptotic effects on endothelial cells. *Thromb Haemost* 2008; 100: 878–85.
- 10 83. Aharon A, Katzenell S, Tamari T, et al. Microparticles bearing tissue factor and tissue factor  
11 pathway inhibitor in gestational vascular complications. *J Thromb Haemost* 2009; 7: 1047–50.
- 12 84. Aharon A, Sabbah A, Ben-Shaul S, et al. Chemotherapy administration to breast cancer  
13 patients affects extracellular vesicles thrombogenicity and function. *Oncotarget* 2017; 8:  
14 63265–80.
- 15 85. Gheldof D, Mullier F, Chatelain B, et al. Inhibition of tissue factor pathway inhibitor increases  
16 the sensitivity of thrombin generation assay to procoagulant microvesicles. *Blood Coagul  
17 Fibrinolysis Int J Haemost Thromb* 2013; 24: 567–72.
- 18 86. Hellum M, Franco-Lie I, Øvstebø R, et al. The effect of corn trypsin inhibitor, anti-tissue factor  
19 pathway inhibitor antibodies and phospholipids on microvesicle-associated thrombin  
20 generation in patients with pancreatic cancer and healthy controls. *PloS One* 2017; 12:  
21 e0184579.
- 22 87. Dolo V, Ginestra A, Ghersi G, et al. Human breast carcinoma cells cultured in the presence of  
23 serum shed membrane vesicles rich in gelatinolytic activities. *J Submicrosc Cytol Pathol* 1994;  
24 26: 173–80.
- 25 88. Ginestra A, Monea S, Seghezzi G, et al. Urokinase plasminogen activator and gelatinases are  
26 associated with membrane vesicles shed by human HT1080 fibrosarcoma cells. *J Biol Chem*  
27 1997; 272: 17216–22.
- 28 89. Dolo V, Ginestra A, Cassarà D, et al. Selective localization of matrix metalloproteinase 9, beta1  
29 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by  
30 8701-BC breast carcinoma cells. *Cancer Res* 1998; 58: 4468–74.
- 31 90. Dolo V, D'Ascenzo S, Violini S, et al. Matrix-degrading proteinases are shed in membrane  
32 vesicles by ovarian cancer cells in vivo and in vitro. *Clin Exp Metastasis* 1999; 17: 131–40.
- 33 91. Ginestra A, Miceli D, Dolo V, et al. Membrane vesicles in ovarian cancer fluids: a new potential  
34 marker. *Anticancer Res* 1999; 19: 3439–45.
- 35 92. Graves LE, Ariztia EV, Navari JR, et al. Proinvasive properties of ovarian cancer ascites-derived  
36 membrane vesicles. *Cancer Res* 2004; 64: 7045–9.
- 37 93. Millimaggi D, Festuccia C, Angelucci A, et al. Osteoblast-conditioned media stimulate  
38 membrane vesicle shedding in prostate cancer cells. *Int J Oncol* 2006; 28: 909–14.

- 1 94. Ginestra A, La Placa MD, Saladino F, et al. The amount and proteolytic content of vesicles shed  
2 by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer Res* 1998; 18:  
3 3433–7.
- 4 95. Angelucci A, D’Ascenzo S, Festuccia C, et al. Vesicle-associated urokinase plasminogen  
5 activator promotes invasion in prostate cancer cell lines. *Clin Exp Metastasis* 2000; 18: 163–  
6 70.
- 7 96. Jung T, Castellana D, Klingbeil P, et al. CD44v6 dependence of premetastatic niche preparation  
8 by exosomes. *Neoplasia N Y N* 2009; 11: 1093–105.
- 9 97. McCready J, Sims JD, Chan D, et al. Secretion of extracellular hsp90alpha via exosomes  
10 increases cancer cell motility: a role for plasminogen activation. *BMC Cancer* 2010; 10: 294.
- 11 98. Lacroix R, Sabatier F, Mialhe A, et al. Activation of plasminogen into plasmin at the surface of  
12 endothelial microparticles: a mechanism that modulates angiogenic properties of endothelial  
13 progenitor cells in vitro. *Blood* 2007; 110: 2432–9.
- 14 99. Dejouvencel T, Doeuvre L, Lacroix R, et al. Fibrinolytic cross-talk: a new mechanism for  
15 plasmin formation. *Blood* 2010; 115: 2048–56.
- 16 100. Lacroix R, Plawinski L, Robert S, et al. Leukocyte- and endothelial-derived microparticles: a  
17 circulating source for fibrinolysis. *Haematologica* 2012; 97: 1864–72.
- 18 101. Lacroix R, Dignat-George F. Microparticles: new protagonists in pericellular and intravascular  
19 proteolysis. *Semin Thromb Hemost* 2013; 39: 33–9.
- 20 102. Kwaan HC, Rego EM. Role of microparticles in the hemostatic dysfunction in acute  
21 promyelocytic leukemia. *Semin Thromb Hemost* 2010; 36: 917–24.
- 22 103. Durrieu L, Bharadwaj A, Waisman DM. Analysis of the thrombotic and fibrinolytic activities of  
23 tumor cell-derived extracellular vesicles. *Blood Adv* 2018; 2: 1054–65.
- 24 104. Al-Nedawi K, Szemraj J, Cierniewski CS. Mast cell-derived exosomes activate endothelial cells  
25 to secrete plasminogen activator inhibitor type 1. *Arterioscler Thromb Vasc Biol* 2005; 25:  
26 1744–9.
- 27 105. Al Saleh HA, Haas-Neill S, Al-Hashimi A, et al. Thrombotic characteristics of extracellular  
28 vesicles derived from prostate cancer cells. *The Prostate* 2018; 78: 953–61.
- 29 106. Lacroix R, Dubois C, Leroyer AS, et al. Revisited role of microparticles in arterial and venous  
30 thrombosis. *J Thromb Haemost JTH* 2013; 11 Suppl 1: 24–35.
- 31 107. Vallier L, Cointe S, Lacroix R, et al. Microparticles and Fibrinolysis. *Semin Thromb Hemost*  
32 2017; 43: 129–34.
- 33 108. Cointe S, Harti Souab K, Bouriche T, et al. A new assay to evaluate microvesicle plasmin  
34 generation capacity: validation in disease with fibrinolysis imbalance. *J Extracell Vesicles* 2018;  
35 7: 1494482.
- 36 109. Lacroix R, Judicone C, Poncelet P, et al. Impact of pre-analytical parameters on the  
37 measurement of circulating microparticles: towards standardization of protocol. *J Thromb*  
38 *Haemost JTH* 2012; 10: 437–46.

- 1 110. Coumans FAW, Brisson AR, Buzas EI, et al. Methodological Guidelines to Study Extracellular  
2 Vesicles. *Circ Res* 2017; 120: 1632–48.
- 3 111. Shpacovitch V, Hergenröder R. Optical and surface plasmonic approaches to characterize  
4 extracellular vesicles. A review. *Anal Chim Acta* 2018; 1005: 1–15.
- 5 112. Laroche M, Dunois C, Vissac AM, et al. Update on functional and genetic laboratory assays for  
6 the detection of platelet microvesicles. *Platelets* 2017; 28: 235–41.
- 7 113. Poncelet P, Robert S, Bailly N, et al. Tips and tricks for flow cytometry-based analysis and  
8 counting of microparticles. *Transfus Apher Sci Off J World Apher Assoc Off J Eur Soc*  
9 *Haemapheresis* 2015; 53: 110–26.
- 10 114. Nolan JP, Jones JC. Detection of platelet vesicles by flow cytometry. *Platelets* 2017; 28: 256–  
11 62.
- 12 115. Hisada Y, Auriemma AC, Alexander W, et al. Detection of tissue factor-positive extracellular  
13 vesicles by laser scanning confocal microscopy. *Thromb Res* 2017; 150: 65–72.
- 14 116. Exner T, Joseph J, Low J, et al. A new activated factor X-based clotting method with improved  
15 specificity for procoagulant phospholipid. *Blood Coagul Fibrinolysis Int J Haemost Thromb*  
16 2003; 14: 773–9.
- 17 117. Jy W, Horstman LL, Jimenez JJ, et al. Measuring circulating cell-derived microparticles. *J*  
18 *Thromb Haemost JTH* 2004; 2: 1842–51.
- 19 118. Hemker HC, Giesen P, AlDieri R, et al. The calibrated automated thrombogram (CAT): a  
20 universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb* 2002;  
21 32: 249–53.
- 22 119. Hellum M, Øvstebø R, Trøseid A-MS, et al. Microparticle-associated tissue factor activity  
23 measured with the Zymuphen MP-TF kit and the calibrated automated thrombogram assay.  
24 *Blood Coagul Fibrinolysis Int J Haemost Thromb* 2012; 23: 520–6.
- 25 120. Hisada Y, Alexander W, Kasthuri R, et al. Measurement of microparticle tissue factor activity in  
26 clinical samples: A summary of two tissue factor-dependent FXa generation assays. *Thromb*  
27 *Res* 2016; 139: 90–7.
- 28 121. Tatsumi K, Antoniak S, Monroe DM, et al. Evaluation of a new commercial assay to measure  
29 microparticle tissue factor activity in plasma: communication from the SSC of the ISTH. *J*  
30 *Thromb Haemost* 2014; 12: 1932–4.
- 31 122. Vallier L, Bouriche T, Bonifay A. New specific and highly sensitive procoagulant test to  
32 measure tissue factor activity on microparticles. *Res Pr Thromb Haemost Berlin*; 2017; 1:1087.
- 33 123. Hisada Y, Mackman N. Measurement of tissue factor activity in extracellular vesicles from  
34 human plasma samples. *Res Pr Thromb Haemost [Internet]* 2018 [cited 2018 Dec 8]; 0: .  
35 Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1002/rth2.12165>
- 36 124. Biró E, Sturk-Maquelin KN, Vogel GMT, et al. Human cell-derived microparticles promote  
37 thrombus formation in vivo in a tissue factor-dependent manner. *J Thromb Haemost* 2003; 1:  
38 2561–8.

1 125. Berckmans RJ, Sturk A, van Tienen LM, et al. Cell-derived vesicles exposing coagulant tissue  
2 factor in saliva. *Blood* 2011; 117: 3172–80.

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