Circulating Extracellular Vesicles in Patients with Cancer and Venous Thromboembolism

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Abstract

Venous thromboembolism (VTE), defined as deep vein thrombosis and/or pulmonary embolism is the second leading cause of mortality in cancer patients, second only to cancer itself. A number of reports suggest that circulating extracellular vesicles (EVs) may be increased in cancer patients with VTE. The aim of this study was to examine circulating EVs in high-risk ambulatory cancer patients, determine if levels are associated with hematological outcomes (VTE, major bleeding event), and to assess the impact of prophylactic antithrombotic therapy (Apixaban). We hypothesized that elevated levels of circulating large EVs will be predictive of cancer associated VTE and/or bleeding events and that treatment with Apixaban will reduce EV levels and incidence of cancer VTE. Plasma samples from patients at baseline, and 90-days follow-up from the Apixaban for the Prevention of Venous Thromboembolism in High-Risk Ambulatory Cancer patients (AVERT) trial were investigated. Total EVs were quantified by their pro-coagulant activity using the Zymuphen MP-Activity kit. Platelet, endothelial and tissue-factor EV levels were quantified by flow cytometry. We observed that circulating EVs exhibited significant associations with sex, age, and cancer type, however we did not observe any relationships with clinical outcomes. Thus, it appears that circulating EVs may not have a role in risk stratification for VTE in in high-risk ambulatory cancer patients.

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List of Abbreviations

APC	Allophycocyanin
ASCO	American Society of Clinical Oncology
ASH	American Society of Hematology
AVERT	Apixaban for the Prevention of Venous Thromboembolism in High-Risk Ambulatory Cancer patients
BMI	Body Mass Index
CAT	Cancer-Associated Thrombosis
CATS	Vienna Cancer and Thrombosis Study
CCI	Charlson Comorbidity Index
CI	Confidence Interval
CP	Cancer Procoagulant
CRNMB	Clinically Relevant Non-Major Bleeds
CRP	C-reactive protein
DNA	Deoxyribose Nucleic Acid
DOAC	Direct Oral Anticoagulants
DVT	Deep Vein Thrombosis
EC	Endothelial Cell

EC-EV	Endothelial Cell-derived Extracellular Vesicle
EM	Electron Microscopy
EMP	Endothelial Microparticles
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Extracellular Vesicle
EV-TF	Extracellular vesicle-tissue factor
EV-TRACK	Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research
F1+2	Prothrombin fragment 1+2
FI	Factor I (fibrinogen)
FII	Factor II (thrombin)
FIII	Factor III (tissue factor)
FIV	Factor IV (calcium ion)
FITC	Fluorescein Isothiocyanate
FLS	Forward Light Scatter
FV	Factor V
FVII	Factor VII
FX	Factor X (Stuart factor)

FXI	Factor XI
FXII	Factor XII (Hageman Factor)
GLA	Gamma-Carboxyglutamic Acid
GBM	Glioblastoma Multiforme
HR	Hazard Ratio
ICAM-1	Intracellular Cell Adhesion Molecule 1
ILV	Intraluminal Vesicles
IMiD	Immunomodulatory Imide Drugs
ISEV	International Society of Extracellular Vesicles
ITAC	Initiation on Thrombosis and Cancer
LMWH	Low Molecular Weight Heparin
MiRNA	MicroRNA
MISEV	Minimal Information for Studies of Extracellular Vesicles
MP	Microparticle
MV	Microvesicle
MVB	Multivesicular Bodies
NCCN	National Comprehensive Cancer Network
NET	Neutrophil Extracellular Traps

NNT	Number Needed to Treat
NTA	Nanoparticle Tracking Analysis
OR	Odds Ratio
PAI-1	Plasminogen Activator Inhibitor-1
PDCD6IP	Programmed Cell Death 6 Interacting Protein (ALIX)
PE	Phycoerythrin
PE	Pulmonary Embolism
PMP	Platelet Microparticles
PolyP	Polyphosphate
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PTG	Peak Thrombin Generation
RNA	Ribonucleic Acid
RPS	Resistive Pulse Sensing
RR	Relative Risk
SLS	Side Light Scatter
sP-selectin	Soluble P-selectin
TF	Tissue Factor

TF-EV	Tissue Factor-bearing Extracellular Vesicle
TF-MP	Tissue Factor Microparticles
TG	Thrombin Generation
TSG101	Tumor Susceptibility Gene 101
UFH	Unfractionated Heparin
VEGF	Vascular Endothelial Growth Factor
VKA	Vitamin K Antagonist
VTE	Venous Thromboembolism
VWF	Von Willebrand Factor
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Venous Thromboembolism and Cancer

Venous thromboembolism (VTE) is a complex multifactorial disease defined as deep vein thrombosis (DVT) and/or pulmonary embolism (PE).[1,2] Clinically, approximately two-thirds of VTE episodes manifest as DVT and one-third as PE with or without DVT.[2] Every year, VTE affects about 100,000 Canadians, causing 10,000 deaths.[3] In addition, the health care burden of VTE is quite substantial with the total cost of VTE in Canada being at least 600 million dollars per year,[4] and in the United States, VTE costs the healthcare system $7-10 billion each year.[2,5–7]

Patients with cancer are six times more likely to develop VTE than noncancer patients, making VTE the second leading cause of death in cancer patients.[8] This association between malignancy and a hypercoagulable state that can manifest as VTE has been known for around a century and can sometimes be referred to as Trousseau syndrome.[2] Cancer patients are generally in a prothrombotic state, and they usually present with abnormalities in each component of Virchow's triad (Figure 1) and thereby contribute to thrombosis.[9–11] The triad consists of a stasis of blood flow, endothelial injury, and hypercoagulability (abnormalities in the coagulation and fibrinolytic pathway and platelet activation).[2] In people with cancer, thrombosis can affect multiple venous systems including the splanchnic, visceral and cerebral veins, and the arterial system leading to other health complications.[2] The risk of VTE in cancer patients is ~15% per year but it varies widely depending on cancer type and concomitant therapeutic interventions.[12] Furthermore, VTE is the leading cause of morbidity and mortality among hospitalized patients with cancer.[13,14] In a Danish registry study, cancer patients diagnosed simultaneously with VTE had a significantly lower one-year survival rate (12% vs 36%; $p<0.001$) and significantly more distant metastases

compared with cancer patients without VTE.[15] However, the treatment of cancer-associated thrombosis (CAT) remains challenging with the risks of recurrent thrombosis and bleeding being higher among patients with cancer than among those without.[16] Current guidelines from the American Society of Hematology (ASH)[17], American Society of Clinical Oncology (ASCO)[18], Initiation on Thrombosis and Cancer (ITAC)[19], and the National Comprehensive Cancer Network (NCCN)[20] all recommend assessing the risk of VTE and bleeding in hospitalized medical and surgical patients with cancer.[17] Routine pharmacological thromboprophylaxis is recommended for patients with a high risk for thrombosis and a low risk for bleeding, however timing, initiation and type of thromboprophylaxis differ from one guideline to another.[17] Overall, risk stratification of cancer patients is a common theme in CAT management and guidelines, due to the unclear and multi-faceted nature of mechanisms that promote thromboembolic events in cancer patients.[21,22]

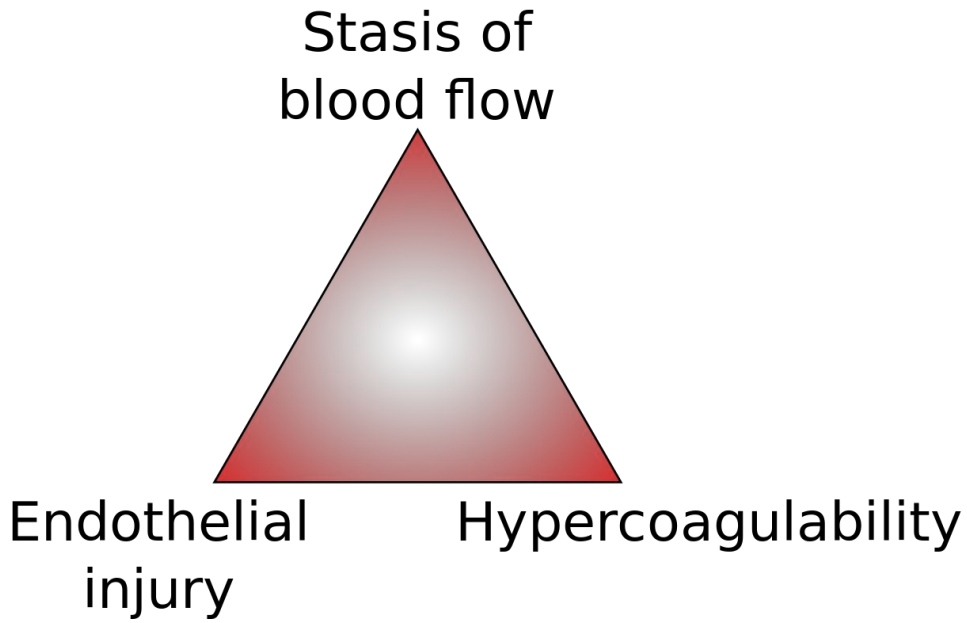


Figure 1.1. Virchow's triad describing the three broad categories of factors that contribute to thrombosis.

1.2 Risk Factors for VTE

The most well-known and established risk factor for VTE is cancer, however, there are several other factors known to increase a patient's risk of developing thrombosis. These can be divided into four categories (Figure 2) patient-related factors (age, sex, race, immobility, previous VTE); cancer-related factors (cancer type, stage, histological grade); treatment-related factors (surgery, chemotherapy, radiotherapy); and candidate biomarkers (platelet count, leukocyte count, D-dimer, soluble P-selectin, extracellular vesicle-tissue factor activity, and extracellular vesicles).[23–25]

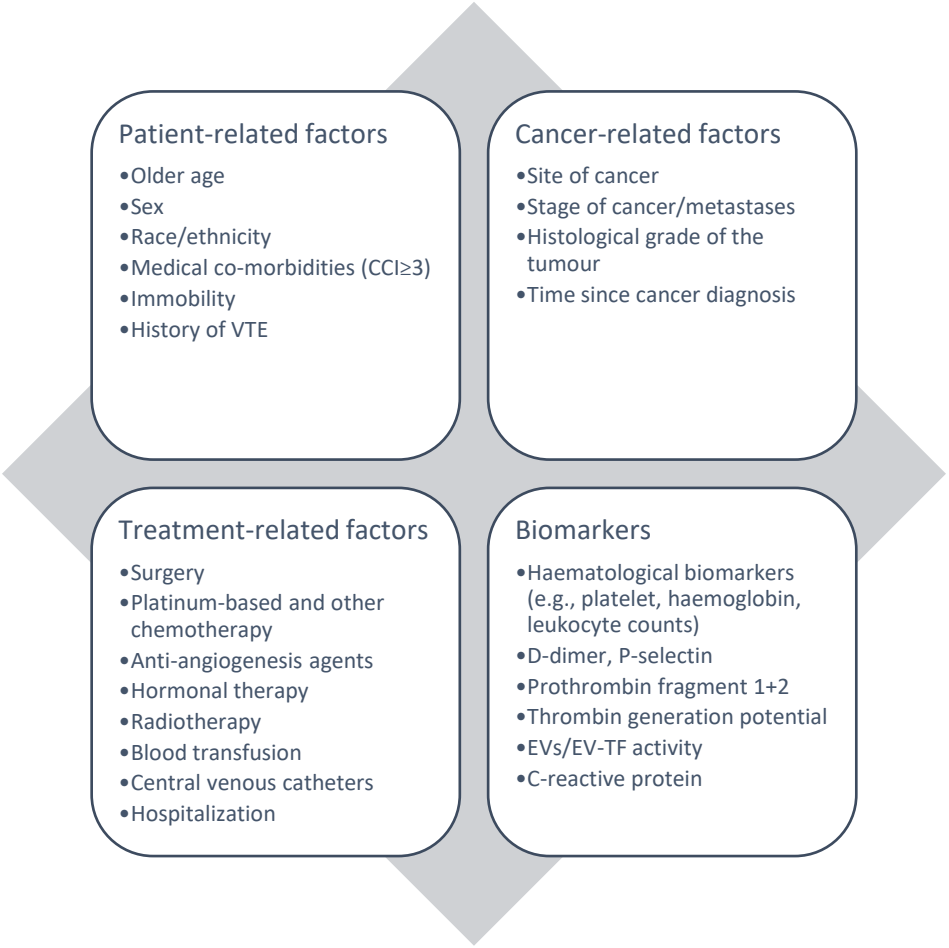


Figure 1.2. Risk factors for cancer associated VTE. Adapted from Ay C., and colleagues.[26]

1.2.1 Patient-related Risk Factors for VTE

In the cancer population, increasing age is a risk factor for VTE. A retrospective cohort study by Vergati and colleagues found that the elderly patients (≥70 years) had an almost 2-fold increased risk of developing VTE compared to young and middle-aged patients.[2,11] Several factors may contribute to this increased risk with procoagulant factors such as factor VIII, factor VII, homocysteine, and fibrinogen increasing naturally with age.[2] However, other studies found that in hospitalized patients with cancer, older age (≥65 years) was associated with a slightly elevated risk of VTE, odds ratio [OR] 1.1; (95% CI 1.0-1.1), while in the ambulatory setting, age was not a significant risk factor for patients with good performance status.[27] Regardless of the presence or type of cancer, increasing age is associated with factors that increase the risk of thrombosis, including increased immobility, and systemic activation of coagulation which should always be considered when evaluating the risk of VTE.[21] Age also plays a role in the risk of VTE by sex.[2] The incidence of VTE is greater in women than in men during middle-aged child-bearing years due to increased endogenous estrogen with exogenous hormonal therapies further increasing this risk.[2] However, past the childbearing years, incidence of VTE is seen to be greater in men than in women.[2] Many studies, were also unable to find sex differences in VTE rates[28–30], however a study of hospitalized patients reported an overall increased risk of VTE in women [OR] 1.1; (95% CI: 1.1-1.2).[31] Race and ethnicity have also seen to play a role in the incidence of VTE. In a large retrospective study by Khorana *et al.*[31] they found a higher incidence of VTE among black patients (5.1%), followed by white and Hispanic patients (4.0%) amongst adult cancer patients admitted at any of the 133 U.S academic medical centers. The lowest incidence of VTE was observed in Asian/Pacific Islander patients (3.3%). These findings were consistent with other epidemiological studies.[32–34] However, Zakai and colleagues concluded that the low rates in North Americans of

Asian descent may be partially explained by the lack of surveillance for VTE, lack of clinical suspicion in 'low-risk' populations, and lack of access to medical care.[32]

Comorbid conditions (i.e., obesity, heart disease, acute infection) have been identified as significant risk factors of cancer associated thrombosis. In a study by Khorana and colleagues, the Charlson Comorbidity Index (CCI), which is an overall measure of the severity of illness, was found to be associated with a greater risk of VTE. In a multivariate analysis, patients with a CCI score of 3 or 4 had an OR of 3.60 (95% CI: 2.72-4.77) while patients with a CCI score ≥5 had an OR of 4.38 (95% CI: 3.33-5.78) indicating a link between disease severity and cancer associated VTE.[27,35] The association between obesity and VTE is also fairly well established. A retrospective case-control study consisting of 732 individuals found that obesity was associated with a 6.2-fold increased risk for VTE.[36] In addition, a retrospective chart review consisting of 7602 cancer patients receiving thromboprophylaxis, found that BMI was a significant predictor of VTE (OR = 1.094, 95% CI 1.021–1.172, p = 0.011).[36] Each one kg/m^2 increase above overweight BMI (25.0-29.0 kg/m^2) increased the odds of VTE by 9.4%.[36]

Lastly, immobility and previous history of VTE also predispose cancer patients to VTE and VTE recurrence. One study found that bed rest greater than 3 days was associated with higher rates of VTE OR = 4.4 (95%: 2.5-7.8), due to the stasis of venous blood flow.[37,38] While a previous history of VTE leads to a 6-fold increase of VTE recurrence when compared with cancer patients with no history of VTE.[38,39]

1.2.2 Cancer-related Risk Factors for VTE

Cancer-related factors are frequently identified as a risk factor for VTE with the primary site of cancer playing a major role in CAT. Patients with pancreatic cancer have the highest risk of VTE (11.7-36%)[40,41], whereas patients with brain (~20%)[42], colorectal (3.1-8.9%)[43,44], gastric (8.7-13.6%)[45,46], bladder (8.2%)[41], renal (1.2-6%)[47] and lung (1.6-13.6%)[45] cancer have lower incidence rates. Another study suggests that the highest incidence rates occur in mucin-producing adenocarcinomas of the pancreas, lung and gastrointestinal tract.[11] In contrast, the rates of increased VTE in cancer patients may also be a reflection of the frequency of cancer within the population as a whole. Higher incidences of VTE have been seen in women with ovarian, breast and lung cancer and a higher incidence in men with prostate, colorectal, and lung cancer.[11] Predictive models now use cancer type as a variable in stratifying an individual patient's risk of developing VTE.[48] The most widely used systems: the Khorana score and Vienna Cancer and Thrombosis Study (CATS) score, consider pancreatic and gastric tumors as very high risk and lung, gynecologic, lymphoma, bladder and testicular cancer as high-risk tumors.[48] Regardless of the different cancer types seen, the incidence of VTE is not the same for all suggesting a specific mechanism driven by cancer.

Cancer stage also impacts the risk of developing VTE with patients at advanced-stage cancer showing greater incidences of cancer-associated VTE. For example, in a population-based cohort study, the risk of cancer patients developing VTE increased with cancer stage, with the calculated adjusted relative risks (RR) for stage I (2.9), II (2.9), III (7.5), and IV (17.1) cancer.[11] Similarly, a cohort study with 6,324 ovarian cancer patients found that the odds of VTE were higher in stage III/IV versus stage I/II, pooled odds ratio [OR] 2.73; (95% CI 1.84-4.06; I^2=64%). Another study from the Netherlands involving multiple tumor types also showed that patients with

distant metastases had a 1.9-fold increased risk (relative risk [RR]: 1.9; (95% CI: 1.6-2.3) of developing VTE.[49] However, many current predictive models do not include cancer stage as a component for CAT, and incorporation of this may improve predictive value in the future.[41,50,51]

Lastly, the initial period after diagnosis of cancer is when the risk of VTE is highest. In a population-based study, the risk of VTE in the first 3 months was greatest OR = 53.5 (95% CI: 8.6-334.3), which later declined in the period after from 3 months to 1 year OR = 14.3 (95% CI: 5.8-35.2) and 1 to 3 year intervals OR = 3.6 (95% CI: 2.0-6.5).[21,27] It is also important to consider that many therapeutic interventions occur during this initial period, which likely contributes to the risk. However, even in patients receiving primarily chemotherapy, the risk is highest in the immediate period of treatment. For example, patients with transitional-cell carcinoma and lung cancer undergoing chemotherapy, 77% and 45% of vascular events, respectively, occurred during the first two cycles of therapy.[52,53] This increased incidence of VTE in the time period following initial diagnosis of malignancy is thought to comprise of effects of the tumor, such as humoral and mechanical effects, which are likely to be highly active in recently diagnosed cancer.[54] Although, this may also be reflective of the intensity of cancer treatments following initial diagnosis with treatments including surgery, radiotherapy, hormone therapy, and chemotherapy which are well-known risk factors of VTE.[54]

1.2.3 Treatment-associated Risk Factors for VTE

Another important aspect when determining the risk of VTE is cancer-treatment-associated risk factors. The prothrombotic state of cancer can be provoked by cancer therapies and treatments, including surgery. VTE is a common complication of patients post-operation, with cancer surgery increasing the risk of postoperative DVT 2-fold and a fatal PE 3-fold, compared to similar

procedures on non-cancer patients.[11] Surgical procedures associated with a high risk of VTE include neurosurgery, major orthopaedic surgery of the leg, renal transplantation, cardiovascular surgery, and thoracic, abdominal, or pelvic surgery for cancer.[11,41] For example, Semrad and colleagues identified 715 cases of VTE and of those, 55% (391) occurred within 2 months of neurosurgery.[48] Similarly, some studies demonstrated that longer times in the operating room, longer times under anesthesia, and need for surgical re-exploration is associated with increased risk of VTE in cancer patients.[39]

Similarly, chemotherapy is an important risk factor for VTE in cancer patients. Cancer patients receiving chemotherapy have a 6- to 7-fold increased incidence of VTE compared to cancer patients not receiving chemotherapy.[11,35,39,41,55] Systemic chemotherapy agents are known to predispose to VTE, with the risk of thrombosis being the highest during the first few months after initiation of treatment. A retrospective observational cohort study of 17,284 cancer patients with various tumor malignancies found that 18% of VTE events occurred within 1 month of starting chemotherapy, while 73% of events occurred within the first 6 months.[35] The mechanisms by which cancer therapies increase the risk of VTE are diverse and vary according to the agent used.[55] The cancer therapeutic agents most commonly associated with VTE were tamoxifen and L-asparaginase.[55] Tamoxifen-associated VTE was highest during the first 2 years upon initiation of the drug and normalizes following discontinuation.[55] This risk was further amplified when other risk factors such as BMI or cigarette smoking were included.[55] Immunomodulatory imide drugs (IMiDs) such as thalidomide and lenalidomide have not yet been found to increase the risk of VTE.[55] However, when used in combination with dexamethasone or cytotoxic chemotherapy in the treatment of multiple myeloma increases the risk of thrombosis.[48,55] The incidence of VTE for multiple myeloma is less than 5% for patients on thalidomide alone, compared to 10-20% for

thalidomide with dexamethasone and 20-40% for thalidomide with chemotherapy.[55–59] Cytotoxic chemotherapy such as cisplatin increases the risk of VTE by damaging endothelial cells and causing apoptosis leading to thrombin generation through tissue-factor dependent mechanisms. Certain chemotherapy agents have also seen to reduce endogenous anticoagulant factors such as protein C and protein S which could affect intercellular interactions and platelet reactivity.[55,59] Other cancer therapies including hormone therapies, aromatase inhibitors, L-Asparaginase, and targeted therapies (e.g. VEGF inhibitors - bevacizumab) have all been associated with VTE.[23,39,50,55]

Central venous catheters (CVC) are vital for many aspects of cancer therapy, including delivery of intravenous drugs and collecting blood samples. However, their use can result in the formation of catheter-related thrombosis which is a significant complication that can interrupt chemotherapy treatment, intravenous medications, and cause serious morbidity such as PE.[21] One study found that the incidence of catheter thrombosis is between 5 and 30%, but it is believed to be underestimated due to clinical signs of catheter thrombosis often being vague and nonspecific.[21,27] Whereas, when the rate of catheter-related DVT is assessed by venography the rate ranges from 27% to 66%.[21,27]

1.2.4 Biomarkers as Risk Factors for VTE

A final risk factor for VTE is alterations in levels of biomarkers. Biomarkers are measurable, biologic parameters which can be used to improve prediction, diagnostic and prognostic models of disease.[24] Biomarkers in the prediction of primary VTE risk have been studied extensively and summarized in a review by Kim and colleagues.[24] Biomarkers assessing primary VTE risk in cancer patients include: hematological biomarkers (e.g., platelet count

>350x10^9/L, leukocyte count >11x10^9/L, haemoglobin level <10g/dL), soluble P-selectin (sP-selectin), prothrombin fragment 1+2 (F1+2), D-dimer, high-sensitivity C-reactive protein (hs-CRP), extracellular vesicles (EVs), tissue factor (TF), extracellular vesicle-tissue factor activity (EV-TF activity), and thrombin generation potential.[26,60] One study reported a 3.5-fold increased risk of VTE in patients with a platelet count ≥443x10^9/L.[61] Other biomarkers such as sP-selectin, is a cell adhesion molecule thought to mediate the adhesion of leukocytes, platelets, and cancer cells in inflammation, thrombosis and cancer cell growth. F1+2 is considered a specific *in vivo* marker of thrombin generation and D-dimer is the primary degradation product of cross-linked fibrin and reflects the activation of haemostasis and fibrinolysis mechanisms. The presence of preoperative D-dimer in colorectal cancer patients was associated with increased risk of VTE during the year post-surgery compared to patients negative for preoperative D-dimer (hazard ratio [HR]: 6.53; (95% CI: 1.58-27.0, p=0.0009). In addition, higher levels of sP-selectin, F1+2, and D-dimer were found in patients with distant metastases compared to patients with localised cancers.[62] However, there are few studies assessing longitudinal changes in cancer-associated VTE biomarkers in cancer patients. A recent longitudinal study over six months in colorectal, lung, pancreatic or brain cancer patients found that patients who developed VTE had significantly elevated haemostasis biomarkers, including sP-selectin and D-dimer compared to those patients who did not develop VTE.[26,63] Thrombin generation (TG) potential reflects the composite of multiple factors that influence blood coagulation. In the Vienna Cancer and Thrombosis Study, authors determined the peak TG (PTG) in 1033 cancer patients and found that 4% of patients without elevated PTG developed VTE during follow-up compared with 11% of patients with elevated PTG resulting to an HR of 2.1 (95% CI: 1.3-3.3).[64]

Tissue factor (TF) is the physiological initiator of coagulation and is expressed in a number of malignancies and solid tumors. Tumor-cell derived TF plays an important role in the generation of thrombin in cancer, however its association with the risk of VTE has only been observed in pancreatic and ovarian cancer.[21] In a small, retrospective study, patients with high TF expression in resected tumor specimens had a subsequent VTE rate of 26.3% compared with 4.5% in patients with low TF expression (P = 0.04).[65] Apart from TF expression in tumours, TF is also present on the surface of extracellular vesicles.[21] Extracellular vesicles (EVs), tissue factor-bearing extracellular vesicles (TF-EVs), and extracellular vesicle-tissue factor activity (EV-TF activity) are increasingly evaluated as biomarkers of cancer-associated VTE. Their role in CAT and primary VTE is controversial but stems from their intrinsic and differential procoagulant activity.[24,66] There are many other biomarkers that are in the early stages of evaluation, including: neutrophil extracellular traps (NETs), polyphosphate (polyP), podoplanin, microRNAs (miRNAs), plasminogen activator inhibitor-1 (PAI-1), cancer procoagulant (CP), and many more inflammatory molecules, however, these warrant further investigation.[24] Biomarkers represent an important factor in understanding CAT and its mechanisms and have opened up a variety of targets for improving risk models and clinical decision making.

Taken together, multiple factors (i.e., age, demographic, cancer, treatment, chemotherapy) associate with higher incidences of VTE in cancer patients.[26,41,48,63,66] Hence, cancer patients have a significantly higher risk of VTE, and patients that do develop VTE have significantly overall shorter survival.[21] However, a high index of suspicion is required for early diagnosis of cancer associated VTE, thus early prevention and treatment is vital to reduce its burden on patients with malignancy.

1.3 Treatment of cancer-associated venous thromboembolism

The standard treatment for VTE consists of anticoagulant prophylaxis with the administration of low molecular weight heparin (LMWH) or intravenous unfractionated heparin (UFH), with early initiation of vitamin K antagonists (VKA) such as warfarin.[67–69] This approach is highly effective in most patients, but oral anticoagulant therapy is often problematic in cancer patients with drug interactions and liver dysfunction leading to unpredictable levels of anticoagulation.[16,70] Randomized clinical trials in cancer patients evaluated the effect of direct oral anticoagulants (DOACs). Oral anticoagulants are divided into those that target thrombin (direct thrombin inhibitor, dabigatran) or activated factor X (factor Xa inhibitors, rivaroxaban, apixaban, and edoxaban). The trial HOKUSAI Cancer that randomized 1050 patients with cancer and acute symptomatic or incidental VTE to LMWH, followed by oral edoxaban or dalteparin.[71] Major bleeding or recurrent VTE in the 12 months after randomization occurred in 12.8% of edoxaban arm patients and 13.5% of dalteparin arm patients.[71] Similarly, the trial Select-D, was a prospective, randomized multicenter pilot trial randomizing 406 cancer patients with acute VTE to dalteparin and rivaroxaban.[72] The 6-month cumulative VTE major bleeding rates were 4% with dalteparin and 6% with rivaroxaban.[72] Overall, they demonstrated a moderate benefit for thromboprophylaxis and anticoagulation and highlighted the need to select a subgroup of high-risk patients who could mostly benefit from primary prophylaxis.[73–75] Therefore, it is currently recommended that DOACs are used for cancer patients with an acute diagnosis of VTE, a low risk of bleeding and no drug-drug interactions with current therapy.[76]

1.4 Prediction Models of Venous Thromboembolism

This has led to the investigation of predictive parameters for the development of VTE and be able to select patients individually based on risk stratification.[77] Several risk assessment models including the Khorana[50], Vienna CATS[51], PROTECHT[78], CONKO[79], ONKOTEV[80], COMPASS-CAT[81], TiC-Onco[82], and CATS nomogram[83] scores have guided clinical decision-making for the prediction of primary VTE; as well as the Ottawa[84] score for recurrent VTE. Among them, the first and most widely used is the Khorana score (Figure 3), which takes into account the site of cancer, thrombocytosis, leukocytosis, low hemoglobin levels, and a BMI $\geq$35kg/m^2, and groups patients into low-, intermediate-, or high-risk for VTE.[50] Consequently, the external validation of the Khorana score was not univocal,[85,86] with criticism that a high proportion of patients (>50%) falling into the intermediate risk category makes it suboptimal in clinical applications.[85,87] A systematic review and meta-analysis found that about one in four (23.4%, 95% CI: 18.4-29.4) of venous thromboembolic events occur in patients with a high-risk Khorana score.[88] This indicates that a significant number of cancer patients with subsequent venous thromboembolic events will not be identified with this form of risk stratification, and thus, not benefit from thromboprophylaxis. Similarly, other scores have attempted to increase the Khorana score predictive performance by introducing soluble biomarkers such as D-dimer and sP-selectin (Vienna CATS score) or the use of specific chemotherapy regiments (PROTECHT score) but have failed to outperform the Khorana score.[24,87–89] The limitation of Vienna CATS was the lack of widespread availability of P-selectin assays, while in the PROTECT score was that the number needed to treat (NNT) to prevent one VTE in high risk patients was 17 versus 15 with the Khorana score.[90] Currently, neither modification has been validated or is recommended by guidelines.[90] Other externally validated risk stratification tools based on the Khorana score are the CONKO

score (included WHO performance status instead of BMI), and the ONKOTEV score (incorporated metastatic disease, vascular/lymphatic compression by tumour and history of VTE).[91] However, a large, multinational prospective cohort study (n=876) compared the discriminatory power of different scores, and found that the PROTECHT and CATS score significantly stratified high- and low-risk patients, whereas the Khorana and CONKO score did not.[91] These findings emphasize the current limitations of risk scoring in predicting CAT. Furthermore, there is a concerning large number of VTE events in patients classified as low risk, indicating the necessity to further investigate potential predictive markers in order to increase the sensitivity.[91]

Among ambulatory setting, the developed risk scores have culminated in clinical applications for the stratified use of prophylactic anticoagulation in patients with cancer. Using the Khorana score as a risk prediction model, two randomized controlled trials: AVERT and CASSINI evaluated DOAC for preventing cancer associated VTE.[91] CASSINI, was a multinational, randomized, controlled trial, where rivaroxaban (10mg once daily) was tested against placebo in ambulatory cancer patients initiating systemic antineoplastic therapy and a Khorana score of $\geq$ 2.[92] They found that VTE was significantly reduced in patients receiving rivaroxaban (11/420, 2.6%) compared to the placebo group (27/421, 6.4%) (HR 0.40, 95% CI: 0.20-0.80).[92]

AVERT, was a multicentre, randomized controlled superiority trial, comparing apixaban (2.5mg twice daily) against placebo in patients with cancer initiating chemotherapy and a Khorana score of $\geq$2.[93] Similar to the CASSINI trial, they found that patients receiving apixaban had a lower number of VTE events (12/288, 4.2%) compared to placebo (28/275, 10.2%) (HR 0.41, 95%CI: 0.26-0.65, $p<0.001$). Based on these trials, recent guidelines recommend primary thromboprophylaxis with DOAC for patients with cancer and a high risk of VTE.[17,19] Despite being able to identify patients who are at an increased risk of CAT, the majority of VTE events still occur

in patients in low- or intermediate-risk categories. Utilizing a threshold of a Khorana score of 2 compared to the original 3 for patients to undergo prophylactic anticoagulation, the VTE rate was reduced from 7% (2.5-month follow-up) to 4.2% (6-month follow-up).[89] However, a systematic review and meta-analysis demonstrated only a modest 1.5-fold higher risk of VTE for patients with a Khorana score of ≥2 than those with a lower score during 6-month follow-up.[89] Furthermore, another study found that when using the guideline-recommended threshold of ≥2, the Khorana score did not risk-stratify patients with hepatobiliary or pancreatic cancer, lung cancer, and gynecologic cancer.[94] This underlies the importance of optimizing established models and finding new risk predictors for VTE in patients currently classified as low risk for CAT.[91,95]

Table 1.1. The Khorana risk score. A predictive model for chemotherapy-associated VTE. Risk categories: low (score 0), intermediate (score 1-2), and high (score ≥3).[50]

Patient characteristic	Risk score
Site of cancer	
Very high risk (stomach, pancreas)	2
High risk (lung, lymphoma, gynecologic, bladder, testicular)	1
Prechemotherapy platelet count 350 × 10^9/L or more	1
Hemoglobin level less than 100 g/L or use of red cell growth factors	1
Prechemotherapy leukocyte count more than 11 × 10^9/L	1
BMI 35 kg/m^2 or more	1

1.5 Extracellular Vesicles

Extracellular vesicles (EVs) are small, cargo-bearing vesicles released by cells into the extracellular space. The pro-coagulant activity of EVs dates to 1946, when Erwin Chargaff and Randolph West found that following a high-speed centrifugation of 31,000 × g, the plasma's ability to clot was suppressed significantly.[96] This suggested that there was a sedimentable component within plasma irrespective to platelets that was responsible for coagulation.[96] However, Peter Wolf

described first physical evidence of EVs amidst blood coagulation research as “platelet dust” in 1967.[97] Following the initial discovery by Wolf, Neville Crawford published images of these vesicles and showed that they contained lipid and carried cargo including ATP and contractile proteins.[98] We now appreciate that EVs are more than inert products of cellular debris but play a functional role in intercellular communication, and biological processes such as coagulation.[99] Currently, EVs are recognized as important signalling molecules in normal cell homeostatic processes or diverse pathological conditions.[100,101] Taken together, it ignited the idea that EVs could be used as biomarkers, have therapeutic applications and play mechanistic roles in disease.[99] In recent years, there have been major advances in understanding the biology of EVs however the definition of EVs has been a large area of debate.[102] There are many reasons why defining EVs has been a difficult endeavour; with one being the ability of many different cell types to release EVs, resulting in various compositions. Furthermore, they are released by a number of pathways via multiple mechanisms resulting in various sizes (30 – 5,000 nm in diameter).[102] This allows for the use of many different analytical methods for the isolation and identification of the EV population.[99] In 2018, the International Society of Extracellular Vesicles (ISEV) published a statement on minimal experimental requirements for the definition of EVs and their functions.[103] Current nomenclature by the ISEV endorses “extracellular vesicle” as the term for particles naturally released by cells, delimited by a lipid bilayer, and cannot replicate.[103] Currently, three main subtypes of EVs have been extensively described: plasma-membrane derived “ectosomes” (microparticles/microvesicles), exosomes and apoptotic bodies.[102–104] However, due to a lack of EV-specific markers that can distinguish subtypes from one another the terms small EV and large EV instead of exosome and microparticle are emerging as preferred nomenclature in the EV community.[103,105]

Medium/Large EVs, also known as microparticles (MPs), 'microvesicles' (MVs), or 'ectosomes' are small (0.1-1μm) fragments of cellular membrane shed from all cell types into the extracellular space.[100,106–108] They can originate from different cells during physiological cell turnover, response to stress, injury, cell activation, or apoptosis.[109–112] Large EVs are generated by the outward budding and fission of the plasma membrane resulting in the exposure of negatively charged phospholipids, specifically phosphatidylserine. Large EVs, however, must be distinguished from the two other EVs released from cells: apoptotic bodies and exosomes.[100,113–115] Apoptotic bodies are the largest among EVs (1-5μm) and arise as a consequence of extensive membrane budding that occurs during apoptosis; exosomes being the smallest member of the group (~20-120nm), also known as small EVs, occur through the inward budding of multi-vesicular bodies (MVBs) and are released from the plasma membrane.[100,102,113–115] These vesicles are distinguished from one another based on subcellular origin, size, and content. EV subtypes are common in the sense that they carry biologically active cytokines, proteins, RNA and other nucleic acids originating from their cell of origin.[102,113,115]

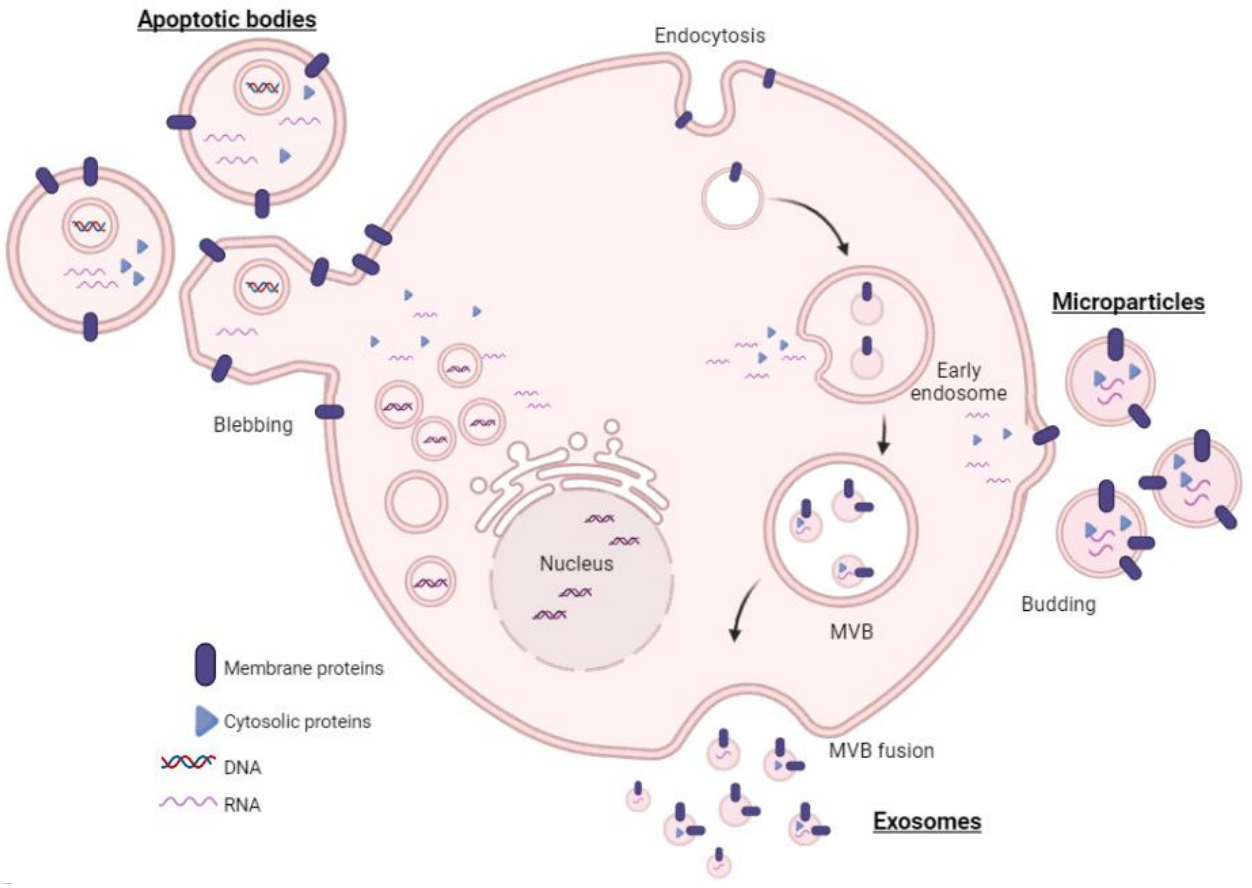


Figure 1.3. Biogenesis and release of EVs. Schematic representation of extracellular vesicle (EV) biogenesis and secretion. Apoptotic bodies are generated upon programmed cell death-induced membrane blebbing. Microparticles/microvesicles are released by the outward budding of the plasma membrane. Exosomes are created by the inward budding of early endosomes and secreted by fusion of these multivesicular bodies with the plasma membrane. Adapted from Hartjes and colleagues.[116]

1.5.1 Biogenesis of Extracellular Vesicles

It is known that the two subpopulations of EVs: microparticles and exosomes arise from different biogenesis mechanisms. The biogenesis of medium/large EVs – specifically microparticles, involves several molecular rearrangements within the plasma membrane.[100] Most if not all, eukaryotic cells release microparticles under both physiological and disease

conditions.[117] Specifically, the onset of disease, such as cancer, leads to aberrant shedding and release of microparticles.[117] In general, a combination of factors will result in the formation of MPs such as the repositioning of phosphatidylserine to the outer leaflet, and contraction of the actin-myosin machinery.[118] Biogenesis occurs via the direct outward blebbing and pinching of the plasma membrane releasing MPs into the extracellular space. Membrane blebbing is influenced by changes to membrane lipid components, protein composition, and Ca^{2+} levels. For example, the initial release of Ca^{2+} from the endoplasmic reticulum activates several Ca^{2+}-dependent enzymes. Calpain, a Ca^{2+}-dependent enzymes, will go on to detach membrane proteins from the intracellular cytoskeleton, and gelsolin bound to actin filaments.[100,119] This leads to further rearrangements of the cytoskeleton enabling blebbing to occur.[100,119] Aminophospholipid translocases (flippases and floppases) and scramblases will drive the asymmetry of membrane phospholipids (exposition of phosphatidylserine from the inner leaflet to the cell surface) which causes physical bending of the membrane.[100,120] High enough translocase activity, results in curvature and eventually vesicular fission.[120] Additionally, cytoskeletal elements and their regulators are required for microparticle fission and release.[121] The activity of Rho family GTPases and, the Rho-associated protein kinase (ROCK) are important regulators of actin and myosin dynamics and induce biogenesis.[120–122] In particular RhoA, phosphorylates myosin light chain (MLC) through a ROCK signaling pathway resulting in the activation of ATP-dependent contractile machinery.[121,122] Once MPs are formed, they pinch off from plasma membrane micro-domains known as lipid rafts or caveolae domains into the extracellular mileu.[119] MPs can then be detected by the presence of phosphatidylserine on the outer leaflet which readily binds to annexin V.[119]

In comparison, small EVs also known as exosomes, are derived from the endosomal system, and are formed as intraluminal vesicles (ILVs) in multivesicular bodies (MVBs).[118,120,123] These MVBs can then either traffic to lysosomes for degradation or fuse with the plasma membrane and release their contents (the ILVs) into the extracellular space.[120] The biogenesis and secretion of ILVs is mainly driven by the endosomal sorting complex required for transport (ESCRT) machinery system. The ESCRT machinery is a cytoplasmic multi-subunit system required for membrane remodelling, allowing vesicle budding and cargo sorting in MVBs.[118] The ESCRT machinery relies on five complexes (ESCRTs 0, I, II, III, and Vps4) in which each have their own role.[117,118,120] ESCRT 0 recognizes ubiquitinated proteins on the outside of the endosomal membrane, and recruits ESCRT I. ESCRT I along with ESCRT II promote endosomal inward budding around clusters of ubiquitinated proteins.[118] ESCRT III is recruited through programmed cell death 6 interacting protein (PDCD6IP) or ALIX and associates with tumor susceptibility gene 101 (TSG101) on the ESCRT I complex.[117,118,120] ESCRT III will then recruit CHMP4 which polymerises as a coil around the neck of the budding ILV. After the addition of CHMP3, the bud is cleaved forming ILVs, followed by ESCRT III disassembly using ATP catalysed by Vps4.[118] This chain of events results in the sequestration of MVB proteins into the ILVs.[117,118,120] In terms of exosome secretion, this involves the Rab family of small GTPases which plays a crucial role in intracellular vesicle transport and MVBs trafficking to lysosomes.[117,118,120] However, modulation of exosome secretion by Rab GTPases is heterogenous and depends on cell-type and cargoes.[124] Overall, the ESCRT pathway is the main mechanism involved in exosome biogenesis. Hence, exosome biogenesis is a finely tuned pathway with multiple molecular players involved in vesicle related physiology.

1.5.2 Isolation and Characterization of EVs

EVs can be isolated from cell culture conditioned medium and a variety of bodily fluids including blood, serum, urine, cerebrospinal fluid, lymphatics, saliva, breast milk, and nasal secretions using various methods of isolation.[120,123] Isolation methods utilize the physical properties of EVs, specifically their buoyant densities, size, and surface compositions.[120] Although there is no consensus on the best method for isolation the most used protocol for isolation/purification is differential centrifugation.[120] Recently, several alternative methods were also introduced for isolation and purification of EVs including density gradient ultracentrifugation, serial filtration, size exclusion chromatography, antibody-coated magnetic beads, and microfluidic devices.[125–127] However, with the currently available techniques, separation is not absolute and there is overlap between subtypes in EV isolations.[120]

Differential centrifugation or ultracentrifugation involves a number of sequential centrifugation steps at different centrifugal forces (g) whose purpose is to remove unwanted components from EVs (MPs or exosomes).[127] In this procedure, EVs are first separated from cells by centrifugal accelerations of typically 200-1500 x g to prepare cell-free supernatant containing EVs.[128] This is followed by subsequent high-speed centrifugation and ultracentrifugation steps, which varies among laboratories (10,000 x g - 20,000 x g), to concentrate and pellet large EVs.[126,129] A final high-speed spin (100,000 x g – 200,000 x g) precipitates small EVs (i.e. exosomes).[126,129] However current limitations of differential centrifugation is that EV sedimentation rate is dependent on shape and mass density, relative to the medium.[128,129] Furthermore, EVs are can bind to cells and removal of cells will result in the removal of other EV (sub)populations.[128,129] Lastly, EVs can be difficult to separate from other ‘membrane structures’ as well by centrifugation.[128] It is also now known that high-speed centrifugation of plasma induces

aggregation of platelet-derived EVs, while EVs of erythrocyte origin have less tendency to aggregate.[128,130,131] Lastly, the reported recovery of EVs by differential centrifugation ranges from 2% to 80% making the study-to-study comparability questionable.[129] EV isolation, however, can be improved as the efficiency of isolation by centrifugation depends on many factors such as acceleration (g), type of rotor and its characteristics (k factor, radius of rotation, and sedimentation path length), and viscosity of the sample.[125] Advantages of differential centrifugation is that only a small set of reagents are needed, and no impacts on EV except gravitational force and pipetting (i.e., no chemicals that might interfere with downstream analysis techniques).[125] Due to these advantages and good reproducibility, differential centrifugation is the most commonly used method.[125] Nonetheless, EV characterization is critically important when it comes to distinguishing between EV populations.[125,126,129]

EV quantification and characterization can be carried out using several methods by evaluating EV size, concentration, and molecular cargo.[132] However, due to the technical challenges in obtaining pure EV fractions free from non-vesicular components the ISEV proposed Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines in 2014,[133] which was later updated in 2018.[103] The ISEV currently recommends that each preparation of EVs be: 1) defined by quantitative measures of the source of EVs; 2) characterized to determine abundance of EVs; 3) tested for elements associated with EV subtypes or EVs generically; and 4) tested for the presence of non-vesicular components, co-isolated components.[103] Quantification of EVs in regard to particle number can be measured using light scattering technologies, such as nanoparticle tracking analysis (NTA); by standard flow cytometry for large EVs or high resolution cytometry for small EVs or by other techniques such as resistive pulse sensing (RPS), and electron microscopy (EM).[103] These techniques allow for measurement of relative concentration of EVs

with or without specific surface markers. However, none of these methods are fully reliable in measuring the absolute number of EVs, because of the complexity of the EV samples (i.e., broad size range, present of other elements with similar size as EVs, inability to detect EVs smaller than 50nm).[132] Therefore, researchers have been using several complementary approaches to analyse EVs in biological or clinical EV samples as is recommended by the EV-TRACK initiative.[134]

EVs can also be quantified by their structural components or cargo including proteins, lipids, and nucleic acids, however, none of these values is perfectly correlated with EV number.[103] Depending on the cell they originate from, EVs express varying surface antigen markers specific to their parental cell. Thus, circulating large EVs can be identified as platelet EVs, endothelial EVs, leukocyte EVs, erythrocyte EVs, or tissue factor-bearing EVs (TF-EVs).[112]

1.5.3 Functional Assays for EV Characterization

Functional assays have been applied to determine the various functions associated with EVs; with common models including coagulation, fibrinolysis and angiogenesis.[129] In the context of coagulation, EVs exhibit both procoagulant and fibrinolytic properties and assays have taken advantage of this to enable their use as a biomarker of thrombosis.[129] Their coagulant properties stem from the exposure of anionic phospholipids, especially phosphatidylserine (PS), and by the exposure of tissue factor (TF), thereby triggering the clotting system.[129,135,136] The presence of PS and TF on EVs ultimately depends on the biogenesis, cellular origin, and process leading to the release of the EVs.[135] Several assays quantify the amount of coagulant PS-positive EVs in plasma samples, based on the ability of annexin V to bind PS in the presence of Ca^{2+}, while others are clot-based and rely on phospholipids to accelerate the conversion of prothrombin to thrombin.[137,138] Annexin V in chromogenic factor Xa (FXa) assays, is dependent on PS-positive

EVs that are able to accelerate the thrombin generation by FXa and thus determine pro-coagulant activity.[138,139] The generation of thrombin, after adding prothrombin, FXa, FVa and calcium is measured via cleavage of a chromogenic thrombin substrate producing absorbance at 405nm.[139] Results are expressed as nanomolar phosphatidylserine equivalent (nM PS) by reference to a standard curve of known concentration.[137–139] However, assays measuring the production of thrombin have a limited contact surface and need to ensure that the phospholipid present on EVs is the rate-limiting step of the reaction in order to effectively quantify coagulant activity.[129,137] Clotting assays on the other hand, involve a more complex and physiological end-point to measure the phospholipid activity of plasma, and not only captured PS-positive EVs.[138] Furthermore, annexin V is not a universal and exclusive EV marker; it has been suggested to identify a large proportion of but not all EVs which can give potentially misleading results.[137,140,141]

Other functional assays measure coagulant activity inherent to EVs expressing TF. Hisada and colleagues give an in-depth overview of two TF-dependent assays measuring pro-coagulant activity of EVs in plasma.[142] Both rely on the ability of TF present on EVs to facilitate FXa generation, with one being "kinetic" and units in fM Xa min^{-1}, while the other is referred to as an "endpoint" assay with units in pg/mL.[142] Functional assays have the advantage of detecting activity irrespective of size, however this can also be a disadvantage when there is interference of soluble antigens, variable quality of antibodies used for antigen capture, and nonexclusion of exosomes.[137]

1.5.4 Flow Cytometry for EV Characterization

Flow cytometry is the 'gold standard' method for EV characterization as it allows high-throughput multi-parametric analysis and quantitation of single large EVs.[134,143] It can detect the origin, size and morphology of EVs using hydrodynamic focusing and fluorescence light scattering

technologies.[134] EVs suspended in fluid flow through a compressed chamber in which a laser beam with a specific wavelength illuminates the sample and the emitted scatter and fluorescence is captured by detectors facing forward and perpendicular to the laser (Figure 4).[134] Light scatter captured on the forward facing detector is reported as forward light scatter (FLS) which is proportional to the diameter while light captured on the perpendicular detector is reported side light scatter (SLS) and denotes morphology and anatomy of the EVs.[134] Due to the heterogeneity of EVs, their size cannot be deconvoluted directly from their side scattering signal.[144] Instead, EV scattering intensity is compared to the scattering intensity of beads with a known size and refractive index to determine the approximate size of EVs within the sample.[144] An alternative approach consists of triggering EV detection on a fluorescence parameter instead of light scattering to increase separation of EV signal from background noise.[145] This enables the ability to detect antigens or surface marker proteins on EVs by applying fluorochrome-conjugated monoclonal antibodies.[145] As a result, the EVs of interest can be quantified and/or classified according to the level of antigen expression.[145] Limitations of flow cytometry do exist however, with the detection of small particles being a considerable challenge.[116,144,145] Forward scatter signal is variable between instruments of different manufacturers and its proper alignment is critical.[145] Furthermore, properties of polystyrene beads significantly differ from those of cells.[116,144,145] Given their low refractive index, lipid-based vesicles scatter much less light than polystyrene beads, resulting in substantial uncertainty when determining EV diameters in relation to beads.[116,144,145] Despite the aforementioned limitations of flow cytometry, it possesses significant advantages in EV analysis. It allows for high-throughput, fast measurements of EVs suspended in a fluid, and identification of specific EV subpopulations.[116,144,145]

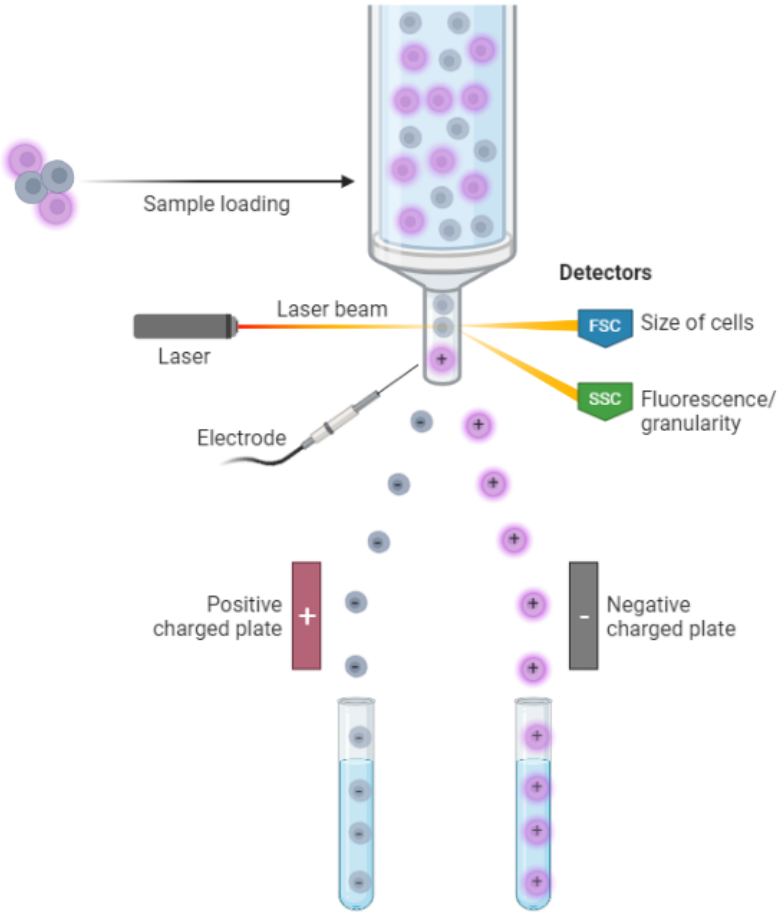


Figure 1.4. Working principle of flow cytometry. The fluidic system in a flow cytometer brings the particle of interest to the interrogation point, where the laser light intercepts the sheath flow stream. The generated scattering and fluorescence signals are collected and analyzed by carefully positioned light detectors.[144]

1.6 Circulating EVs

With the growing knowledge on EVs released from different types of cells, including leukocytes, platelets, endothelial cells – detected from both human and body fluids, have confirmed that EVs play important roles in physiological and pathological conditions.[136,146,147] EVs released into the extracellular space can transfer information to neighboring cells inducing functional and physio-pathological changes.[120,136,147–149] EVs contain biologically active cargo

consisting of proteins, lipids, and nucleic acids which upon transfer can induce transient or persistent modifications in recipient cells.[136,146] Indeed, EVs affect a range of physiological processes from immune signaling, angiogenesis, cell proliferation and apoptosis, tissue homeostasis, inflammation and development depending on the type of cell they are released from.[150–152] For example, EVs secreted by platelets can modulate the clotting response following tissue injury by containing cargo such as α-granules, coagulation factors and growth factors that would ultimately promote wound healing.[97] However, increasing evidence shows that EV biocomposition can shift in response to extrinsic stressors such as heat shock, hypoxia, oxidative stress, and infectious agents, reflecting the physiological state of the cell/tissue of origin.[150,151,153] These changes in EV molecular content can impact signalling cascades to initiate or promote pathological responses associated with malignant, chronic, and infectious disease states.[150] Thus, EVs are important mediators of cell-to-cell communication and are part of the global mechanism to maintain physiological homeostasis, but disturbance of EV roles in homeostasis can result in disease, thereby establishing a new found link between EVs and pathophysiology.[146] With the growing interest in EVs as key mediators of intercellular crosstalk, it has opened a new avenue of research of EVs as diagnostic and prognostic biomarkers.

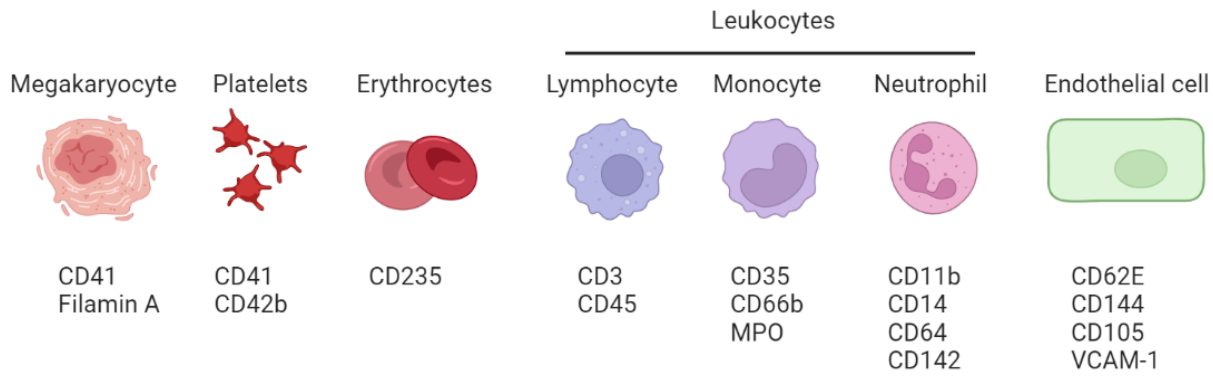


Figure 1.5. Sources of circulating EVs in plasma and their specific cellular markers. MPO: myeloperoxidase; VCAM-1 Vascular cell adhesion protein 1. Adapted from Zarà and colleagues.[136]

Sources of EVs in blood, or circulating EVs, isolated from the plasma of healthy subjects are derived primarily from platelets and/or megakaryocytes, with a subset of EVs originating from erythrocytes, leukocytes, and endothelial cells (Figure 5). However, many studies have shown that the levels of these EV subtypes change under pathological conditions.[136,154–158] Medium/large EVs released from platelets (sometimes referred to as PMPs), can be detected by specific platelet markers such as CD41, CD42, CD61 and CD62.[136,143,159] Platelet-EVs are considered platelet activation markers and have been associated with numerous cardiovascular diseases, thrombotic disorders, autoimmune diseases, type II diabetes, and support thrombin generation, inflammation and autoimmunity.[136,143,159–161] Many platelet-EVs have also been seen to express TF and PS (through fluorescence-conjugated antibodies and annexin V, respectively); thereby supporting their prothrombotic role.[136] Interestingly, some studies have also detected annexin V-negative platelet EVs which have distinct roles other than thrombosis, circulate for a longer period and participate in cell-to-cell communication.[136,162] Additionally P-selectin expressed on platelet-EVs binds leukocyte glycoprotein ligand-1 (PSGL-1), promoting leukocyte aggregation and

accumulation.[136,160] However, to which extent an increase in platelet EVs reflects pathology and disease state is currently unclear.[161]

Medium/large EVs released from endothelial cells (EC-EVs, sometimes referred to as EMPs) can be detected in human plasma using endothelial cell-specific markers including CD54, CD62E, CD62P, CD31, CD106, CD105, CD144, and CD146.[136,143] EC-EVs account for 5-15% of EVs in peripheral blood and have relevant physiological roles in inflammation, endothelial injury and endothelial dysfunction. Furthermore, EC-EVs are mediators of thrombus formation and exhibit pro-coagulant activity which can be partially explained by their increased surface expression of PS and TF.[136,143,163] Endothelial activation or injury can result in an increase in levels; and thus EC-EVs have even been suggested as biomarkers of vascular health.[143,163] For instance, patients with acute and chronic vascular disorders, including acute coronary syndrome[164], severe hypertension[165], end-stage renal failure[166], and pulmonary arterial hypertension[167] have all presented with elevated levels of large EC-EVs.[143,168] However, the role of EC-EVs in the pathogenesis of vascular diseases remains controversial. On one hand, EC-EVs are capable of promoting endothelial cell activation, impairing vasorelaxation, promoting coagulation and thrombosis, but other studies have shown that they can also induce angiogenesis or promote endothelial cell survival.[143,169]

EVs released from immune cells, particularly from monocytes and granulocytes can also engage in the early phases thrombus formation.[136] Leukocyte-derived EVs generally contain inflammatory cytokines (e.g., interleukin 1 beta, intracellular cell adhesion molecules (ICAM-1), P-selectin glycoprotein ligand-1 (PSGL-1), TF, and nucleic acids.[136] However, the plasma membrane and cytosolic protein composition of leukocyte-derived EVs differ amongst the various

subpopulations.[136,163] They have roles in leukocyte activation, trans-endothelial migration, modulating immune responses, inflammatory reactions, and thrombosis.[136]

1.6.1 Circulating EVs in thrombosis

EVs have been associated with thrombosis and coagulation since the 1940s, primarily linked to the surface exposure of negatively charged phospholipids such as PS.[136,147,160] During thrombosis, vascular and blood cells come into contact directly, but they also communicate through EVs released from the different cell types involved in blood clot formation.[163] Circulating EVs within the blood are normally formed in low numbers under tightly regulated conditions and have controlled hemostatic properties, however, circulation of EVs at high concentration could predispose patients to thrombosis.[100,114,170–172] EVs released from activated platelets, leukocytes or from endothelial cells following apoptosis, express high levels of PS (detected by annexin V binding), which can bind coagulation factors, and ultimately enhance fibrin clot formation.[172] Prothrombotic activities of EVs are generally attributable but not restricted to the expression of TF, and the exposure of negatively charged phospholipids, such as PS, on their surfaces.[173–176] Novel studies indicate that the thrombogenicity of EVs is much more complex; involving multiple procoagulant surfaces, cross-linking of different cellular players at the site of injury as well as transfer of activation signals to other cell populations.[163]

EVs that express PS on their surface are considered procoagulant, as PS can facilitate the assembly and activation of coagulation factors (Figure 6).[163] Activated coagulation factors can bind and assemble in the presence of calcium ions, forming the tenase (factors VIIa, IXa, and X) and prothrombinase (factors Va, Xa, and II) complexes, thereby potentiating coagulation (Figure 6).[147,163] PS specifically binds in a reversible and calcium-dependent manner to the γ-

carboxyglutamic acid (GLA) domains located at the N-terminal of coagulation factors FVII, IX, X and II (prothrombin).[177] In addition, EVs expressing PS may directly associate with factor XII and support thrombin generation via activation of the intrinsic coagulation cascade.[163] Although PS is exposed on the surface on most large EVs, particularly of platelet origin, one study detected the presence of EVs staining negative for annexin V, demonstrating the existence of PS-negative EV population.[136]

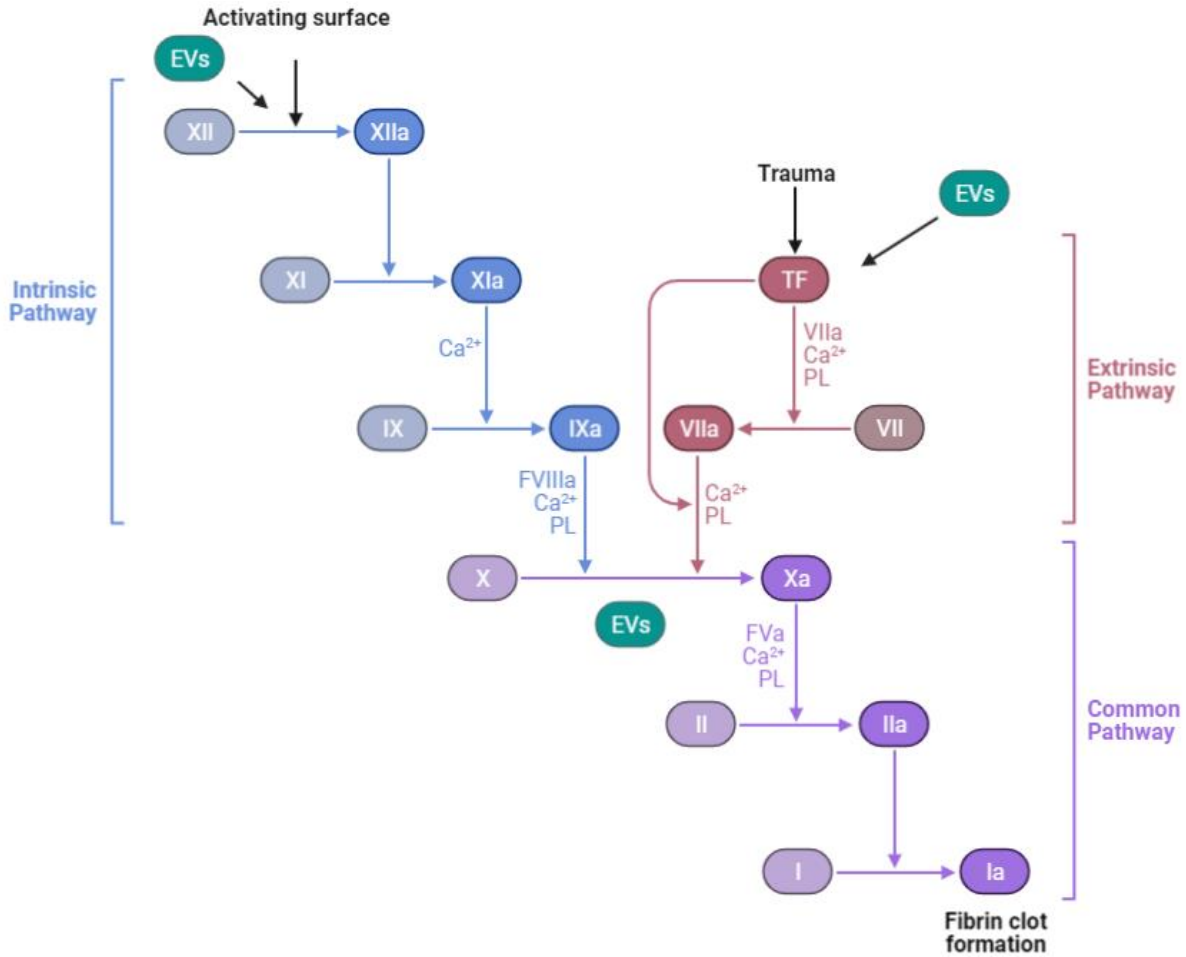


Figure 1.6. Mechanism of blood coagulation and the pro-coagulant role of EVs. The clotting or coagulation cascade consists of the intrinsic pathway which is activated with the exposure of factor XII with the collagens of injured blood vessels; and the extrinsic pathway which is activated by the release of tissue factor from trauma or EVs and activation of factor VII. The role of EVs in the coagulation cascade is shown.

TF also known as factor III (FIII) or CD142 is a 263-amino acid polypeptide consisting of an extracellular N-terminal domain, a transmembrane domain, and a cytoplasmic C-terminus.[135] Upon binding of its extracellular domain with coagulation protease factor VII/VIIa, it forms the TF/FVIIa complex, which triggers the initiation of the extrinsic coagulation cascade, leading to thrombin production which culminates fibrin generation.[135,136,178] The presence of TF on circulating EVs, also referred to as tissue factor-positive EVs (TF-EVs), lead to the proposal that EV-TF activity would reflect prothrombotic state of cancer patients.[136,178,179] Exposure of TF is observed on EVs deriving from platelets, erythrocytes, neutrophils, granulocytes, and monocytes.[163] Although under physiological conditions, TF is expressed by cells surrounding blood vessels such as fibroblasts of the tunica adventitia, and is not constitutively expressed by cells in the circulation.[136] This allows for the formation of a hemostatic envelope preventing circulating FVII/VIIa from interacting with TF, resulting in inappropriate coagulation.[135] However, these cells in contact with the blood such as monocytes and endothelial cells can transiently express it under pathological conditions or upon activation.[135,136,180] Different mechanisms that may regulate the activity of TF expressed on EVs have also been proposed such as post-translational modifications or low (also encrypted) vs. high-activity conformational states. Previous results have also concluded that TF-EVs participate in coagulation activation but not in coagulation propagation.[181] This suggests that EVs from different cellular sources may have different procoagulant activities.[182] One study found that TF-expressing monocyte-derived EVs had the highest ability to induce spontaneous clotting but a weak effect on coagulation propagation, compared to platelet-EVs, EC-EVs, and erythrocyte-EVs.[183] Contrastingly, platelet-EVs and erythrocyte-EVs induce clotting only through the contact pathway which is much weaker, and contribute more to coagulation propagation.[139,163,183]

In addition to TF expression on cells, a low amount of TF is present in the blood of healthy individuals, called "blood-borne TF", and is mainly associated with EVs originating from both vascular and circulating cells.[184] Studies have demonstrated that the physiological levels of blood-borne TF can increase in many disease states and pathological conditions. Therefore, upon vascular injury, the exposure of TF on the vessel wall would play a role in the initiation phase of coagulation, whereas blood-borne TF would be involved in the propagation phase of thrombus formation.[136] The procoagulant role of EVs has also been shown to be increased by the ability of PS to activate tissue factor TF, providing a catalytic surface for the assembly of enzymatic coagulation complexes that initiate and maintain coagulation.[107,115] This function eventually gives rise to the homeostasis as well as the pathological process of thrombosis.[115]

The role of circulating EVs in thrombosis is further complicated by the indirect ability to contribute to thrombosis, independent of TF and PS surface expression, that propagate intercellular communications.[136,163] Other procoagulant molecules and receptors carried by EVs that have been shown to contribute to thrombus propagation include: P-selectin glycoprotein ligand-1 (PSGL-1, CD162) which can mediate interaction of EVs with CD62P expressed on activated platelets and endothelial cells.[185] As well as, glycoprotein GpIb on procoagulant platelet EVs has been shown to bind Von Willebrand factor (vWF), on Weibel-Palade bodies released from injured or activated endothelial cells.[163] Insights into the intrinsic ability of EVs to contribute to thrombosis are complex and multifold involving multiple pathways and interactions with vascular cells and components of the hemostatic system.

1.7 Circulating EVs in cancer associated VTE: clinical studies

Cancer patients that develop VTE have been seen to present with elevated baseline EV levels of varying phenotypes compared to cancer patients that do not develop VTE.[186–188] Additionally, a few studies found an association between elevated EVs (based on author cut-off) and risk of cancer associated VTE, suggesting that elevated EV levels could play a role in identifying patients who may benefit from thromboprophylaxis.[174,189,190]

To date, many studies have investigated the association between circulating EVs, and cancer patients suffering from VTE. While some report on elevated plasma EV levels,[172,190] other focus on EV-surface expression of particular antigens,[186,191] the procoagulant activity[192,193], or a combination of the above,[172,190,194] suggesting that these EVs may be responsible for the thrombotic state associated with cancers.[174,176,187,189,195,196] These studies are further complicated by study design with some being case-control[186,193], cross-sectional[173], retrospective cohort[174,194,195], and prospective cohort studies.[175,188,196,197]

The majority of clinical studies are retrospective and found elevated levels of TF-EVs and TF-EV activity in cancer patients with VTE compared with those without VTE.[186,194,195] There is a significant lack of large prospective clinical trials analyzing the predictive value of EVs for future VTE and those that have bear conflicting results. For example, Zwicker *et al.,* found that elevated levels of tissue factor-bearing microparticles (TF+MPs) were associated with VTE in cancer patients of different histology (adjusted OR 3.72, 95% CI 1.18-11.76, P=0.01).[174] Similarly, Faille *et al.,* found that biomarkers D-dimer and microvesicle-tissue factor (MV-TF) activity were significant risk factors for VTE in pancreatic adenocarcinoma,[188] whereas another study in patients with multiple myeloma did not find such an association.[187] Cumulative probability of VTE at 6

months was also found to be significantly higher in patients with elevated D-dimers and in patients with high MV-TF activity.[188] A third case-control study by Wang and colleagues, found that the proportion of MPs and TF+MPs were significantly more elevated in patients with VTE than in those without VTE ($P<0.05$ for both).[197] However, other studies have failed to show increased MPs as predictive biomarkers of future thrombosis.[175,187,198] Thaler *et al.* determined MP-TF activity in pancreatic, gastric, colorectal and brain cancer patients but were unable to find a significant association of MP-TF activity with the risk of VTE in any of the cancer types.[175] Likewise, Hernandez *et al.* performed a prospective cohort study and were unable to find a statistically significant association for TF activity of isolated MPs with the risk of VTE.[198] However, one has to keep in mind that these studies do not provide an answer to the question whether increased presence of coagulant TF+EVs directly contributes to cancer-associated VTE, or is merely a consequence of the thrombotic state.[199] The reason for the discrepancy between studies is unclear, but may be related to variability in thrombotic risk with different malignancies, stages of cancer, cancer treatment but also methodological discrepancies, and a significant lack of standardization.[115,178,199] Also pre-analytical variables such as blood withdrawal, EV isolation, and EV preparation are not consistent across the studies.[178,199] One should also take into consideration the relationship between EV levels and physiological variations, such as fluctuations according to time, age, and sex, needs to be further explored. A recent meta-analysis, including two case-control studies and four cohort studies, found that TF-bearing MPs were associated with increased risk of VTE in patients with cancer but concluded that more well-designed studies and more comprehensive adjustments for confounders were required to affirm the association.[200] Regardless of these differences, evidence primarily in pancreatic cancer indicates that EVs bearing TF might play an important role in the pathogenesis of VTE.[199,201]

Despite the emerging evidence supporting a role of EVs in predicting VTE, the utility of EVs beyond that of current prediction tools is not clear and the utility of EVs in assessing response to prophylactic antithrombotic therapy has not been studied. We therefore measured levels of circulating EVs in a cohort of high-risk ambulatory cancer patients recruited in the Apixaban for the Prevention of Venous Thromboembolism in High-Risk Ambulatory Cancer patients (AVERT) trial.[93]

Rationale and Background

Patients with active cancer have substantially increased risk of venous thromboembolism (VTE) which is associated with significant morbidity and mortality in an already high-risk population.[35,202] Our collaborator Dr. Wells, and others have shown that parenteral and oral prophylactic treatment can reduce the risk of VTE in patients undergoing chemotherapy[93,203]. However, routine thromboprophylaxis is not recommended for ambulatory cancer patients because the reduction in risk of VTE is not great enough to outweigh the moderately increased risk of bleeding.[89] The number needed to treat (NNT), to prevent one episode of symptomatic VTE of about 30 is judged to be too low.[89] Also, many patients and oncologists do not consider VTE to be of major importance when their primary aim is focused on treating the cancer. Therefore, strategies to identify those at greatest risk of VTE may help to target individuals who would benefit most from prophylaxis while minimizing impact on those not at risk (i.e., a lower NNT). The most well-known risk stratification tool is the Khorana score, which was introduced in 2008.[50] Over 50 studies have evaluated its score since its publication, but concluded results were conflicting with most events occurring outside the high-risk group, and the estimated risk being considerably lower for lung cancer patients and hematologic malignancies.[88] Thus, there have been calls by many,

including the developer of the Khorana score, to find new biomarkers that can refine the score and increase its accuracy.[77,95]

One emerging biomarker that has shown promise in cancer associated VTE is circulating large EVs, or MPs. The contribution of EVs, in thrombotic events are mainly due to their procoagulant surface made up by anionic phospholipids, such as PS, and expression of highly coagulant proteins, such as TF.[136] Clinical studies have demonstrated that platelet EVs[186,188], endothelial EVs[186], TF-EVs[190,196], and the TF-activity[175,194,195] of EVs are all increased in cancer patients with VTE compared to patients without VTE, with some studies demonstrating cancer patients with higher TF-EV levels have a higher risk of developing VTE.[149,151] Despite, these promising results, many others have failed to demonstrate their role as predictive biomarkers in small cell lung, stomach or colorectal cancer, and in multiple myeloma patients[175,204–206], with significant associations only observed in pancreatic cancer.[142] This suggests that EVs directly and indirectly enhance thrombosis, however, whether they can predict VTE outcomes amongst cancer patients already deemed to be high-risk for VTE has yet to be determined. Furthermore, levels of EVs in cancer patients that develop bleeding events and in response to prophylactic antithrombotic therapy has not yet been assessed.

Objective

The objective of this study is to determine whether levels of circulating large extracellular vesicles (EVs) are associated with hematological outcomes (venous thromboembolism, major bleeds, and clinically relevant non-major bleeds (CRNMB)) in ambulatory cancer patients, and to assess the impact of prophylactic antithrombotic therapy (Apixaban). We have three specific aims:

1. To determine whether the level of EVs (endothelial, platelet or TF+) predict risk of VTE in patients already determined to be at high risk such that they enable selection of a very high-risk group.
2. To determine if EVs select a very high-risk group, if bleeding risk is independent of EV levels, enabling EV levels to select patients with a higher benefit/risk ratio while taking antithrombotic therapy.
3. To determine whether reductions in circulating EVs are indicative of response to prophylactic antithrombotic therapy (Apixaban).

Hypothesis

We hypothesized that elevated levels of circulating large EVs will be predictive of cancer associated VTE and/or bleeding events and that treatment with Apixaban will reduce EV levels and incidence of cancer VTE.

CHAPTER 2: METHODOLOGY

2.1 Study participants and ethics

We examined plasma samples from the Apixaban for the Prevention of Venous Thromboembolism in High-Risk Ambulatory Cancer patients (AVERT) trial. Details of the clinical study protocol have been previously published.[93] In brief, it was a randomized, placebo-controlled, double-blind clinical trial assessing the efficacy and safety of apixaban (2.5 mg twice daily) for thromboprophylaxis in ambulatory patients with cancer who were at intermediate-to-high risk for venous thromboembolism (Khorana score ≥2) and were initiating chemotherapy. Randomization occurred up to and including 5 days prior to the administration of the first anti-cancer treatment. All enrolled patients were followed for the entire 7 months (180 days on study drugs and an additional 30 days of follow-up) in which blood samples were drawn at baseline, 1, 3, 6, and 7-month visits. The primary efficacy outcome was objectively documented venous thromboembolism over a follow-up period of 180 days. The main safety outcome was a major bleeding episode. We conducted a secondary analysis on 660 plasma samples from 330 cancer patients. Only paired samples available from collections at baseline (0 days) and 3-months follow-up were studied. The study was approved by the Ottawa Hospital Research Ethics Board and samples were analyzed in a blinded fashion.

2.2 Determination of VTE and bleeding events

The efficacy outcome of VTE was defined as a first episode of objectively documented major venous thromboembolism (proximal deep-vein thrombosis or pulmonary embolism) within the first 180 days. VTE was defined as any symptomatic or incidentally detected proximal deep-vein thrombosis of the lower or upper limbs, any nonfatal symptomatic or incidental pulmonary

embolism, and pulmonary embolism-related death. Patients who presented with symptoms of VTE during the follow-up period underwent diagnostic imaging for confirmation of the primary outcome.

The safety outcome of major bleeding was defined by the International Society on Thrombosis and Hemostasis as overt bleeding that was associated with a decrease in the hemoglobin level of 2 g per deciliter or more, led to the transfusion of 2 or more units of packed red cells, occurred in a critical site, or contributed to death. Other safety outcomes included clinically relevant non-major bleeding and overall survival during the trial period. Adherence to the trial regimen was estimated with the use of a pill count recorded by patients in a medication diary.

2.3 Measurement of pro-coagulant EV-activity

The ZYMUPHEN MP-activity functional assay kit (Figure 2.1) was used for the measurement of large EV, or microparticle (MP), procoagulant activity in human plasma. In brief, a microtiter plate pre-coated with annexin V-streptavidin captures EVs from the diluted assayed plasma sample, supplemented with calcium, Factor Xa and thrombin inhibitors. Phosphatidylserine (PS) on the surface of EVs binds to the plate, exposes their phospholipid surface, allowing the pro-thrombinase complex to cleave prothrombin into thrombin. The phospholipids concentration is then the limiting factor and there is a direct relationship between the phospholipid's concentration and the amount of thrombin generation. Thrombin generation is measured by a specific chromogenic substrate, giving an indication of the amount of PS-expressing EVs in the sample.[139]

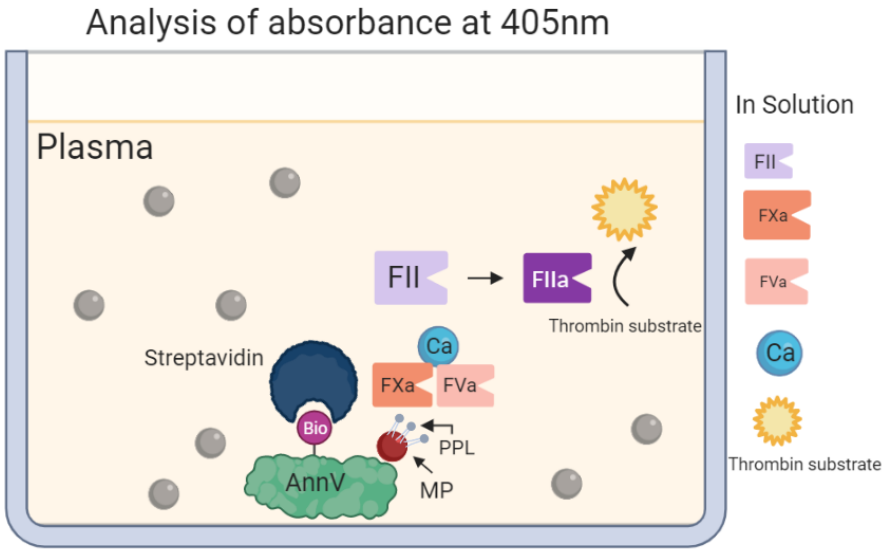


Figure 2.1 ZYMUPHEN MP-activity kit schematic.

The platelet-poor plasma samples and kit controls were diluted 1:20 in the kit sample diluent, which is supplemented with calcium Factor Xa (FXa) and thrombin. A calibrator curve, diluted controls and diluted samples were added to the microtiter plate and incubated for 1 hour at 37°C. The plate was washed five times, then 100 µL of Reagent A (FXa-FVa) and 50 µL of Reagent 2 (prothrombin) were added and incubated for 10 min at 37°C, then 50 µL of Reagent 3 (thrombin substrate) was added the plate was read at 405 nm, subtracting the blank. Results were calculated from the calibrator curve and are expressed as nanomolar (nM) phosphatidylserine equivalent.

2.4 Isolation and immunolabeling of EVs from plasma

We isolated circulating large EVs from archived platelet-free plasma as described previously.[207] Plasma samples were thawed quickly in a water bath at 37°C. One-hundred eighty microliter of samples were centrifuged at 12,000 x g for 2 min at 4°C. One-hundred fifty microliter

of the supernatant was transferred to fresh tubes and centrifuged at 20,000 x g for 20 min at 4℃. The supernatant was discarded, and the large EV pellets were re-suspended in 150 μL of Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4, 0.1 μm filtered). In fresh tubes, 50 μL of samples were diluted in Annexin V binding buffer to a final volume of 200 μL for platelet labelling. The platelet samples labelling was performed using anti-CD41-APC (1:100) and Annexin V- FITC (1:50) as labels. Tissue factor labelling was conducted by diluting 100 μL of resuspended samples in Annexin V binding buffer to a final volume of 200 μL. Anti-CD142-APC (1:100) and Annexin V- FITC (1:50) as labels were used to label tissue factor-bearing EVs. As a negative (isotype) control, a subpopulation of EVs were resuspended in Annexin V binding buffer containing an APC-conjugated IgG control antibody. After labelling, samples were re-centrifuged at 20,000 x g for 20 min at 4℃ after a 2-hour incubation in the dark at room temperature. The supernatant was removed, and the pellet was preserved and re-suspended in 300 μL of Annexin V binding buffer. The samples were transferred to flow cytometry tubes for analysis containing counting beads (AccuCount Blank Particles – 1 x 10^6 particles/mL, Spherotech) as reference particles for calibrating EV concentration (EVs/mL) in each tube. All isotype controls, antibodies and Annexin V were purchased from BioLegend (San Diego, USA). Diagram of EV isolation and immunolabeling for flow cytometry is shown in Figure 2.2.

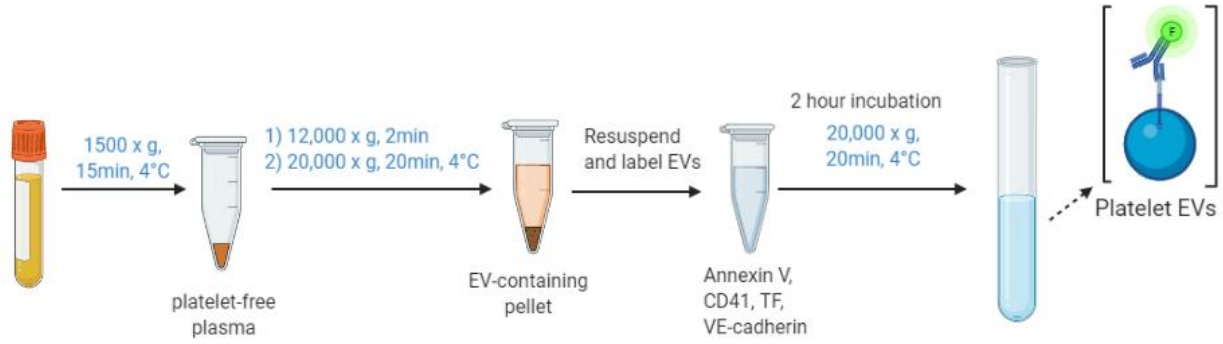


Figure 2.2. Diagram of EV isolation and immunolabeling for flow cytometry. Differential centrifugation was used to isolate medium/large EVs, followed by immunolabeling with fluorescent markers specific for platelet (CD41), TF- (CD142), and total EVs (annexin V).

2.5 Quantitation of circulating EVs by nanoscale flow cytometry

Circulating large EVs were quantified at the University of Ottawa Flow Cytometry and Virometry Facility using a Beckman Coulter CytoFLEX S with CytExpert Version 2.3.0.84. ApogeeMix beads (Apogee Flow Systems, Hertfordshire, UK) were used to establish a size gate of ~100-1000 nm. Large EVs were identified as ~100-1000 nm particles in size staining positive for the membrane marker Annexin V compared to negative controls. Results are expressed as the number of annexinV+ (Total), annexinV+ and CD41+ (platelet), and annexinV+ and CD142+ (tissue factor) EVs/mL plasma. Samples labelled with isotype controls, antibodies alone in buffer, and unlabelled samples were analyzed as controls. FlowJo version 7.6.5 (FlowJo, Ashland, OR) was used for analysis.

2.6 Gating strategy

The gating strategy is illustrated in Figure 2.3. for identifying and analyzing EV populations of interest stained for platelet marker CD41, tissue factor marker CD142, and total EVs with the phosphatidylserine marker annexin V. The first gating strategy employs initial gating on apogee beads to establish size limit for identifying EVs in the range of 100-1000nm. This may be achieved by plotting side scatter (SSC) in logarithmic mode and forward scatter (FSC) in logarithmic mode. Include all events with a FSC less than or equal to that of the 1um and greater than or equal to 0.1um microspheres (Region 1 in Fig. 2.3A). This allows for the discrimination of medium/large EVs from background and other vesicles.

Region 1 in Figure 2.3A is taken to establish the population of EVs staining positive for the platelet marker CD41 and phosphatidylserine marker annexin V. Specific gating of platelet and TF-EVs is shown in Figure 2.3B to discriminate from all medium/large EVs. This is established by plotting APC in logarithmic mode against FITC in logarithmic mode. Platelet EVs or TF-EVs can be quantified from fluorescent readings in Fig. 2.3B, quadrant 2 (Q2), while total EVs are quantified using both Q1 and Q2. Fluorescent readings from Q1 and Q2 in labelling buffer controls and isotype controls are subtracted from sample readings, followed by calibration with counting beads using Figure 2.3C, Region 2.

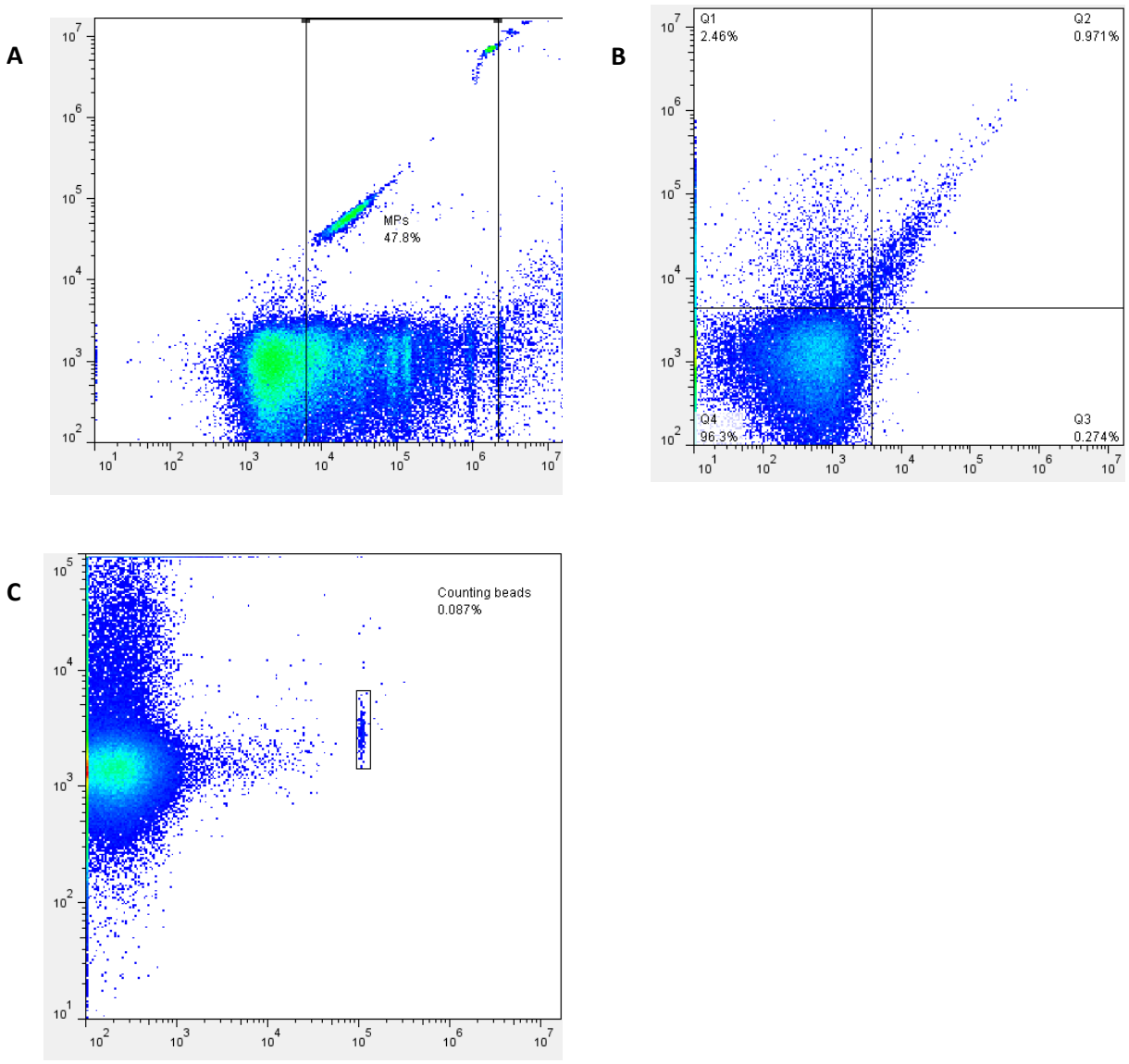


Figure 2.3. Gating strategy for isolation and analysis of EVs on FlowJo. A. Gating of initial medium/large EVs from background. B. Gating of EV subpopulations (platelet and TF-EVs). C. Gating of counting beads.

2.7 Data and Statistical Analysis

Results are expressed mean ± SEM. Statistical analyses were carried out by nonparametric Mann-Whitney U independent t-test (as distribution of sample data was not of Gaussian form), linear regression with R value, one-way ANOVA (Kruskal-Wallis) with Dunn's multiple post hoc comparisons test, and repeated measures ANOVA with Bonferroni post hoc comparisons test. The software packages used were Python Jupyter Notebook (version 6.4.4), and JASP (JASP Team, version 0.16.3) as indicated. Python Jupyter Notebook was used to generate figures, while all statistical tests and analyses were conducted with JASP. Statistical significance was inferred at $p < 0.05$.

CHAPTER 3: RESULTS

3.1 Patient characteristics of measured pro-coagulant EV-activity

Circulating pro-coagulant EV-activity was analyzed in a total of 660 plasma samples from 330 cancer patients using the Zymuphen MP-Activity Assay at baseline and 3-months follow-up timepoints. Out of 330 patients, 28% (92) had lymphoma, 27% (90) gynecologic cancer, 11% (37) pancreatic cancer, 9% (30) lung cancer, 6% (19) brain cancer, 5% (16) stomach cancer, 4% (13) myeloma, 4% (12) breast cancer, 2% (5) colon cancer, 1% (2) bladder cancer, 4% other cancers. The median age was 62 years (IQR: 55-69), 42% (139) were male and, 51% (169) patients received Apixaban. Out of the hematological outcomes: 5% (18) had a VTE event, and 8% (27) had a bleeding event (both major bleed and CRNMB combined) within the first 180 days after randomization (Table 3.1).

Table 3.1. Baseline characteristics and outcomes of cancer patients from the AVERT trial in which procoagulant MP-activity was determined.

Cancer Type	Patients, n (%)	Age, years	Sex, male, n (%)	Received Apixaban, n	First VTE event, n	Bleeding event, n
Lymphoma	92 (28)	63 (53-70)	53 (58)	47	2	8
Gynecologic	90 (27)	61 (54-68)	0 (0)	46	5	10
Pancreatic	37 (11)	63 (59-68)	22 (59)	21	4	2
Lung	30 (9)	62 (56-73)	15 (30)	16	0	1
Brain	19 (6)	60 (53-66)	14 (74)	11	4	1
Stomach	16 (5)	62 (60-67)	10 (63)	9	2	1
Myeloma	13 (4)	71 (64-75)	8 (62)	6	0	1
Breast	12 (4)	59 (46-68)	0 (0)	4	0	1
Colon	5 (2)	58 (56-62)	3 (60)	1	0	0
Bladder	2 (1)	47 (42-53)	1 (50)	0	0	0

Other	14 (4)	67 (59-72)	10 (71)	8	1	2
TOTAL	330	62 (55-69)	139 (42)	169	18	27

3.2 Pro-coagulant EV-Activity at baseline

The baseline pro-coagulant EV-activity was significantly greater in male patients than in female (15.48 ± 1.27 vs. 11.54 ± 0.87 nM; $p<0.05$; Figure 3.2.1) and in patients 55 years of age or older compared with those younger (13.86 ± 0.86 vs. 11.18 ± 1.43 nM; $p<0.05$; Figure 3.2.2B). Linear regression analysis of baseline EV-activity also demonstrated an increase in EV-activity as patients age ($R^2=0.022$, $p<0.007$) (Figure 3.2.2A). There was also a significant difference in baseline pro-coagulant EV-activity between the different cancer types (Figure 3.2.3; Supplementary table 2). Dunn's multiple post hoc comparisons test revealed significant increases in baseline EV-activity for lymphoma, lung and other cancer patients compared to brain and gynecologic cancer patients (17.25 ± 1.57, 18.98 ± 3.67, and 16.60 ± 2.17 vs. 6.24 ± 0.76 and 8.14 ± 0.88). Other cancer patients included: chronic lymphocytic leukemia, jejunum, gastroesophageal, distal oesophageal, cholangiocarcinoma, testicular, hypopharynx, peritoneum, hematologic, spleen, mesenteric root mass and unknown. Lastly, baseline EV-activity was found to be significantly increased in patients with new cancers compared to patients with recurrent cancer (13.84 ± 0.84 vs. 9.85 ± 1.23, $p<0.05$; Figure 3.2.4).

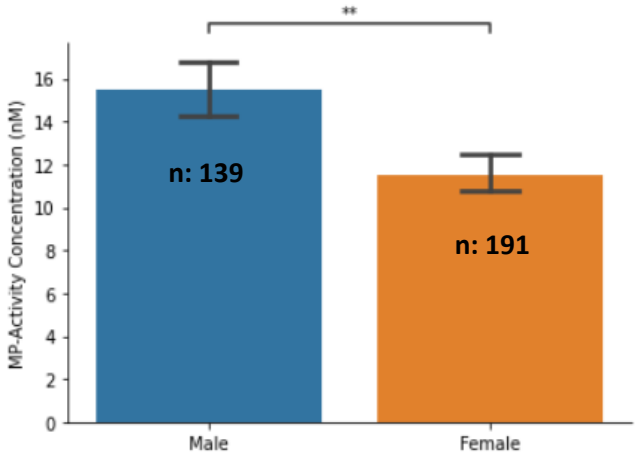


Figure 3.2.1. Pro-coagulant EV-activity was assessed was assessed at baseline for paired patient samples. MP-activity concentration (nM) was measured by sex (males and females). Data presented as Mean +/- SEM, **P<0.01, N values are displayed. Mann-Whitney U independent samples t-test.

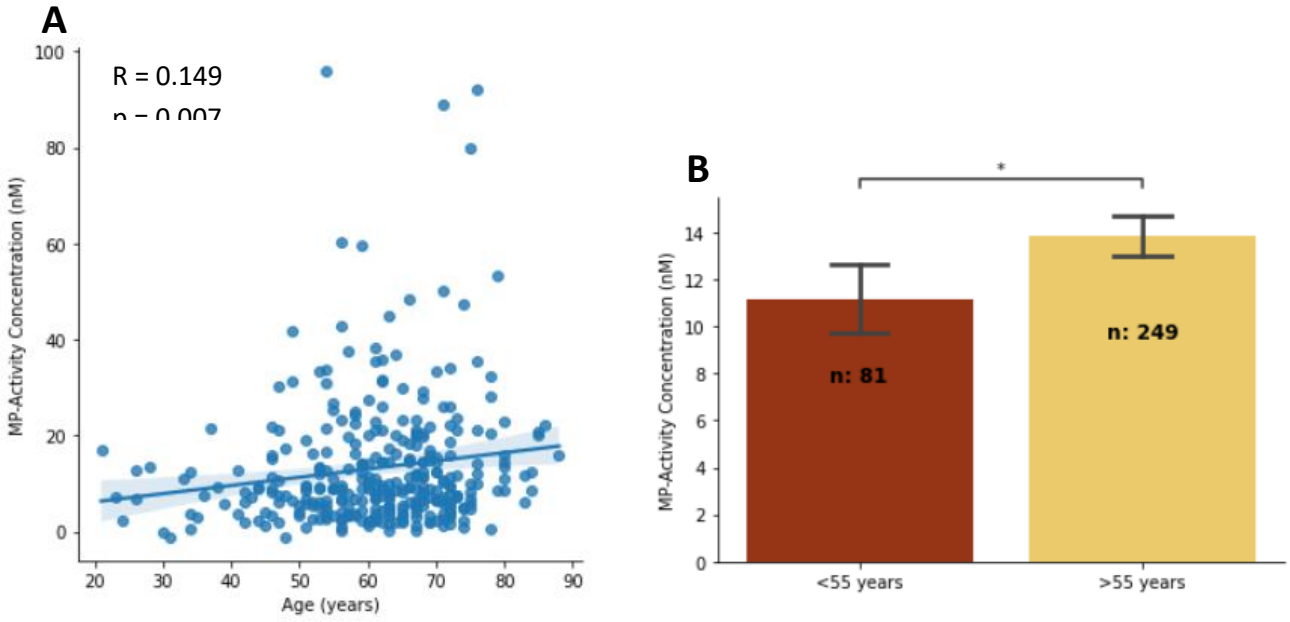


Figure 3.2.2. Pro-coagulant EV-activity (MP-activity concentration (nM)) was assessed at baseline for paired patient samples. (A) Linear regression equation was found to be F(1,328)= 7.417, p = 0.007, with an, R of 0.149. (B) EV-activity was also measured by patients above 55 years of age. Data presented as Mean +/- SEM, *P<0.05, N values are displayed. Mann-Whitney U independent samples t-test.

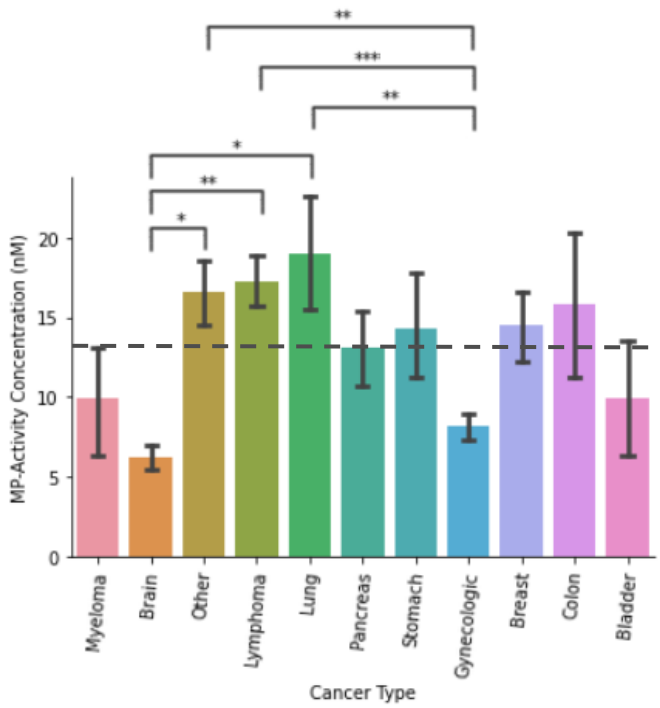


Figure 3.2.3. Pro-coagulant EV-activity (MP-activity concentration (nM)) at baseline was measured by cancer type, Data presented as Mean +/- SEM, *P<0.05,**P<0.01, ***P<0.001. Dashed line represents the mean of the mean for each cancer type. One-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparison post test.

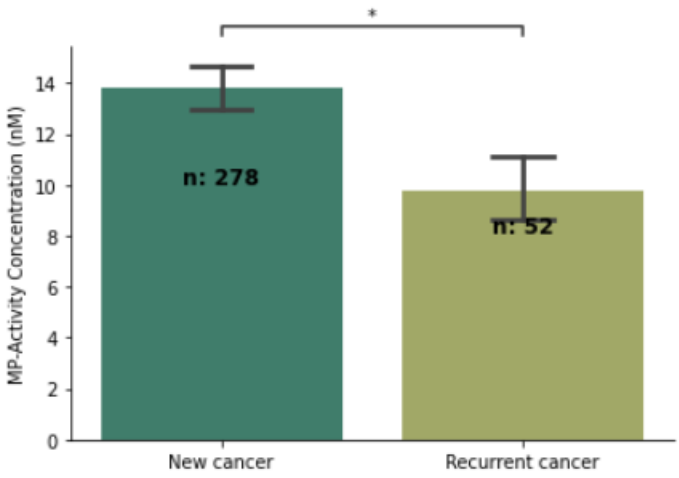


Figure 3.2.4. Pro-coagulant EV-activity was assessed at baseline for paired patient samples. MP-activity concentration (nM) was measured by new or recurrent cancers. Data presented as Mean +/- SEM, *P<0.05, N values are displayed. Mann-Whitney U independent samples t-test.

There was no significant difference in baseline pro-coagulant EV-activity in the 18 patients that had a VTE event compared to the 312 patients that did not (14.45 ± 2.17 vs. 13.13 ± 0.78 nM; Figure 3.2.5). Baseline pro-coagulant EV-activity was also not significantly different in the 27 patients that had major and non-clinically relevant bleeding events than the 303 that did not (16.35 ± 3.16 vs. 12.92 ± 0.76 nM; Figure 3.2.6).

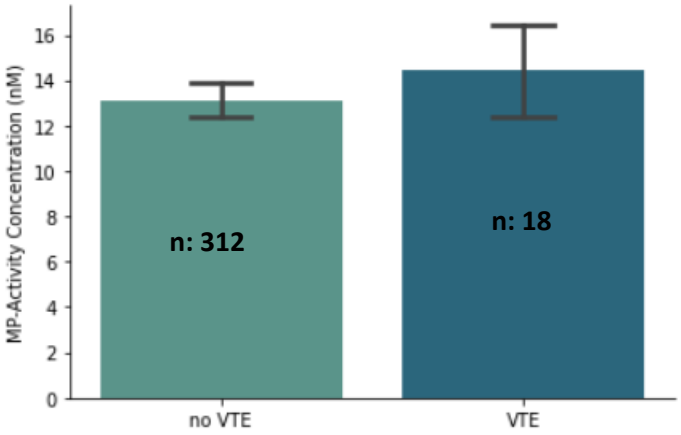


Figure 3.2.5. Pro-coagulant EV-activity (MP-activity concentration (nM)) at baseline for paired patient samples was measured by VTE status, Data presented as Mean +/- SEM, p=0.57. N values are displayed. Mann-Whitney U independent samples t-test.

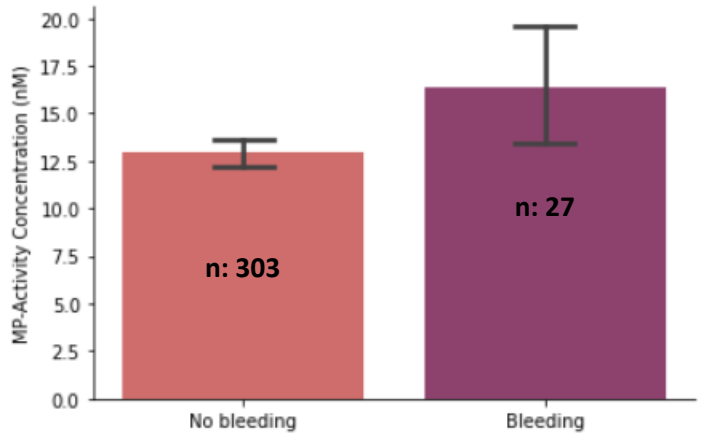


Figure 3.2.6. Pro-coagulant EV-activity (MP-activity concentration (nM)) at baseline for paired patient samples was measured by bleeding event, Data presented as Mean +/- SEM, p = 0.30. N values are displayed. Mann-Whitney U independent samples t-test.

3.3 Flow Cytometry

Circulating EVs were also quantified using flow cytometry as a second approach in the same pool of paired 660 plasma samples at baseline and 3-months follow-up from 330 cancer patients. Circulating EVs were quantified as platelet EVs, tissue factor-bearing EVs or total EVs. However, due to instrument limitations, quantification of EVs by flow cytometry has yet to be completed; and an interim analysis was conducted. For platelet and total EVs, EVs were quantified in 56 paired patient samples, and for TF-EVs, EVs were quantified in 51 paired patient samples. Out of the 56 patients, 30% (17) gynecologic cancer, 23% (13) had lymphoma, 9% (5) myeloma, 9% (5) pancreatic cancer, 5% (3) brain cancer, 7% (4) lung cancer, 7% (4) stomach cancer, 2% (1) bladder cancer, 4% (2) breast cancer, 2% (1) colon cancer, and 4% (2) other cancers. The median age was 63 years (IQR: 54-71), 45% (25) were male and, 50% (28) patients received Apixaban. Out of the hematological outcomes: 4% (2) had a VTE event, and 6% (3) had a bleeding event (both major bleed and CRNMB combined) within the first 180 days after randomization (Table 3.2).

Table 3.2. Baseline characteristics and outcomes of cancer patients from the AVERT trial in which total EVs was quantified by flow cytometry.

Cancer Type	Patients, n (%)	Age, years	Sex, male, n (%)	Received Apixaban, n	First VTE event, n	Bleeding event, n
Gynecologic	17 (30)	68 (56-71)	0 (0)	7	1	2
Lymphoma	13 (23)	55 (53-69)	8 (62)	6	0	1
Myeloma	5 (9)	63 (57-71)	3 (60)	3	0	0
Pancreatic	5 (9)	62 (59-63)	3 (60)	3	0	0
Brain	3 (5)	54 (53-63)	2 (67)	1	0	0
Lung	4 (7)	62 (60-65)	3 (75)	2	0	0
Stomach	4 (7)	61 (55-62)	3 (75)	3	1	0

Breast	2 (4)	71	0	2	0	0
Bladder	1 (2)	42	1	0	0	0
Colon	1 (1)	67	0	0	0	0
Other	2 (4)	76 (74-78)	2 (100)	1	0	0
TOTAL	56	63 (54-71)	25 (45)	28	2	3

3.4 Platelet EVs

Platelet EV levels measured at baseline were not significantly increased in males compared to females (5.31 ± 1.11 x 10^7 vs. 3.69 ± 0.50 x 10^7 /mL; p=0.624; Supplementary Figure 3.4S1). Patients did not show any significant correlations between baseline platelet EVs and age ($F(1,55)=3.483$, $R = 0.244$, $p = 0.067$); nor was there a difference in platelet EVs between patients >55 compared to <55 years of age (3.98 ± 0.67 x 10^7 vs. 5.40 ± 1.06 x 10^7 /mL; p = 0.148; Supplementary Figure 3.4S2A and B, respectively). A one-way ANOVA (Kruskal-Wallis) revealed significant differences between the different cancer types $F(1,46) =1.921$, $p=0.036$ (Figure 3.4.1). Baseline platelet EVs in bladder and colon cancer were not included in the statistical analysis as there was only one patient in each group. Dunn's post hoc comparisons test indicated significant increases in stomach (8.86 ± 3.17 x 10^7) and lymphoma (6.50 ± 1.41 x 10^7) cancer types compared to myeloma (1.35 ± 0.59 x 10^7), gynecologic (3.36 ± 0.78 x 10^7), and other cancers (1.63 ± 0.70 x 10^7) (Supplementary Table 3). Platelet EVs at baseline compared between new and recurrent cancer types also did not show any significant differences (4.77 ± 0.67 x 10^7 vs. 2.83 ± 0.69 x 10^7 /mL; p = 0.245).

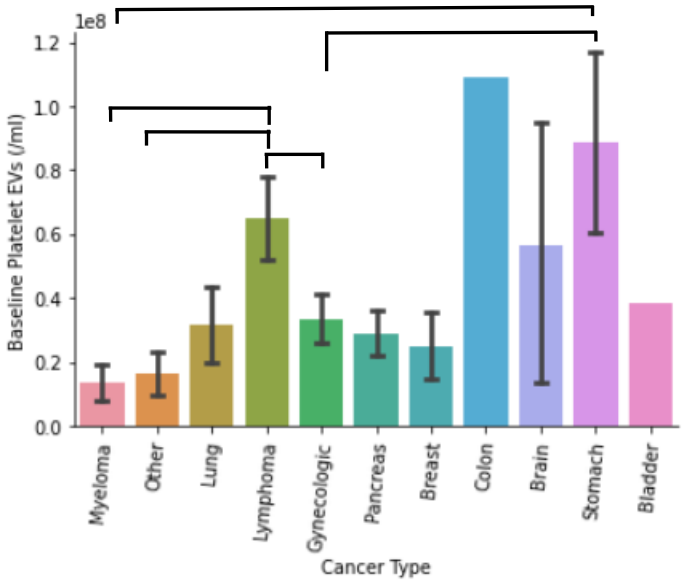


Figure 3.4.1. Platelet EVs (/mL) at baseline were measured by cancer type. Data presented as Mean +/- SEM. One-way ANOVA (Kruskal-Wallis) with Dunn's post hoc comparisons test; F=(1,46) =1.921, p=0.036.

The difference between platelet EVs at baseline in patients presenting with or without VTE and bleeding events were also compared. The two patients presenting with VTE had baseline platelet EVs of 2.53 ± 0.93 x 10^7 /mL which was lower than the 55 patients that did not present with VTE (4.47 ± 0.59 x 10^7 /mL, p=0.680; Supplementary Figure 3.4S5). Baseline platelet EVs were also not significantly different in the 3 patients that had a bleeding event compared to the 54 that did not (3.47 ± 1.03 vs. 4.48 ± 0.68 x 10^7 /mL; p = 0.84; Supplementary Figure 3.4S6).

3.5 Tissue factor-EVs

TF-EVs measured at baseline showed no difference in male versus female patients (6.04 ± 1.77 x 10^5 vs. 8.41 ± 4.76 x 10^5 /mL; p=0.158; Supplementary Figure 3.5S1) nor in patients above 55 years of age compared to patients below 55 years of age (4.46 ± 1.75 x 10^5 vs. 14.42 ± 8.45 x 10^5 /mL; p = 0.362; Figure 3.5.1B). However, there was a significant decrease in baseline TF-EVs in aging $F(1,50)= 4.293$, p = 0.044, R = 0.284, Figure 3.5.1A. One-way ANOVA of baseline TF-EVs assessed amongst the different cancer types was seen to be the greatest in patients with lymphoma (11.80 ± 6.15 x 10^5 /mL), gynecologic (10.22 ± 9.03 x 10^5 /mL) and stomach (6.06 ± 5.59 x 10^5 /mL) cancer although this increase was not statistically different compared to the remaining cancer types $F(1,40) = 0.224$, p=0.977. TF-EVs were measured for only a single bladder, colon, and other cancer patient and thus were removed from the statistical analysis. Measured TF-EVs in the cancer types are listed as follows: lung (3.58 ± 2.95 x 10^5 /mL), breast (1.42 ± 0.69 x 10^5 /mL), colon (2.36 x 10^5 /mL), myeloma (1.79 ± 0.31 x 10^5 /mL), brain (1.99 ± 0.40 x 10^5 /mL), pancreatic (3.42 ± 1.65 x 10^5 /mL), bladder (1.04 x 10^5 /mL), and other (0.31 x 10^5 /mL) (Supplementary Figure 3.5S2). Furthermore, there was no significant difference in baseline TF-EVs in new compared to recurrent cancers (8.28 ± 3.30 x 10^5 vs. 2.61 ± 0.78 x 10^5 /mL; p=0.638; Supplementary Figure 3.5S3).

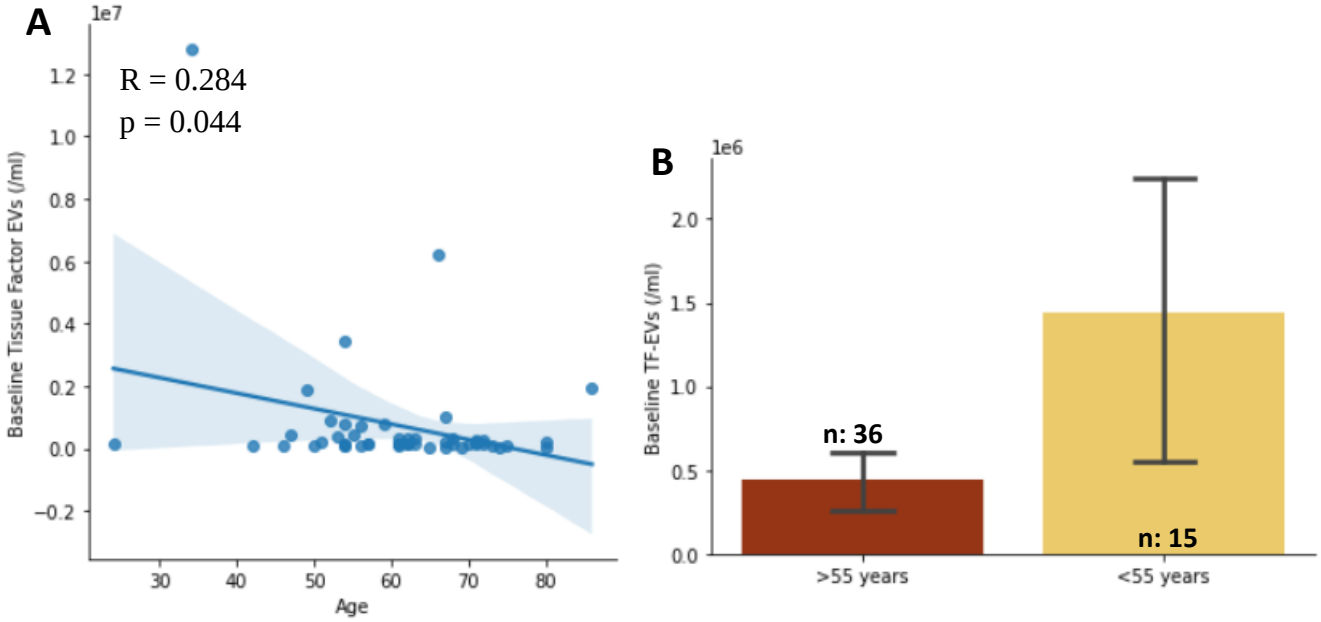


Figure 3.5.1. TF-EVs (/mL) were assessed at baseline for paired patient samples. (A) Linear regression equation was found to be F(1,50) = 4.293, p=0.044, R=0.284. (B) TF-EVs was also compared between patients above and below 55 years of age. Data presented as Mean +/- SEM; p = 0.362, N values are displayed. Mann-Whitney U independent t-test.

The difference between baseline TF-EVs in patients presenting with or without VTE and bleeding events were also compared, however, there was a very low number of samples quantified in the VTE and bleed groups. The two patients presenting with VTE had baseline TF-EVs of 4.31 ± 3.21 x 10^5 /mL which was lower than the mean TF-EVs of the 49 patients that did not present with VTE (7.51 ± 2.91 x 10^5 /mL, p=0.904; Supplementary Figure 3.5S4). Baseline TF-EVs were also not significantly different in the 3 patients that had a bleeding event compared to the 48 that did not (14.50 ± 10.0 x 10^5 vs. 6.94 ± 2.91 x 10^5 /mL; p = 0.129; Supplementary Figure 3.5S5).

3.6 Total EVs

Total AnnV+ EVs measured at baseline showed no difference in male versus female patients ($2.20 \pm 0.37 \times 10^8$ vs. $1.49 \pm 0.20 \times 10^8$ /mL, p=0.296; Supplementary Figure 3.6S1). Comparison of total EVs in aging did not demonstrate a significant trend ($F(1,55)=1.970$, $R = 0.186$, $p = 0.166$, Supplementary Figure 3.6S2A) and was not significantly different in patients > 55 years compared to <55 years of age ($1.61 \pm 0.23 \times 10^8$ vs. $2.25 \pm 0.41 \times 10^8$ /mL, $p = 0.168$; Supplementary Figure 3.6S2B). A one-way ANOVA (Kruskal-Wallis) test revealed significant differences between the different cancer types $F(1,46) = 2.393$, p=0.032 (Figure 3.6.1). Baseline total EVs in bladder and colon cancer were not included in the statistical analysis as there was only one patient in each group. Dunn's post hoc comparisons test indicated significant increases in stomach ($3.52 \pm 0.26 \times 10^8$) and lymphoma ($2.78 \pm 1.40 \times 10^8$) cancer types compared to myeloma ($0.72 \pm 0.90 \times 10^8$), lung ($1.51 \pm 1.68 \times 10^8$), gynecologic ($1.27 \pm 1.29 \times 10^8$), and pancreatic cancers ($1.08 \pm 0.58 \times 10^8$) (Supplementary Table 4). Baseline total EVs were not significantly different between new and recurrent cancers ($1.88 \pm 0.23 \times 10^8$ vs. $1.47 \pm 0.44 \times 10^8$ /mL, $p = 0.322$; Supplementary Figure 3.6S3)

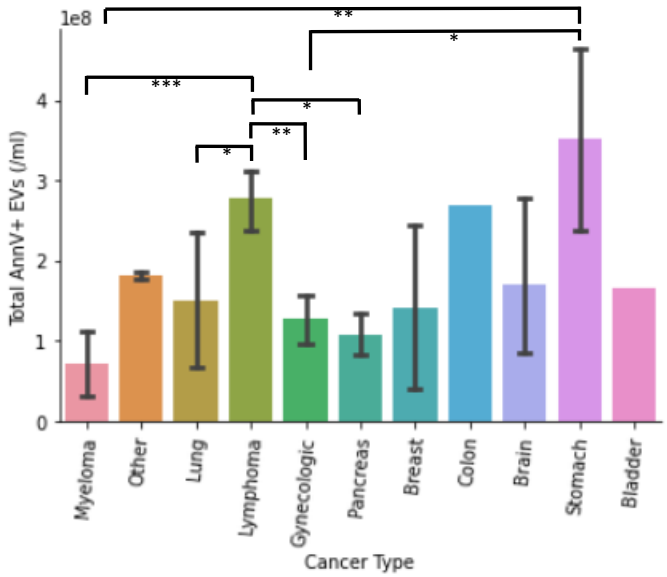


Figure 3.6.1. Total AnnV+ EVs (/mL) at baseline were measured by cancer type. Data presented as Mean +/- SEM. One-way ANOVA (Kruskal-Wallis) with Dunn's post hoc comparisons test; F(1,46)=2.393, p=0.023.

The difference between total AnnV+ EVs at baseline in patients presenting with or without VTE and bleeding events were also compared, however, there was a very low number of samples quantified in the VTE and bleed groups. The two patients presenting with VTE had baseline total EVs of 0.99 ± 0.52 x 10^8 /mL which was not significantly different than the mean total EVs of the 55 patients that did not present with VTE (1.83 ± 0.21 x 10^8 /mL, p=0.501; Supplementary Figure 3.6S4). Baseline total EVs were also not significantly different in the 3 patients that had a bleeding event compared to the 54 that did not (2.20 ± 1.43 x 10^8 vs. 1.78 ± 0.20 x 10^8 /mL; p = 0.900; Supplementary Figure 3.6S5).

3.7 Circulating EVs over time

The change in levels of circulating EVs from 0-months (baseline) to 3-months were assessed in all cancer patients and then stratified by patient- and disease-specific characteristics. The change in circulating EVs over time was assessed by pro-coagulant EV-activity and flow cytometry (platelet, TF-, and total EVs). Initial assessment of changes in circulating EVs over time revealed a significant decrease from 0-months to 3-months in MP-activity concentration (12.84 ± 0.71 nM vs. 11.27 ± 0.66; p = 0.003) but no significant changes over time in platelet, TF-, or total EVs (4.40 ± 0.56 x 10^7 vs. 4.44 ± 0.62 x 10^7 /mL, p = 0.86; 7.39 ± 2.80 x 10^5 vs. 3.00 ± 0.34 x 10^5 /mL, p = 0.90; and 1.80 ± 0.20 x 10^8 vs. 1.97 ± 0.25 x 10^8 /mL, p = 0.56), respectively (Figure 3.7.1).

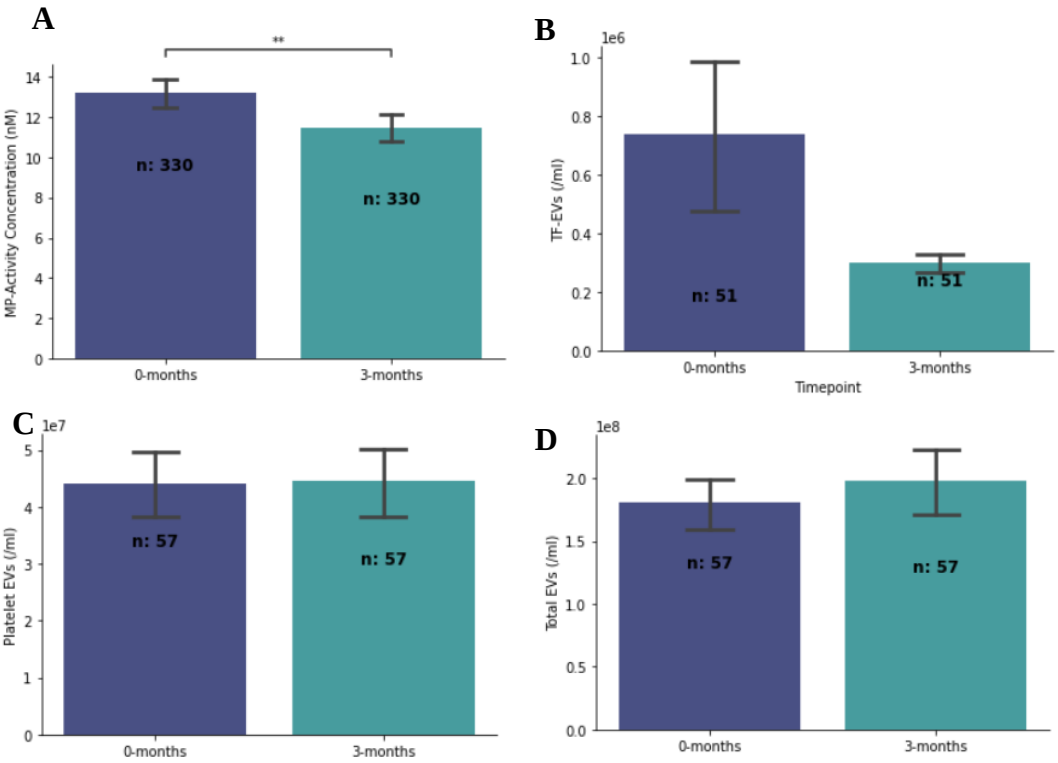


Figure 3.7.1. Circulating EVs over time in all patients was measured by MP-activity concentration (nM) and flow cytometry (platelet, TF-, and total EVs (/mL)) at 0-months and 3-months. Data presented as Mean +/- SEM; **p <0.01, N values are displayed. Mann-Whitney U independent t-test.

We further assessed changes in circulating EVs over time to determine if there were any associations between the sexes (males and females). Circulating EVs measured by MP-activity and flow cytometry (platelet, TF-, and total EVs) were compared across time. Repeated measures ANOVA between the sexes and MP-activity revealed a significant main effect of timepoint $F(1,328) = 5.493$, $p = 0.020$, $\eta_p^2 = 0.016$ and sexes $F(1,328) = 7.731$, $p=0.006$, $\eta_p^2 = 0.023$ but no significant interaction effect between timepoint and sexes $F(1,328) = 0.507$, $p = 0.477$, $\eta_p^2 = 0.002$. Bonferroni post-hoc comparisons test showed significantly increased MP-activity for males, 0-months compared to females, 3-months (mean difference = 5.222 ± 1.448, p=0.002; and compared to females, 0-months (mean difference = 3.940 ± 1.448, p = 0.040). Repeated measures ANOVA was repeated for platelet, TF- and total EVs but revealed no significant main effects or interaction effects between timepoint and between the sexes (p>0.05) (Supplementary Figure 3.9S1).

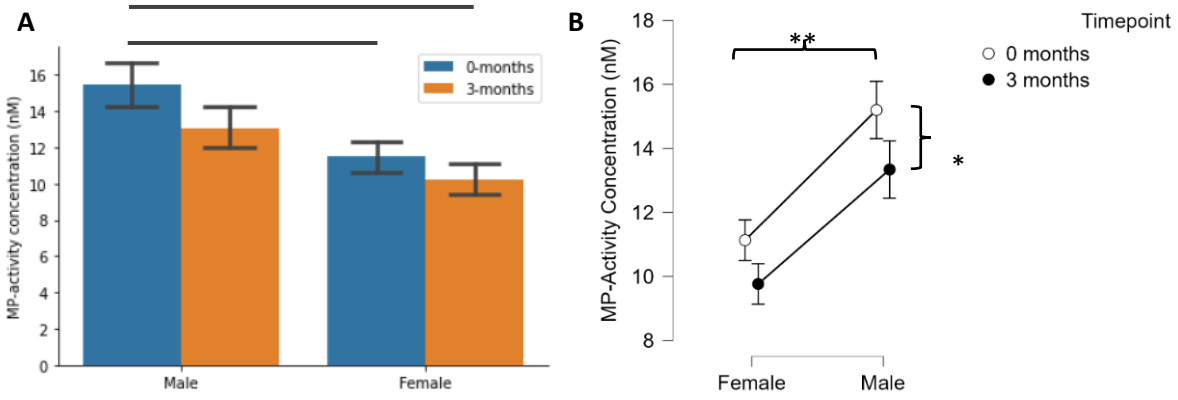
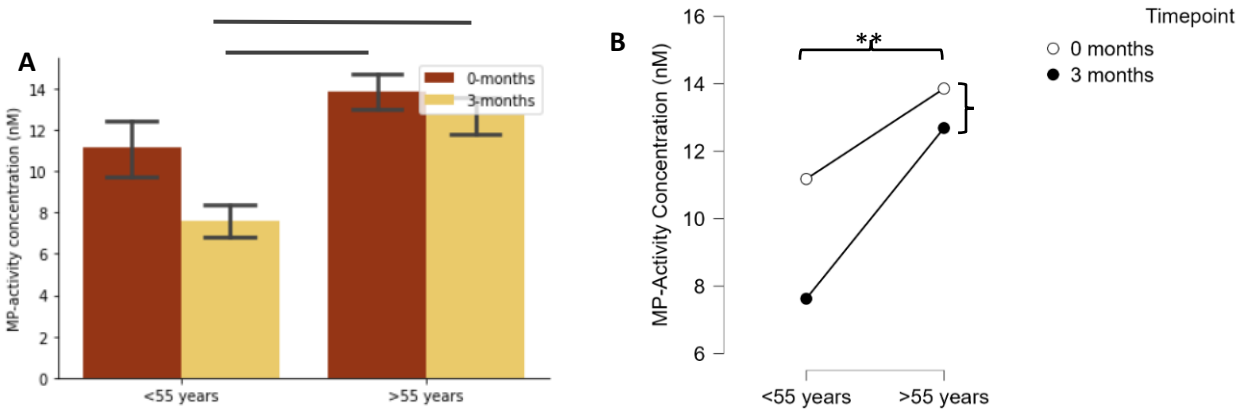


Figure 3.7.2. Circulating EVs over time in males and females was measured by MP-activity concentration (nM) at 0-months and 3-months. Data presented as Mean +/- SEM; **p <0.01, *p<0.05. Repeated measures ANOVA with Bonferroni post hoc test was conducted.

Circulating EVs over time was also assessed in aging to determine whether changes in EV levels over time is dependent upon age. Associations between patients above and below 55 years of age and the interaction with EV levels at 0-months and 3-months were determined by repeated measures ANOVA. Results from MP-activity concentration revealed a significant main effect of timepoint $F(1,328) = 6.87$, $p = 0.009$, $\eta p^2 = 0.021$ and age $F(1,328) = 7.691$, $p = 0.006$, $\eta p^2 = 0.023$ but no significant interaction effect between timepoint and sexes $F(1,328) = 1.745$, $p = 0.187$, $\eta p^2 = 0.005$. Bonferroni post-hoc comparisons test showed significantly increased MP-activity for patients >55 years, 0-months compared to <55 years, 3-months (mean difference = 6.228 ± 1.66, $p = 0.001$); and increased MP-activity for >55 years, 3-months compared to <55 years, 3-months and (mean difference = 5.06 ± 1.66, $p = 0.015$). Additionally, platelet and total EVs decreased over time in patients <55 years, while patients >55 years of age had an increase from 0 months to 3-months. Repeated measures ANOVA for platelet EVs and total EVs revealed a significant interaction effect between timepoints and age $F(1,55) = 5.167$, $p = 0.027$, $\eta p^2 = 0.086$; and $F(1,55) = 5.724$, $p = 0.020$, $\eta p^2 = 0.094$, respectively No interaction effect was observed for TF-EVs, and no main effects for timepoint or age was observed in platelet, TF-, or total EVs (Figure 3.7.3).



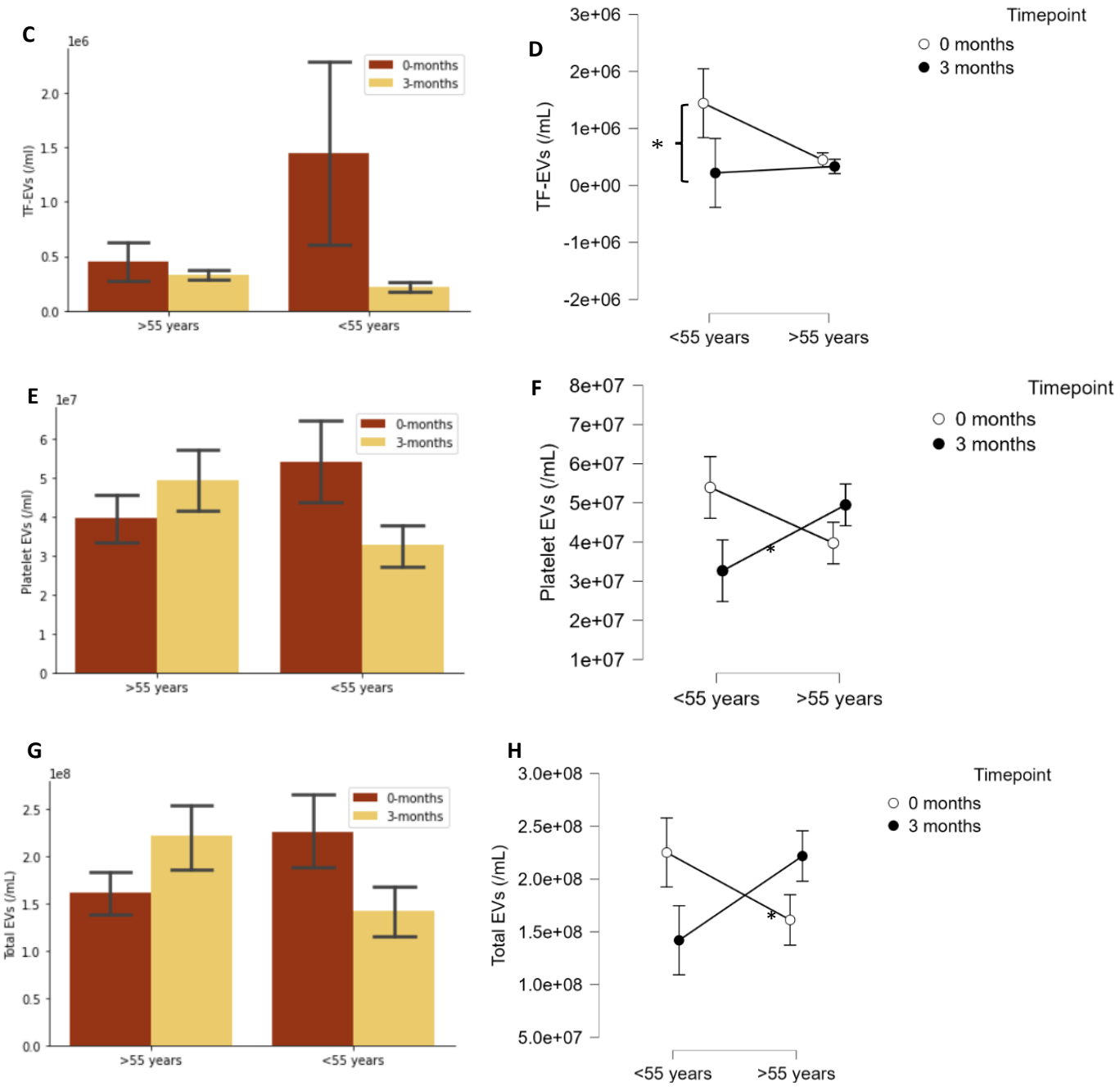


Figure 3.7.3. Circulating EVs over time in patients <55 or >55 years of age were measured by A, B. MP-activity concentration (nM); C, D. TF-EVs (/mL); E, F. platelet EVs (/mL); and G, H. total EVs (/mL) at 0-months and 3-months. Data presented as Mean +/- SEM; **p <0.01, *p<0.05. Repeated measures ANOVA with Bonferroni post hoc test was conducted.

Assessment of circulating EVs over time across primary cancer types also showed significant differences. Circulating EVs measured by MP-activity concentration and flow cytometry (platelet, TF-, and total) were analysed in each cancer type at timepoints 0-months and 3-months. Repeated measures ANOVA for circulating EVs measured by EV-activity revealed a significant main effect of cancer type $F(1,319) = 3.207$, $p < 0.001$, $\eta_p^2 = 0.091$ but no main effect for timepoint, nor an interaction effect between cancer type and timepoint. Bonferroni post hoc comparisons for results averaged over timepoint showed increased EV-activity in lung vs. brain (mean difference = 11.053, p = 0.026); lung vs. gynecologic (mean difference = 8.209, p = 0.017); lymphoma vs. brain (mean difference = 9.354, p = 0.031); and lymphoma vs. gynecologic (mean difference = 6.511, p=0.003) cancer patients. Additionally, Bonferroni post hoc comparisons for cancer type and timepoint together showed increased EV-activity in lung, 0 months vs. gynecologic, 0 months (mean difference = 10.840, p = 0.015); lymphoma, 0 months vs. gynecologic, 0 months and 3 months (mean difference = 9.106 and 8.306, $p < 0.001$ and p=0.003, respectively); and lymphoma, 0 months vs. brain, 3 months (mean difference = 12.094, p = 0.043) (Figure 3.9.4, and Supplementary Table 5). The analysis of repeated measures ANOVA was also conducted for circulating platelet, TF-, and total EVs measured by flow cytometry. No significant main effect was seen for timepoint, or cancer type, nor was there a significant interaction effect between timepoint and cancer type ($p>0.05$) (Supplementary Figure 3.7S2).

Circulating EVs over time were also assessed between patients with new and recurrent cancers, however no significant differences were seen. Repeated measures ANOVA for circulating EVs measured by EV-activity, platelet, TF-, and total EVs did not reveal any significant associations between new and recurrent cancers, timepoints 0 months and 3-months or an interaction effect between new/recurrent and timepoints ($p>0.05$) (Supplementary Figure 3.7S3).

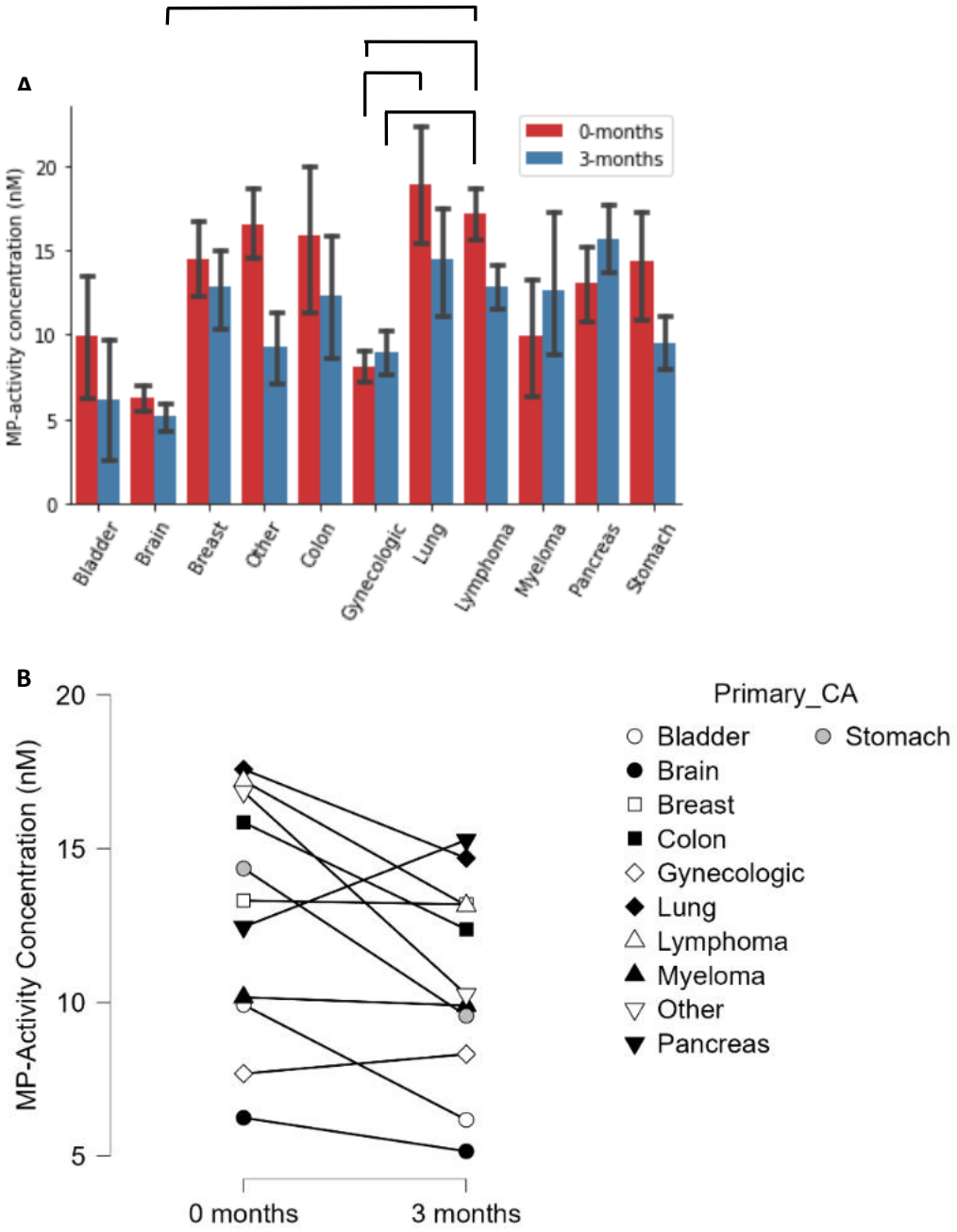


Figure 3.7.4. Circulating EVs over time in each patient cancer type were measured by MP-activity concentration (nM) at 0-months and 3-months. Data presented as Mean +/- SEM; ***p <0.001, *p<0.05. N values in order of cancer type shown: 2, 19, 12, 14, 5, 90, 30, 92, 13, 37, 16. Repeated measures ANOVA with Bonferroni post hoc comparisons test.

Furthermore, circulating EVs over time based on VTE or bleeding outcomes were also compared. Circulating EVs measured by MP-activity concentration and flow cytometry (platelet, TF-, and total) were analysed based on VTE status or bleeding event at timepoints 0-months and 3-months. Repeated measures ANOVA for circulating EVs measured by MP-activity based on VTE status did not reveal a significant main effect for timepoint, VTE or the interaction between timepoint and VTE (Figure 3.7.5). This analysis was repeated for platelet, TF- and total EVs, which also did not reveal any significant differences (Supplementary Figure 3.7S4). Repeated measures ANOVA for EV-activity and bleeding event did not show any significant effect of timepoint, bleeding event or the interaction between timepoint and bleeding (Figure 3.7.6). Further analysis of platelet, TF- and total EVs also did not show any significant differences between timepoint, bleeding and the interaction effect between timepoint and bleeding (Supplementary Figure 3.7S5).

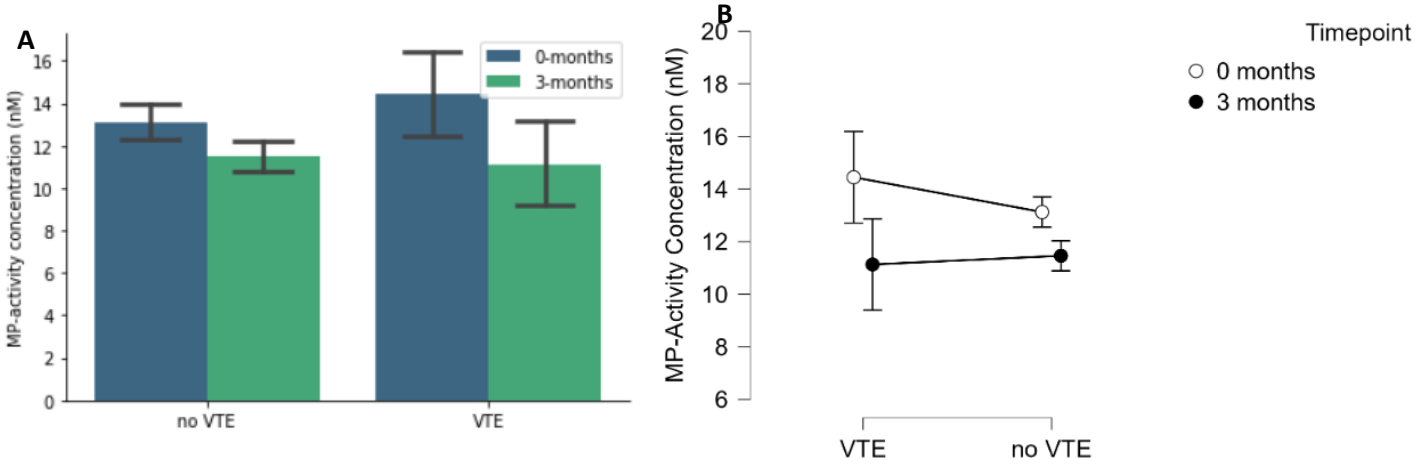


Figure 3.7.5. Circulating EVs over time in patients with or without VTE events were measured by MP-activity concentration (nM) at 0-months and 3-months. Data presented as Mean +/- SEM. N values: no VTE (312), VTE (18). Repeated measures ANOVA with Bonferroni post hoc comparisons test.

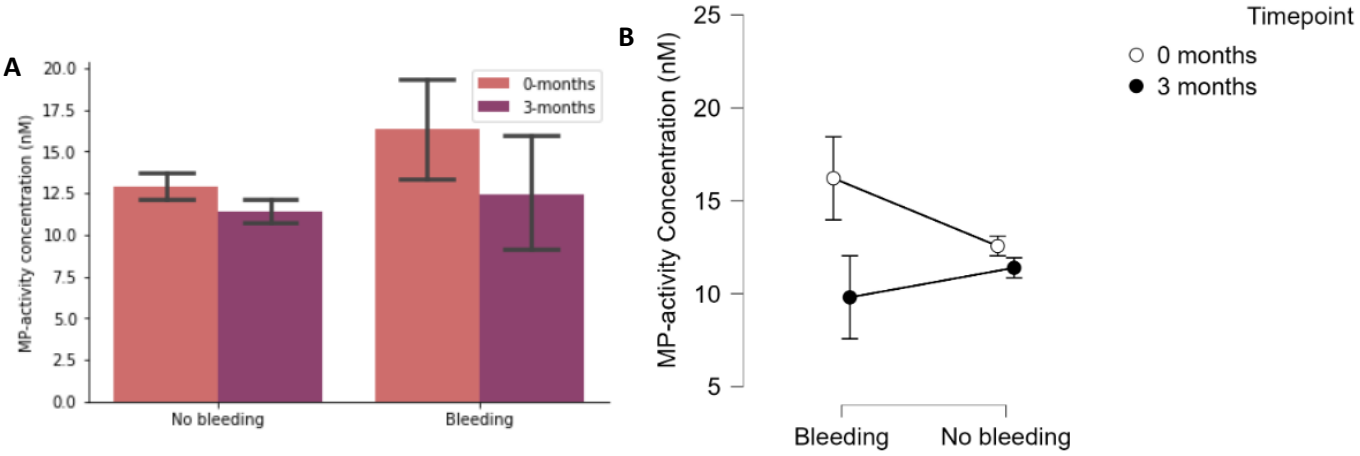


Figure 3.7.6. Circulating EVs over time in patients with or without bleeding events were measured by MP-activity concentration (nM) at 0-months and 3-months. Data presented as Mean +/- SEM; N values: no bleeding (303), bleeding (27). Repeated measures ANOVA with Bonferroni post hoc comparisons test.

Lastly, circulating EVs over time in response to prophylactic antithrombotic therapy with Apixaban were measured by MP-activity and flow cytometry (platelet, TF-, and total EVs). The change in circulating EVs from timepoints 0-months to 3-months were compared in patients that either received Apixaban or Placebo. Repeated measures ANOVA for circulating EVs measured by MP-activity revealed a significant main effect of timepoint $F(1,328) = 5.131$, $p = 0.024$, $\eta_p^2 = 0.015$ but there was no significant effect of treatment with Apixaban or an interaction effect between timepoint and treatment ($p>0.05$). Additionally, Bonferroni post-hoc test did not show any significant mean differences between the groups (Figure 3.7.7). Repeated measures ANOVA conducted for platelet, TF-, and total EVs showed no significant main effects of timepoint or treatment with apixaban; nor were there any significant interaction effects between timepoint and treatment ($p>0.05$) (Supplementary Figure 3.7S6).

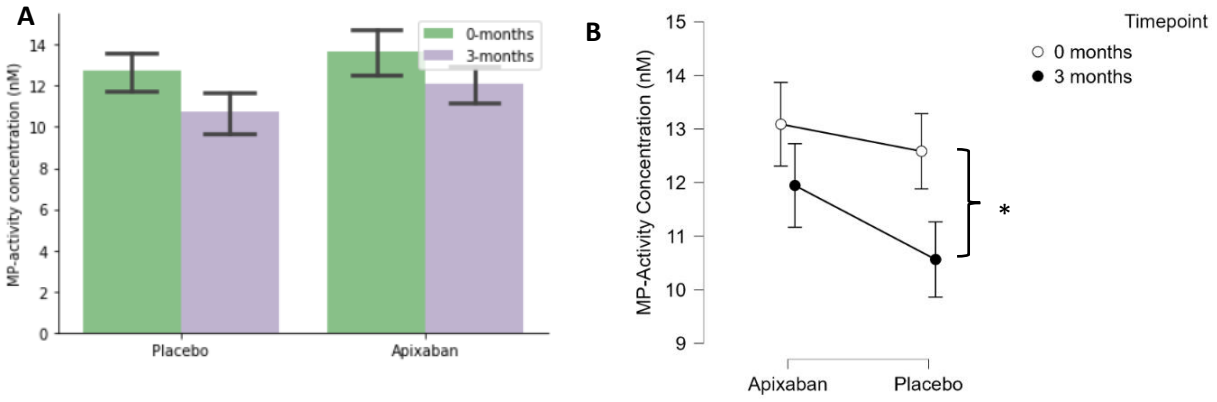


Figure 3.7.7. Circulating EVs over time in patients receiving treatment (apixaban or placebo) was measured by MP-activity concentration (nM) at 0-months and 3-months. Data presented as Mean +/- SEM; *p<0.05. N values: placebo (161), apixaban (169). Repeated measures ANOVA with Bonferroni post hoc comparisons test.

CHAPTER 4: DISCUSSION

The objective of this thesis was to determine whether elevated circulating EVs could predict VTE or bleeding outcomes in cancer patients, and to determine if changes in circulating EVs are indicative of response to prophylactic antithrombotic therapy. We thus, investigated pro-coagulant EV-activity, platelet EVs, TF-EVs, and total EVs in the plasma of patients that were recruited in the AVERT trial at 0-months (baseline) and 3-months follow-up. We did not find an association between EVs and VTE, or bleeding outcomes in cancer patients. However, we identified several associations with patient- and disease-related characteristics including age, sex, cancer type and treatment with prophylactic antithrombotic therapy over time. We further demonstrated that levels of platelet EVs, TF-EVs and total EVs had similar associations with age and cancer type as pro-coagulant EV-activity, however, we were unable to detect any associations with hematological outcomes, or treatment. These results suggest that pro-coagulant EV-activity and levels of platelet, TF, and total EVs may not represent novel biomarkers for intermediate-to-high risk (Khorana ≥2) cancer patients for VTE.

VTE is a frequent complication in cancer patients and is a significant cause of morbidity and mortality.[208] The risk of cancer-associated VTE is multifactorial, and dependent on a variety of patient- and disease-related factors like age, sex, cancer type, histology, treatment and biomarkers.[209] Currently, the incidence of VTE among the cancer population is estimated to be ~15% and has significantly increased over the past 2-decades.[208] Despite the high risk and increased incidence of VTE, routine thromboprophylaxis is not recommended in all cancer patients due to greater risk of bleeding complications during anticoagulant treatment.[209–211] Major bleeding events occurred in 4% of cancer patients during the first three months of anticoagulant treatment, while the 1-year cumulative rate of major bleeding was found to be as high as 10-15%.[209]

Therefore, the clinical decision to provide cancer patients with thromboprophylaxis is based on a risk assessment of the reduction in VTE and the risk of bleeding. This has led to the development of risk assessment models for the prediction of cancer associated VTE, which aim to stratify high-risk patients only.[39,80–82,86] As well as the development of models assessing bleeding risk in the context of VTE prophylaxis, however these are not regularly used in the ambulatory cancer patient setting.[212] The most widely used VTE risk assessment tool: the Khorana risk score, combines clinical variables through a point system to classify cancer patients into low, intermediate, and high-risk categories.[50] Originally, patients were considered for thromboprophylaxis if they were deemed to be high-risk with later trials including both intermediate and high risk cohorts.[89,213] However, contradicting results have been reported regarding the positive predictive value and substantially variable performance among cancer types.[89,213] Over the past decade, many studies have aimed to improve the Khorana score by assigning additional points for chemotherapy[78], patient performance status[79], laboratory biomarkers[64], vascular compression[80], and more. These modified risk scores, are not externally validated, only showed slightly improved prediction, or include biomarkers that are not readily available in each medical institute.[214] Thus, the need to identify novel predictive biomarkers which could be used for VTE risk assessment and targeted prophylaxis has become increasingly clear.[2,95,178,214,215]

In this regard, EVs found in the circulation, specifically those expressing TF or PS on their surface have emerged as potential biomarkers of cancer associated VTE.[21,136,178,199] Current evidence on experimental and cross-sectional studies suggests that their might be a role for platelet-, endothelial-, and TF-EVs in the prothrombotic state found in cancer patients.[199] However, results from prospective studies in evaluating EV-TF activity as a biomarker for the prediction of VTE in cancer patients are conflicting and inconsistent, with the association

appearing to be strongest in pancreatic cancer.[199,216] Furthermore, the change in EV levels or procoagulant EV-activity throughout the course of disease, and whether there is an association between thromboprophylaxis and EVs has not been studied.

4.1 Circulating EVs between the sexes and aging

Pro-coagulant EV-activity was measured at baseline and 3-months follow-up after the initiation of chemotherapy and Apixaban (2.5 mg daily) in intermediate-to-high risk cancer patients for VTE. Baseline measurements of EV-activity were significantly elevated in male patients compared to females, and increased with age, which may be reflective of the increased prothrombotic state of this population. Currently, the existence of sex and age differences for incident VTE remains controversial, however, men have been shown to be 50% more likely to suffer recurrent VTE than women.[217] The precise mechanisms are not fully understood, but is thought to be partly in regards to the hormonal influences on thrombosis, particularly estrogen and growth hormone.[217] Further studies measuring coagulation factors, inhibitors and activations markers observed significant effects of age, sex, menopause and hormone use.[217] Specifically, increases in levels of factors VII, VIII and IX and decreased levels of protein C – a coagulation inhibitor, were associated with increasing age, especially in men.[217] This imbalance may be related to increased activation of coagulation, and hence increased risk of VTE. In our study we found that baseline EV-activity in males at 0 months was significantly lower than EV-activity in females at 0 months and at 3-months. Furthermore, when we stratified patients on whether they were <55 or >55 years of age, those that were younger had significantly lower EV-activity at 3-months compared to patients that were older at both 0 month and 3-month timepoints. Baseline measurements of circulating EVs assessed by flow cytometry did not have any significant associations between the sexes. However, platelet, TF-, and total EVs all demonstrated significant

associations with age. Baseline TF-EVs showed a significant decrease in levels as patients increased in age but was not significantly different between patients >55 years compared to <55 years of age. TF-EVs were also significantly increased at baseline compared to 3-months when levels were averaged between patients >55 and <55 years of age. Platelet EVs and total EVs were not significantly different over time or between the age groups, but rather demonstrated an interaction effect between timepoint and whether patients were <55 or >55 years of age. Patients that were >55 years had an increase in platelet and total EVs from 0 months to 3-months while patients that were <55 years of age had a decrease of levels over time.

Whether these increases represent the hypercoagulable state of the male sex and increasing age, or are specific to the EV profiles of these patients cannot be concluded. The amount, size and molecular composition of EVs between the sexes and aging remains unclear[146,218], and the few studies that do examine sex- and age-related differences are conflicting and vary between healthy[219] and disease populations.[220] One study found that the concentration of EVs in plasma decreased with age in healthy individuals, however, they concluded that this was in part due to enhanced EV uptake and internalization.[218] In healthy individuals, studies have also shown that circulating EVs are more abundant in women than men of the same age,[221] while other studies have found no difference.[172] Another study found significantly higher PMPs in female than in male cancer patients ($p<0.037$), but were unable to find differences in EMPs between male and female, nor any correlations with age.[186] However, in age-related cardiovascular diseases, EVs released from senescent cells in the plasma (i.e. endothelial cells) have heterogenous roles promoting calcification, inflammatory markers, and oxidative stress through release of miRNA or other molecules.[222] Therefore, circulating EVs are definitely altered with age and sex however, whether this association is predictive of disease state has yet to be determined.

4.2 Circulating EVs and cancer type

Comparison of pro-coagulant EV-activity in cancer types were vastly different, among patients demonstrating the significance of malignancy and its effect on circulating EVs. In addition to circulating cells (e.g., platelets, endothelial cells, leukocytes) whose populations can vary widely between cancers and mode of treatment, tumor cells also release several types of EVs which also differ in size, biogenesis, and molecular composition.[223] It has been shown that that the level of EVs circulating in blood is significantly elevated in cancer patients.[186,224,225] In this study, we found that baseline EV-activity, was significantly increased in lung, lymphoma and a combination of other cancer patients compared to brain, and gynecologic cancer patients. Similar increases were also seen when circulating EVs across the different cancer types were assessed by platelet and total EVs. Specifically, lymphoma and stomach cancer patients had greater levels platelet and total EVs compared to myeloma, gynecologic and other cancer patients. No significant differences were seen when we compared TF-EV levels between the different cancer types.

We also assessed circulating EVs over time from 0 months to 3-months between the different cancer types. EV-activity at baseline was significantly increased in patients with lymphoma compared to EV-activity in gynecologic cancer patients at both timepoints, and for EV-activity in brain cancer patients at 3-months. The analysis was repeated for circulating EVs assessed by flow cytometry, however, there were no significant associations over time for platelet, TF-, and total EVs between the various cancers. In studies with multiple cancer types, an increase in procoagulant EV-activity was associated with different cancers such as pancreatic, colorectal, gastric, lung, breast, testicular, brain and hematopoietic.[226] Similarly, other authors observed increased total MPs in gastric, lung, ovarian and breast cancers compared to healthy patients.[226] Kim and colleagues reported elevated platelet MPs in gastric cancer compared to healthy

individuals.[227] The most extensive study comparing different types of patients with cancer (60 pancreatic, 43 gastric, 126 colorectal and 119 brain) found higher levels of MP-TF activity in pancreatic and gastric in comparison to colorectal and brain cancers.[175] Overall, studies comparing EV levels across different tumor types are infrequent, but it is evident that tumor cells release increased numbers of EVs compared with healthy cells and are exposed to large quantities of EVs secreted by various cell types.[228] Tumor-derived EVs can be distinguished from normal cell-derived EVs due to the presence of specific oncogenic surface proteins that they carry.[229] For example, EVs derived from melanoma cancer cells were found to be enriched in PD-L1 (major immune response regulator), CD63 and caveolin-1, when compared with healthy donor cells.[229,230] Additionally, EVs derived from human colorectal cancer were found to carry the oncoprotein mutant K-Ras. Other markers of tumor-derived EVs include specific miRNA signatures such as miR-373 for breast cancer, or miR-21 for squamous cell carcinoma.[230] However, further work is necessary to evaluate whether specific malignancies release EVs more frequently compared to others, and whether this increase can predict malignancy itself.

In addition to cancer type, we also evaluated differences in circulating EVs assessed by EV-activity and flow cytometry (platelet, TF- and total EVs) in patients presenting with new or recurrent cancers. There was a significant increase in baseline EV-activity when we compared patients with new cancers vs. recurrent cancers, however this increase was not seen in platelet, TF-, or total EVs. Additionally, when we analysed circulating EVs over time (0 months and 3-months) between new and recurrent cancers we did not find any significant associations in either EV-activity, platelet, TF-, or total EVs. Direct comparison of circulating EV concentrations between new and recurrent cancers has not been studied in the current literature. However, one study compared EV concentrations from glioblastoma (GBM) patients facing a relapse with EV

concentrations from either preoperative GBM plasma samples or matched postoperative GBM samples. They found that the level of plasma EVs in recurrent GBMs was nearly 40% higher than in the primary GBM samples immediate post resection, but no difference between recurrent and preoperative GBM samples.[231] Thus, whether circulating EVs could be a basis as a biomarker capable of describing tumor status, warrants further investigation.

4.3 Circulating EVs in cancer associated VTE and bleeding

Several studies have evaluated the presence of circulating TF-EVs or EV-TF activity and VTE occurrence in cancer patients. Increased circulating TF-EVs have been associated with VTE in pancreatic cancer patients[188,194,232], while there are conflicting findings in breast cancer patients.[194,233] Other studies have failed in finding associations between elevated TF-EVs and VTE in soft tissue sarcoma, lymphoma, colorectal, breast, stomach, lung and pancreatic cancers.[175,187,193,234,235] Two studies evaluating MP-activity were also contrastingly different. In the first study, Sartori and colleagues found increased MP activity levels between glioblastoma patients with and without VTE[176]; however, in another study elevated MP levels (nM PS) were not predictive of VTE.[236] In our study, we were unable to find associations with circulating EVs and VTE with the majority of patients presenting with lymphoma, gynecologic, pancreatic and lung cancers. When we evaluated circulating EVs over time from 0 months to 3-months we also were not able to find any significant associations between the different timepoints or VTE. Flow cytometry analysis of platelet EVs, TF-EVs and total EVs did not show any differences between patients that did and did not develop VTE, although this could be due to the low number of samples analyzed. The reason for the discrepancy in our results compared to the literature requires further testing but could be explained partially by the multifactorial aspect of VTE, that is highly dependent on several risk factors such as immobilization, tumor subtype, tumor size,

chemotherapy and many other acquired or inherited risk factors.[236] In addition, procoagulant EV-activity may not be an appropriate method of quantifying EVs to demonstrate a predictive role for the occurrence of VTE in pre-selected intermediate-to-high risk cancer patients.

The association between circulating EVs in cancer associated bleeding risk has not been well studied. Many cancer patients at risk for VTE experience bleeding events due to prophylaxis treatment to avoid thrombosis.[236] The frequency of bleeding varies with cancer type and is observed in 15-20% of patients with blood malignancy and ~7% of patients with solid tumors.[236] One study found that among acute leukaemia patients with elevated EV-TF activity (>2 pg/mL), two patients experienced bleeding.[236] In our study, we did not see any significant associations at baseline or over time with pro-coagulant EV-activity and patients that developed bleeding, nor with platelet, TF- or total EVs. The incidence of bleeding in cancer associated VTE is correlated with anticoagulant thromboprophylaxis that patients are receiving. One study found a bleeding incidence of 12.2% with apixaban, 5.7% with dabigatran, and 14.7% with rivaroxaban.[237] Therefore, circulating EVs do not seem to have any correlations with bleeding outcomes which most likely is related to treatment and malignancy types.

4.4 Circulating EVs in prophylactic antithrombotic therapy

Circulating EVs in response to prophylactic antithrombotic therapy has not been studied in cancer and we sought to determine whether treatment with the DOAC, apixaban would lead to reductions in circulating EVs over time. Analysis of circulating EVs over time for patients receiving apixaban or placebo revealed a significant main effect of timepoint. There was a significant decrease in EV-activity from 0-months to 3-months when patients receiving apixaban and placebo were averaged together, however no difference between apixaban and placebo. In

flow cytometric analyses of EVs over time, the change in platelet, TF-, or total EVs over time did not differ from 0 months to 3-months or between patients that received placebo versus apixaban. A sub-study of the RASTEN trial which measured EV-TF activity at baseline, during treatment, and at follow-up found small variations between timepoints that appeared to be independent of LMWH treatment.[206] Another study, evaluated circulating EV concentration from nonvalvular atrial fibrillation (NVAF) patients receiving rivaroxaban or warfarin. They found that rivaroxaban significantly reduced levels of circulating EVs compared with warfarin controls, suggesting that rivaroxaban attenuates the proinflammatory state of NVAF patients.[238] One reason for this discrepancy between the literature and our results could be explained by the difference in EV measurement. Our study as well as the RASTEN trial measured EV-activity while the second study used flow cytometry, in which our sample size was limited for. The difference in the patient population could also explain the results, since we determined EV levels within patients already considered intermediate-to-high risk for VTE. Further investigation is required to conclude whether a decrease in circulating EVs reflect antithrombotic prophylactic treatment in cancer patients.

4.5 Study limitations

The primary objective of this study was to identify whether circulating EVs identify risk of VTE amongst cancer patients already considered to be intermediate-to-high risk (Khorana ≥2). This would allow for the translation of circulating EVs into risk assessment tools that would guide clinical decision making within the ambulatory cancer patient setting. Current work on circulating EVs suggest an association between elevated TF-EVs (including EV-TF activity as well TF-bearing EVs) and VTE in pancreatic cancer patients, however, most other cancer types have not been as strongly associated.[136,163,178,199] Additionally, conflicting outcomes could be explained by

the variety of techniques used for measurement of EVs (antigen or activity), EV purification methods, pre-analytical variables (e.g., timing of blood sampling, blood withdrawal, storage), and other methodological discrepancies and overall lack of standardization. Other important considerations are different types, stages and treatments of cancer, with limited data from prospective studies. The incidence of VTE differs greatly across different cancer types, which limits the feasibility of EVs as predictive factors of VTE risk in cancer patients.[136,163,178,199,215] Additionally, many chemotherapy drugs used to treat cancer have been associated with an increased risk of thrombotic events, with combinatorial therapies further increasing this risk.[21] In our study, we measured pro-coagulant EV-activity with a functional assay in a large cohort of patients with different cancer types, receiving various chemotherapy agents. We also measured platelet EVs, TF-EVs, and total EVs using flow cytometry. Detection techniques such as flow cytometry allow to establish the cellular origin of EVs but provide no information on their thrombotic activity. Conversely, functional assays incorporate high sensitivity, and ease of use, but do not allow determination of cellular origin.[215] However, due to technological limitations, detection of circulating EVs by flow cytometry was incomplete. The plasma samples we received were from a previous clinical trial conducted, therefore our study had a retrospective design, and we were unable to control for pre-analytical variables, or inclusion of patients. In this regard, the ISEV has been aiming to improve many of these considerations by establishing guidelines for detection and isolation of EVs, although further efforts are still required.[103,134] Lastly, the role of circulating EVs in cancer associated thrombosis is still poorly understood with many complex factors contributing to the occurrence of VTE. Initially, it was understood that the thrombotic activity of EVs stemmed from the presence of negatively charged phospholipids and tissue factor. However, researchers have now demonstrated that the role of EVs is far more complex, and

multifactorial with mechanisms involving crosstalk between tumor-derived EVs, and vascular cells that may promote positive feedback.[178] Therefore, it is important to acknowledge that proper standardization must be established within the community before their role in a multifactorial disease such as cancer associated thrombosis can be validated and used to guide clinical decision-making.

CHAPTER 5: FUTURE RESEARCH DIRECTONS

The current study provides significant evidence that there are significant associations for circulating EVs at baseline and over time in sex, age, and cancer type within cancer patients at intermediate-to-high risk for developing VTE. However, an association between circulating EVs and VTE, bleeding events or treatment with Apixaban was not found. Further prospective studies with standardized methods on EV isolation, detection, and quantification should be considered to confirm circulating EVs as a prognostic marker of cancer associated VTE. Despite these findings, there exists a significant information gap in the current understanding of the mechanistic role of circulating EVs within cancer associated VTE and how EV levels and activity could be applied in the development of diagnostic strategies.

Additional experiments such as quantifying circulating EVs and EV-activity throughout the course of cancer and development of VTE at multiple timepoints could be done to understand how levels are impacted by malignancy and stage. This would be conducted by measuring concentration of platelet EVs, TF-EVs, endothelial EVs, and leukocyte EVs as well as EV-TF activity and PS-dependent EV-activity using flow cytometry, functional assays, and nanoparticle tracking analysis. A multi-timepoint, prospective study would allow to investigate the diagnostic potential, which would be more valuable than determining baseline cut-off values which would be variable for each characterization method. Furthermore, since the Khorana score is criticized for including a high proportion of patients in the low/intermediate risk category[88,239], EVs can be measured specifically in this population. EV numbers from various subgroup populations, as well as EV-activity in cancer patients considered at low-risk would be compared to patients at intermediate-risk and high-risk for VTE, enabling better understanding of circulating EVs and its potential role as a prognostic biomarker. Receiving operating characteristic (ROC) curves will be

used to assess the performances of EV levels over their entire range prior to the VTE event. The area under the curve (AUC) will then estimate the discriminative power of EVs for early prediction of VTE outcome. This approach will provide a sensitive and specific analysis of EVs in diagnostics that would contribute to clinical decisions on which cancer populations will receive thromboprophylaxis. Blood should be drawn carefully; and patient care should be documented precisely to control for pre-analytical variables. Additionally, cancer-related treatment should be documented from each patient and an effort should be made to select patients undergoing similar treatment, with similar cancer types and stage. Moreover, a randomized clinical trial study design in which patients with high levels of circulating EVs (cut-off based on previous literature) randomized to either thromboprophylaxis or placebo could be conducted. This would allow further understanding of how circulating EVs respond to anticoagulant therapy.

Aside from the clinical aspect, this could be combined with basic science and high-resolution imaging of isolated EVs using electron microscopy or atomic force microscopy. Immunocompetent mouse strains such as C57BL/6 and BALB/c could be explored to develop allograft models with murine cancer cells[240] in which EV trafficking is specifically inhibited in platelets and endothelial cells through the activity of drugs such as calpeptin – a calpain inhibitor, or Y27632.[241] The latter drug is a highly potent competitive inhibitor of both ROCK1 and ROCK2, which competes with ATP-binding to inhibit EV release.[241] This would be followed by the induction of thrombosis through the infrarenal vena cava (IVC) and longitudinal analysis of clot formation with high frequency ultrasonography. Alternatively, application of ferric chloride ($FeCl_3$) could also be used to induce thrombosis in large and small veins, as well as mesenteric microvessels.[240,242] Additionally, physical characterization of clot formation and circulating EVs in control mice which did not receive EV-inhibitors to determine protein content or signalling

molecules of interest with immunoblotting or immunosorbent assays would allow for profiling of EVs and their cargo in cancer-associated thrombosis. These basic science and characterization techniques would provide further in-depth understanding of the signaling roles of circulating EVs within cancer and how they contribute to thrombosis.

CHAPTER 6: CONCLUSION

This thesis delineates changes in baseline and 3-month follow-up circulating EV levels and activity in cancer patients at intermediate-to-high risk for VTE in Canada. We have demonstrated that procoagulant EV-activity is increased in males, newly diagnosed cancers, and has positive associations with age. We also demonstrated that EV-activity was significantly altered between the cancer types and showed a general decline in all patients over time. Platelet EVs, TF-EVs and total EVs did not recapitulate EV-activity but demonstrated similar trends. On the other hand, we did not find any significant associations between procoagulant EV-activity nor circulating EV levels and VTE, bleeding events or treatment. This highlights the major barriers that still exist in the biomarker use of circulating EVs in cancer associated VTE, specifically the lack of standardization methodologically and the complex, heterogenous nature of EVs. Although significant conclusions, could not be made for hematological outcomes (VTE and bleeding events) in cancer patients, we were able to find several other associations with patient characteristics. Therefore, evaluation of circulating EVs in clinical plasma samples may be a relevant practice to examine their prognostic and diagnostic role within disease. Future studies should focus on incorporating EV characterization into future clinical trials within cancer associated VTE. Specifically, when patient blood is drawn, EV quantification should be another measured parameter, and should be compared with current biomarkers to assess the utility and contribution of EVs to VTE. By quantifying EVs within the cancer population, prior to VTE and throughout the course of treatment, a more accurate representation of circulating EVs will be identified and will allow for novel therapeutic strategies, ultimately improving patient outcomes.

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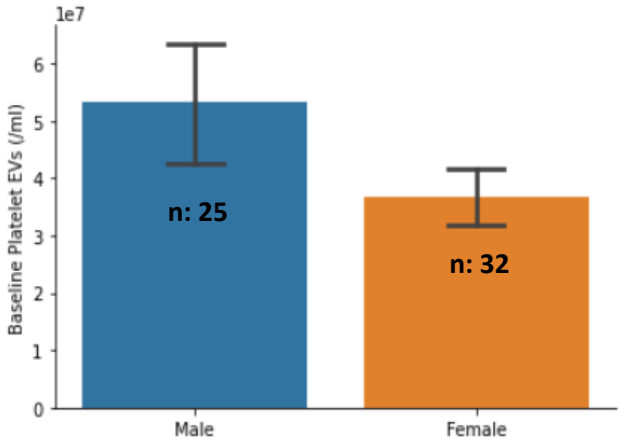
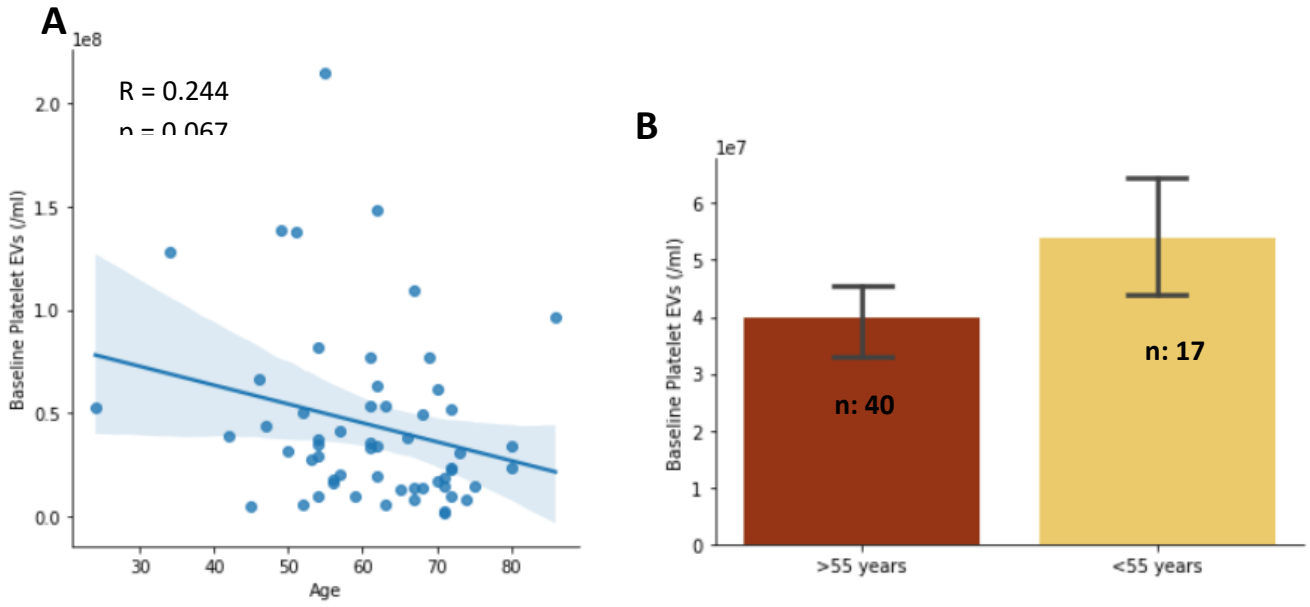
APPENDIX A

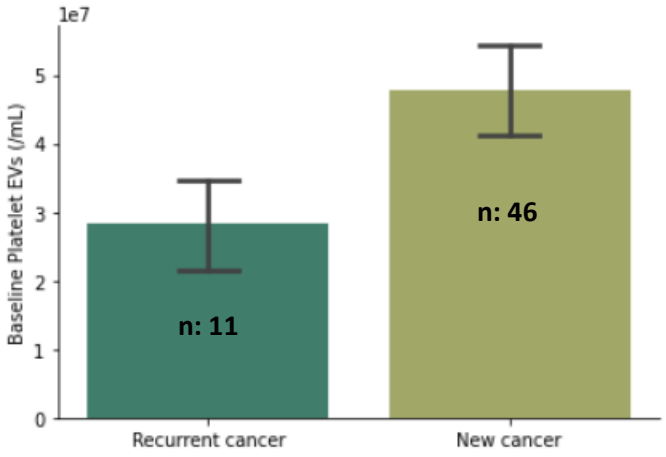
Supplementary Figures



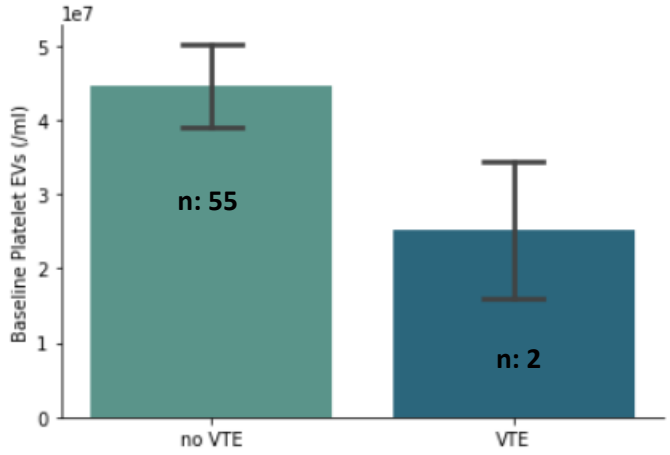
Supplementary Figure 3.4S1. Platelet EVs were assessed at baseline for paired patient samples. Platelet EV concentration (/mL) was compared between the sexes (males and females). Data presented as Mean +/- SEM, p = 0.624, N values are displayed. Mann-Whitney U independent samples t-test was conducted.



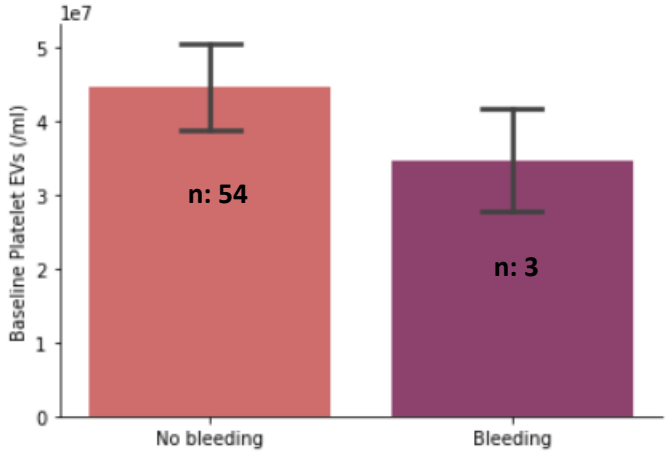
Supplementary Figure 3.4S2. Platelet EVs (/mL) were assessed at baseline for paired patient samples. (A) Linear regression equation was found to be F(1,55) = 3.483, p=0.067, with an, R of 0.244. (B) Platelet EVs was also compared between patients above and below 55 years of age. Data presented as Mean +/- SEM; p = 0.148, N values are displayed. Mann-Whitney-Wilcoxon test two-sided with Bonferroni correction.



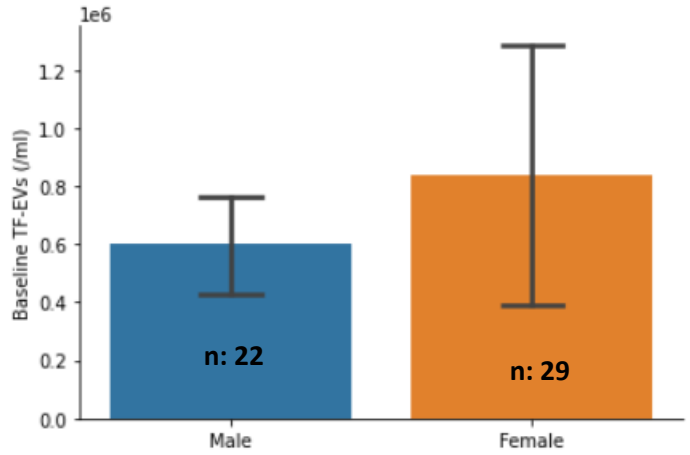
Supplementary Figure 3.4S3. Platelet EVs (/mL) were assessed at baseline for paired patient samples. Platelet EV concentration was assessed between new vs. recurrent cancers. Data presented as Mean +/- SEM, p = 0.245, N values are displayed. Mann-Whitney U independent samples t-test.



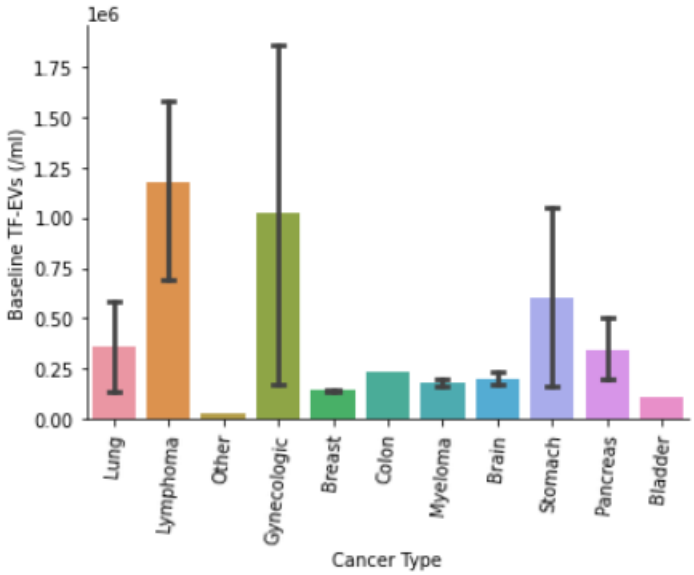
Supplementary Figure 3.4S4. Platelet EVs (/mL) at baseline were assessed by VTE status. Data presented as Mean +/- SEM, p = 0.680, N values are displayed. Mann-Whitney U independent samples t-test.



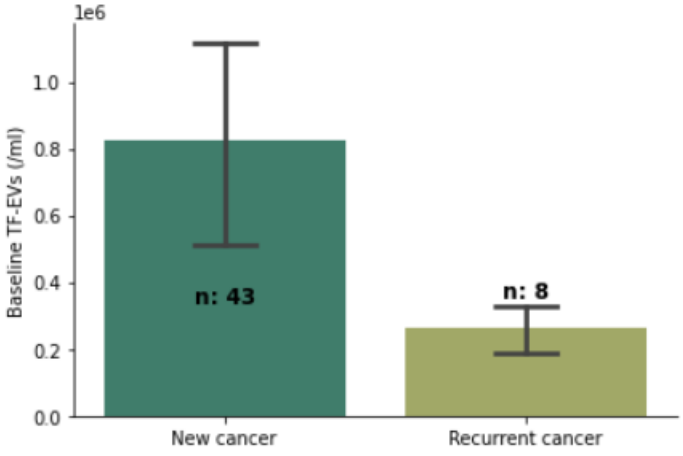
Supplementary Figure 3.4S5. Platelet EVs (/mL) at baseline were assessed by bleeding event. Data presented as Mean +/- SEM, p = 0.90, N values are displayed. Mann-Whitney U independent samples t-test.



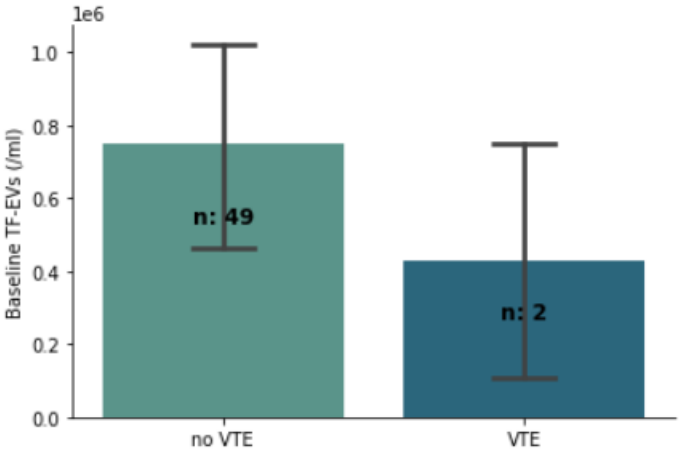
Supplementary Figure 3.5S1. TF-EVs (/mL) were assessed at baseline and compared between the sexes (males and females). Data presented as Mean +/- SEM, p = 0.158, N values are displayed. Mann-Whitney U independent samples t-test.



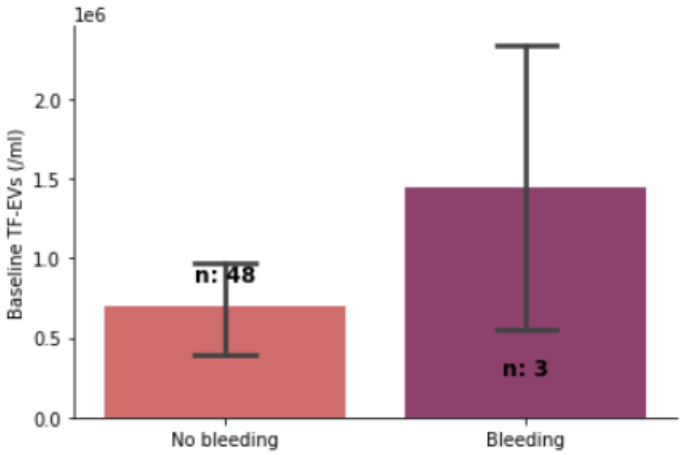
Supplementary Figure 3.5S2. TF-EVs at baseline (/mL) were measured by cancer type. Data presented as Mean +/- SEM. One-way ANOVA. F(1,40)=0.224, p=0.977.



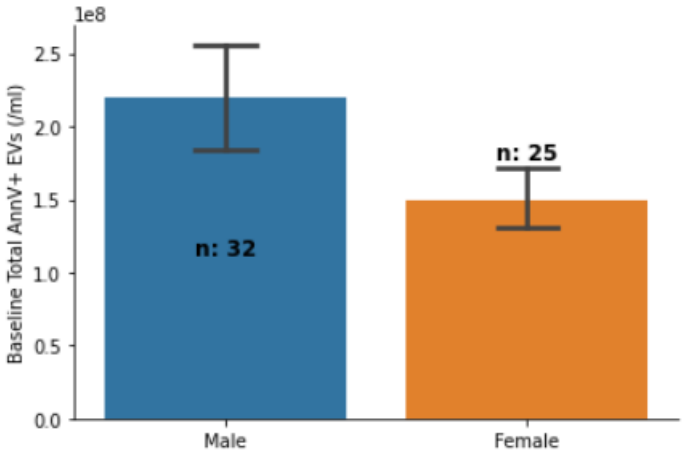
Supplementary Figure 3.5S3. TF-EVs (/mL) were assessed at baseline and compared between the new and recurrent cancers. Data presented as Mean +/- SEM, p = 0.638, N values are displayed. Mann-Whitney U independent samples t-test.



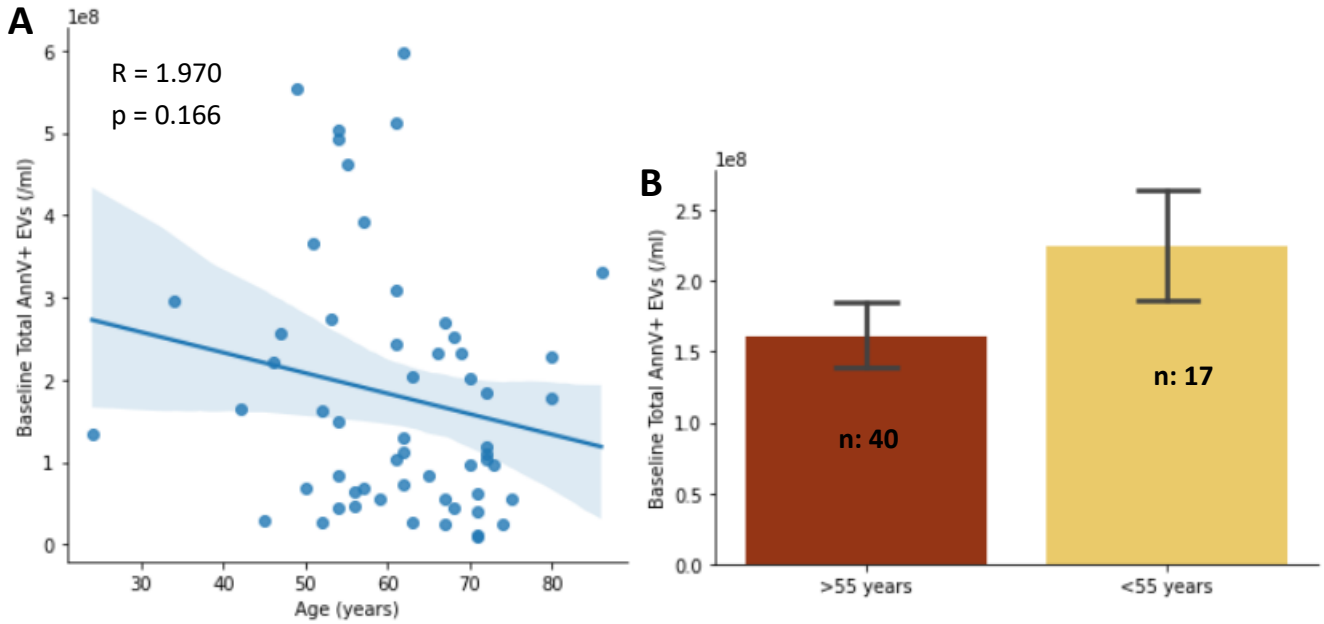
Supplementary Figure 3.5S4. TF-EVs (/mL) at baseline were assessed by VTE status. Data presented as Mean +/- SEM, p=0.904, N values are displayed. Mann-Whitney U independent samples t-test.



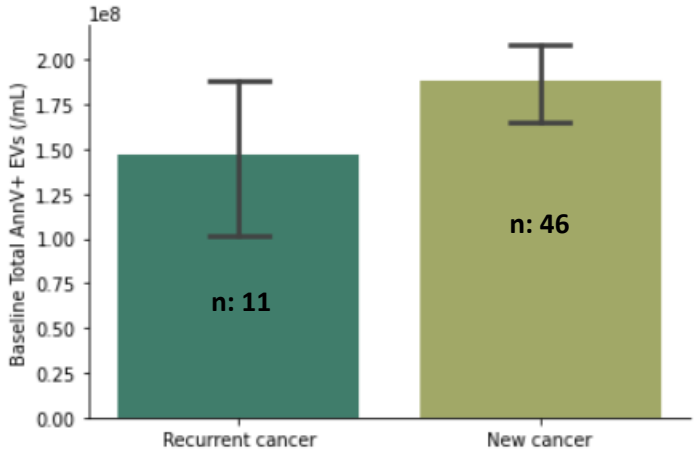
Supplementary Figure 3.5S5. TF-EVs (/mL) at baseline were assessed by bleeding event. Data presented as Mean +/- SEM, p = 0.129, N values are displayed. Mann-Whitney U independent samples t-test.



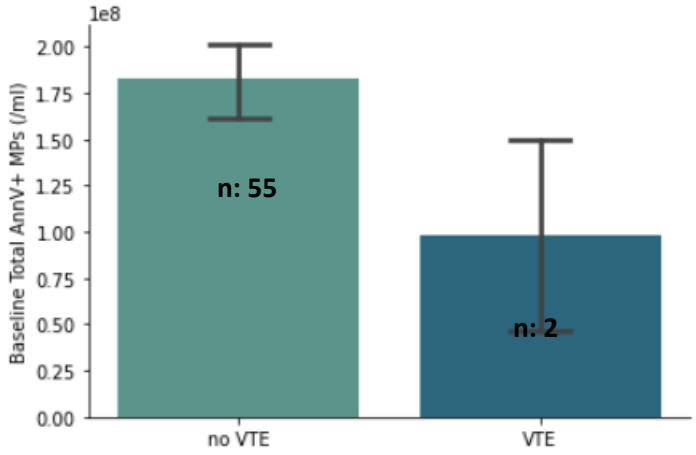
Supplementary Figure 3.6S1. Total AnnV+ EVs (/mL) were assessed at baseline and compared between the sexes (males and females). Data presented as Mean +/- SEM, p = 0.3, N values are displayed. Mann-Whitney U independent samples t-test.



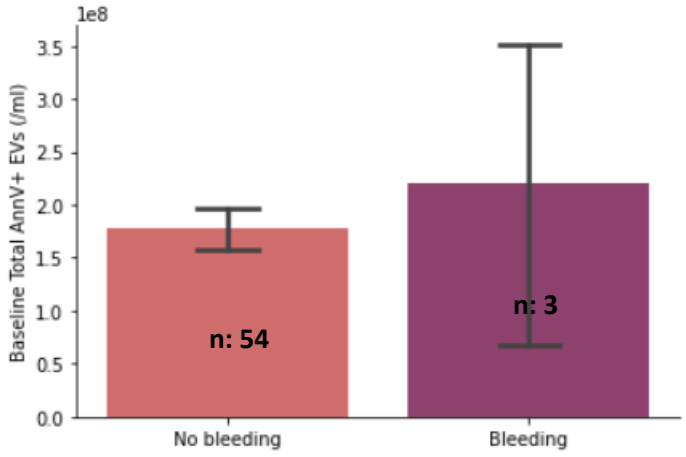
Supplementary Figure 3.6S2 Total AnnV+ EVs (/mL) in aging was assessed at baseline for paired patient samples. (A) Linear regression equation was found to be F(1, 55) = 1.970, p=0.166, R =0.186. (B) total EVs were also compared between patients above and below 55 years of age. Data presented as Mean +/- SEM; p = 0.168. N values are displayed. Mann-Whitney U independent t-test was conducted.



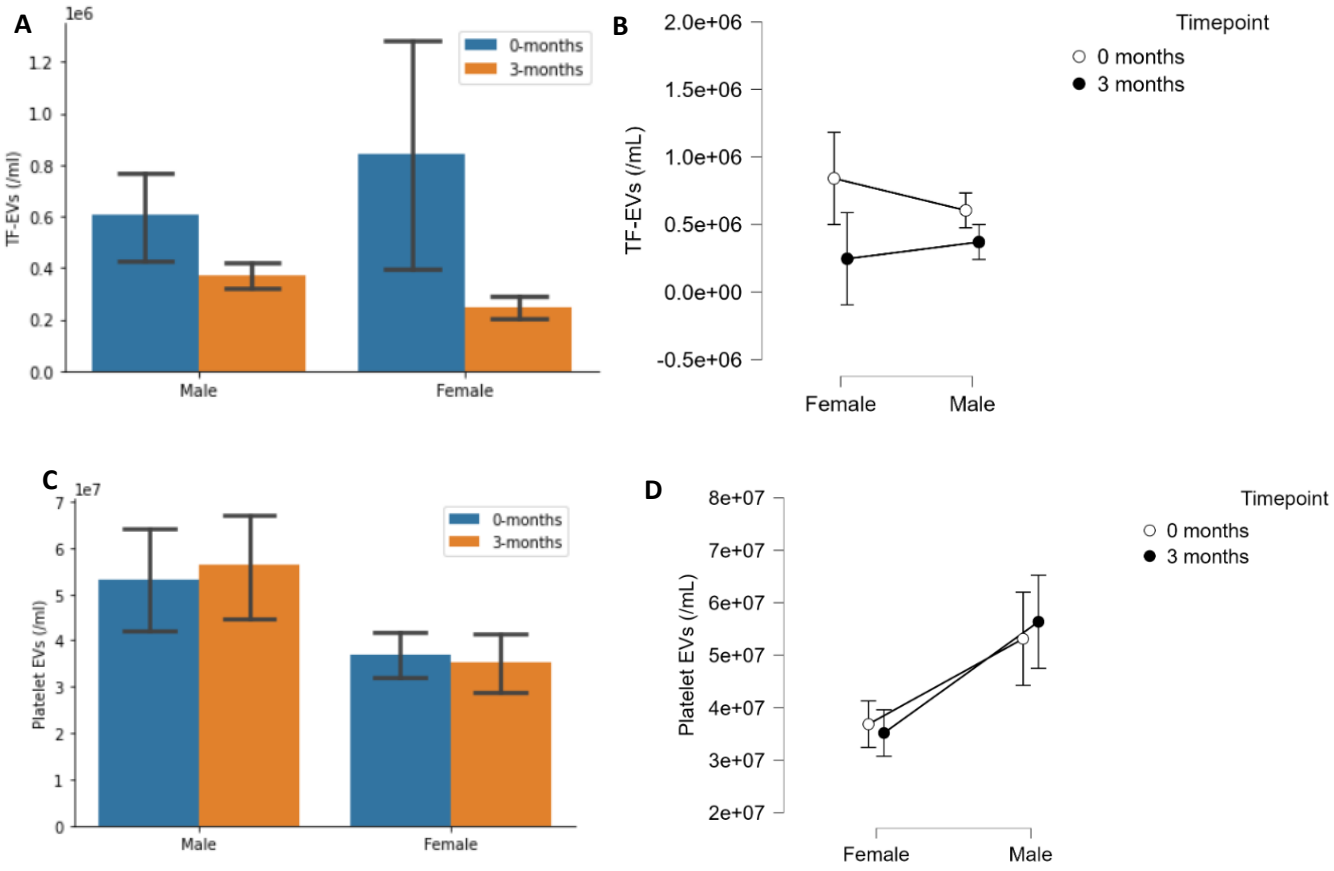
Supplementary Figure 3.6S3. Total AnnV+ EVs (/mL) were assessed at baseline and compared between the new and recurrent cancers. Data presented as Mean +/- SEM, p = 0.322, N values are displayed. Mann-Whitney U independent samples t-test.

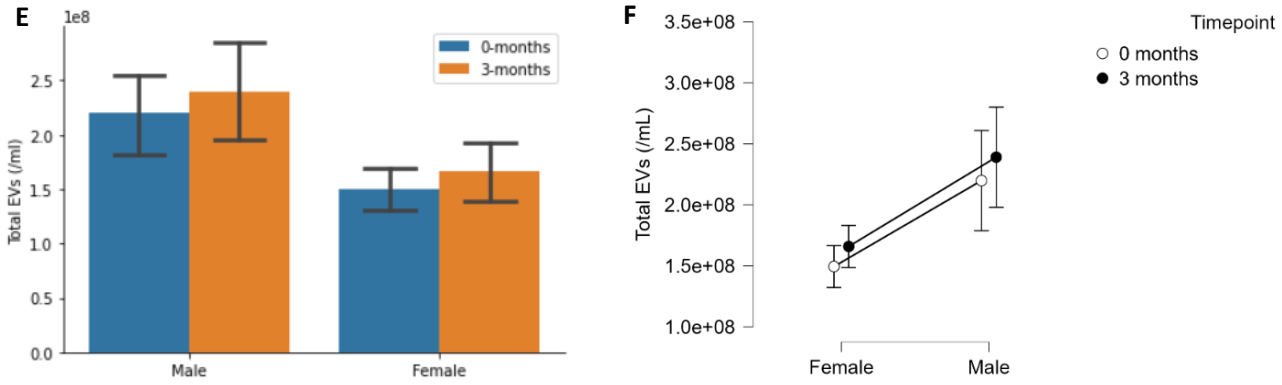


Supplementary Figure 3.6S4. Total AnnV+ EVs (/mL) at baseline were assessed by VTE status. Data presented as Mean +/- SEM, p = 0.501, N values are displayed. Mann-Whitney U independent samples t-test.

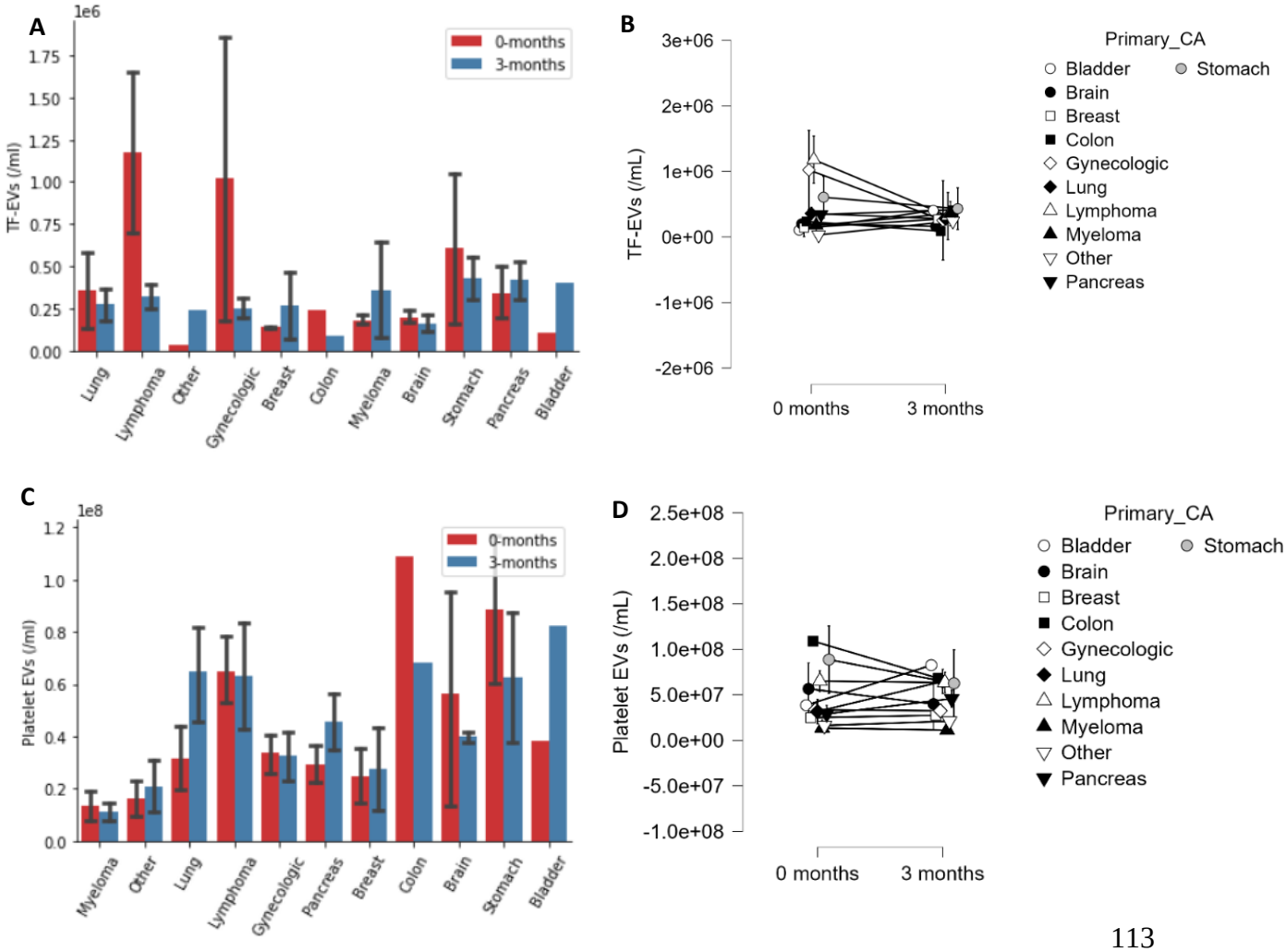


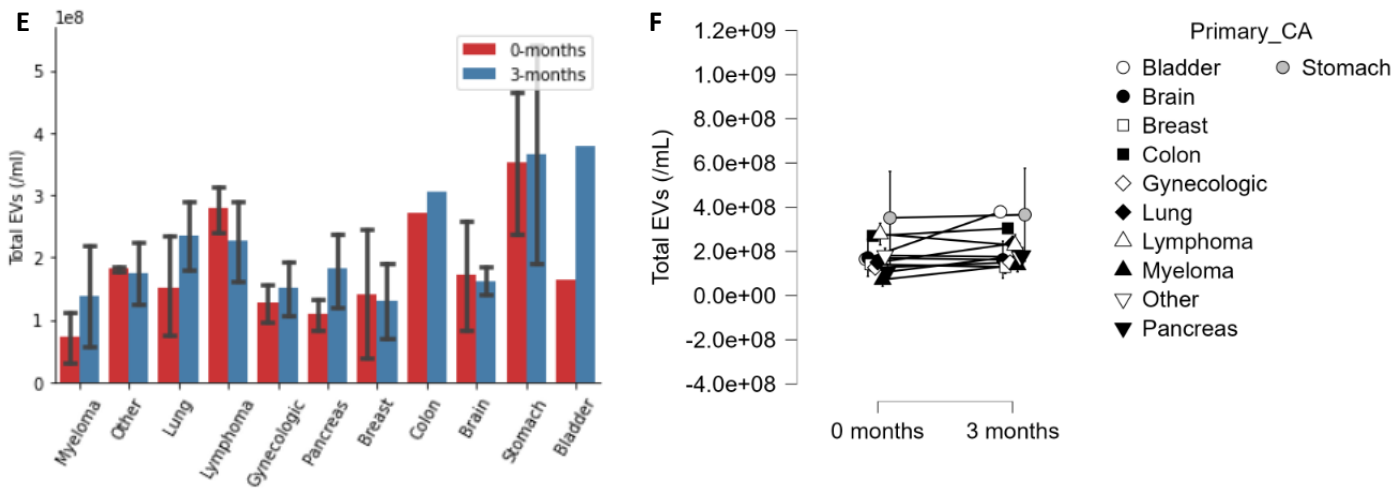
Supplementary Figure 3.6S5. Total AnnV+ EVs (/mL) at baseline were assessed by bleeding event. Data presented as Mean +/- SEM, p = 0.900, N values are displayed. Mann-Whitney U independent samples t-test.



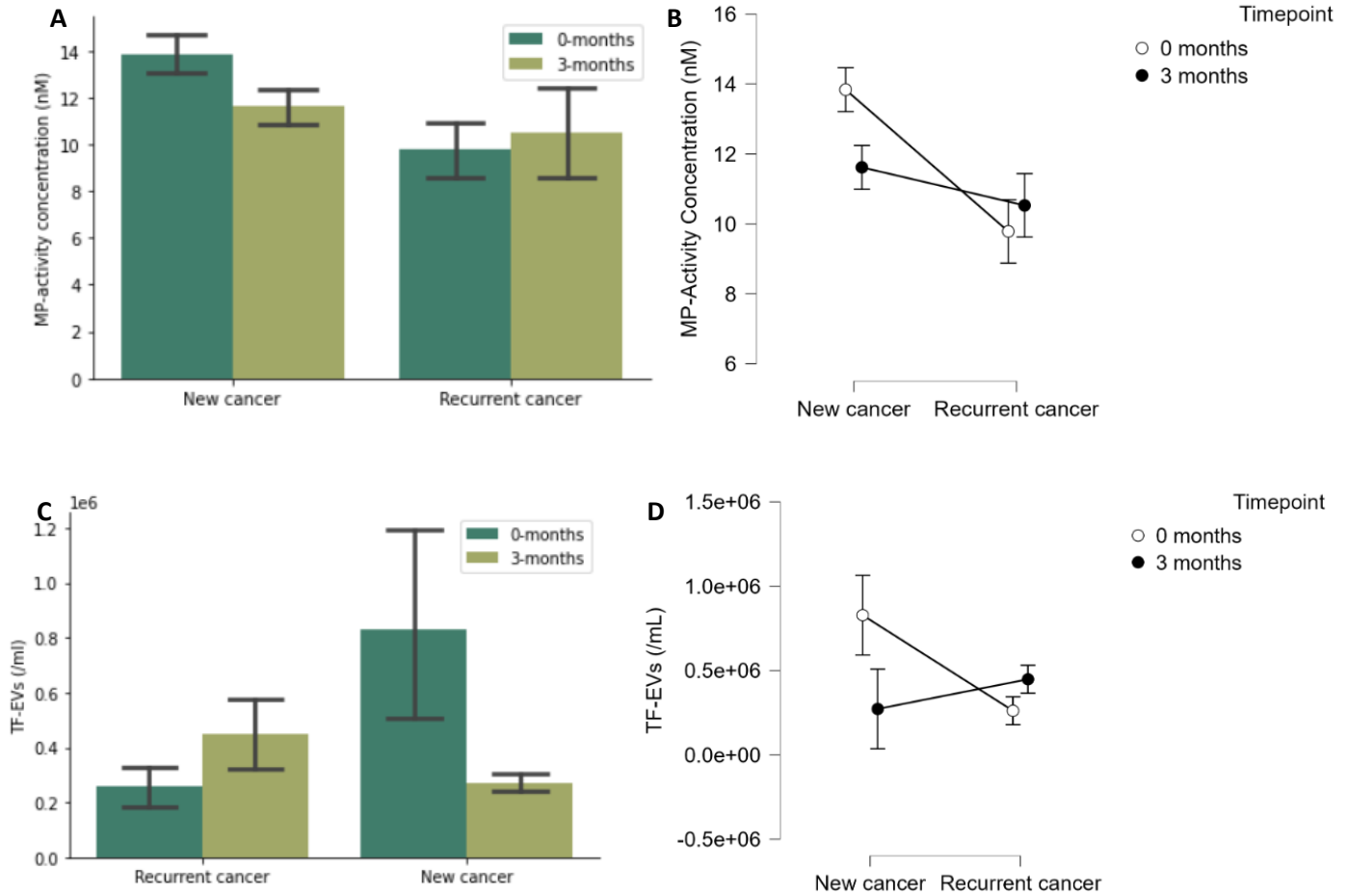


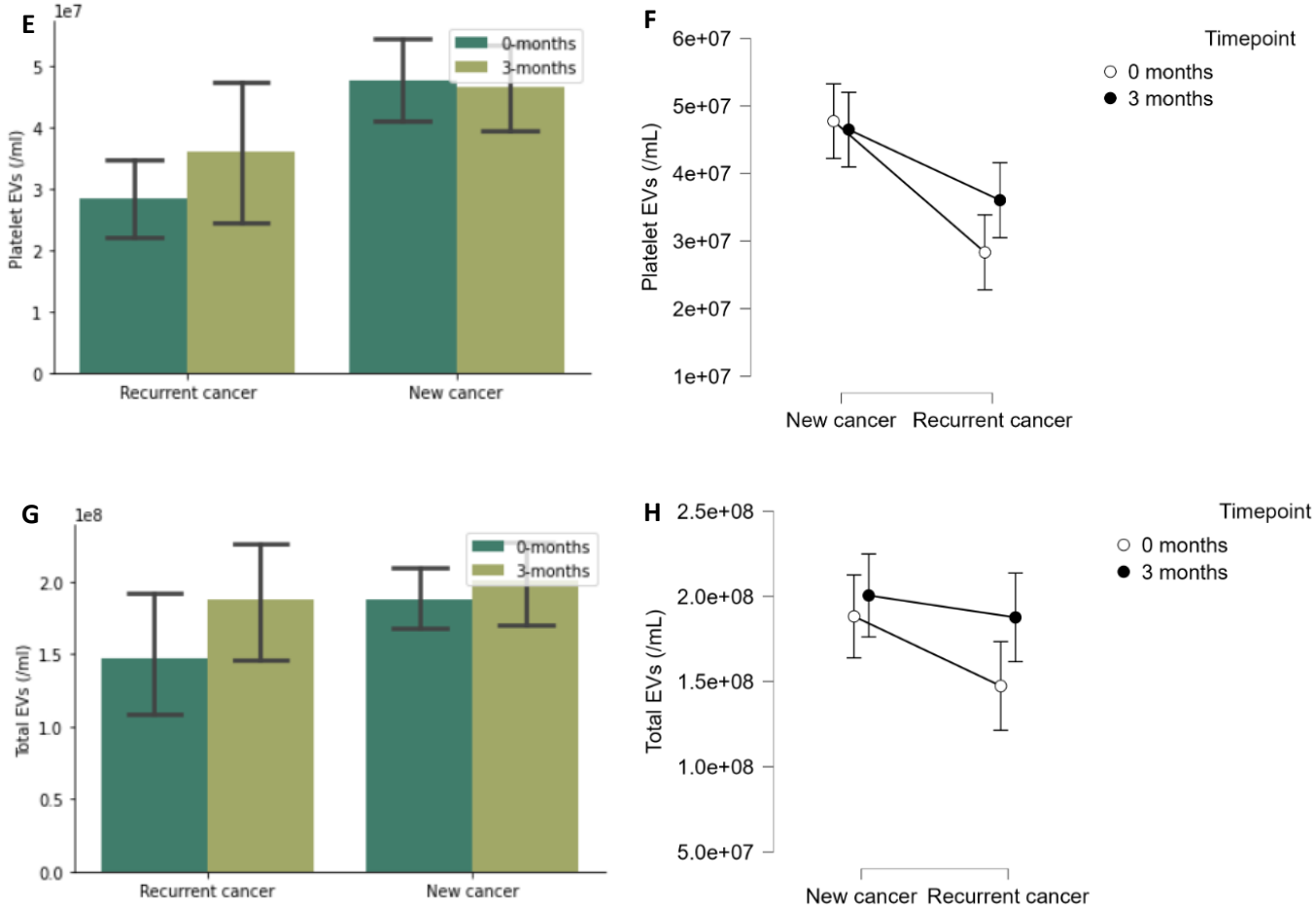
Supplementary Figure 3.7S1. Circulating EVs over time in males and females was measured by flow cytometry at 0-months and 3-months. A, B. TF-EVs (/mL), C, D. platelet EVs (/mL), E, F. total EVs (/mL). Data presented as Mean +/- SEM; Repeated measures ANOVA with Bonferroni post hoc test.



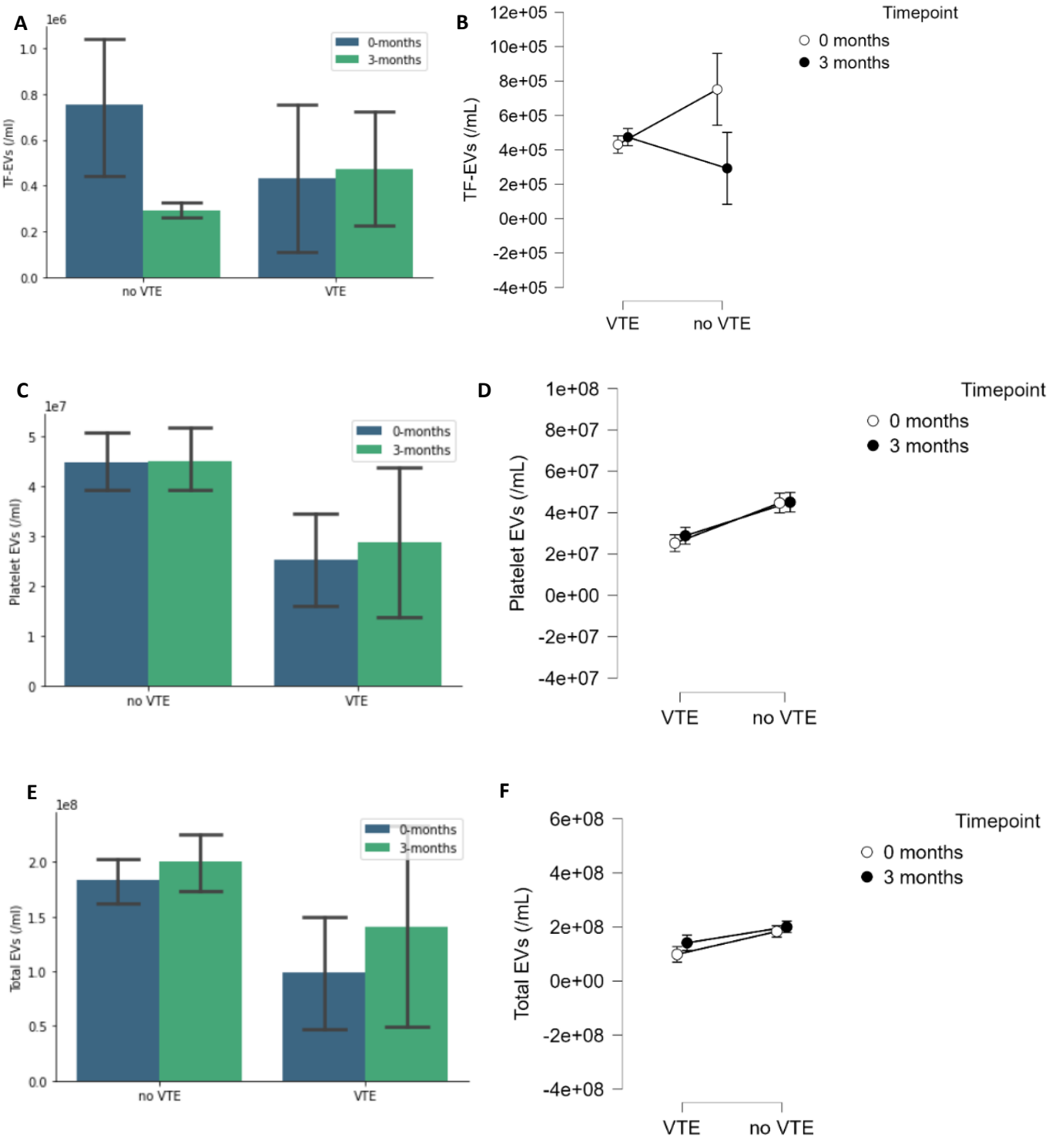


Supplementary Figure 3.7S2. Circulating EVs over time in each patient cancer type were measured by flow cytometry. A, B. TF-EVs (/mL), C, D. platelet EVs (/mL), and E, F. total EVs (/mL) at 0-months and 3-months. Data presented as Mean +/- SEM; Repeated measures ANOVA with Bonferroni post hoc test.

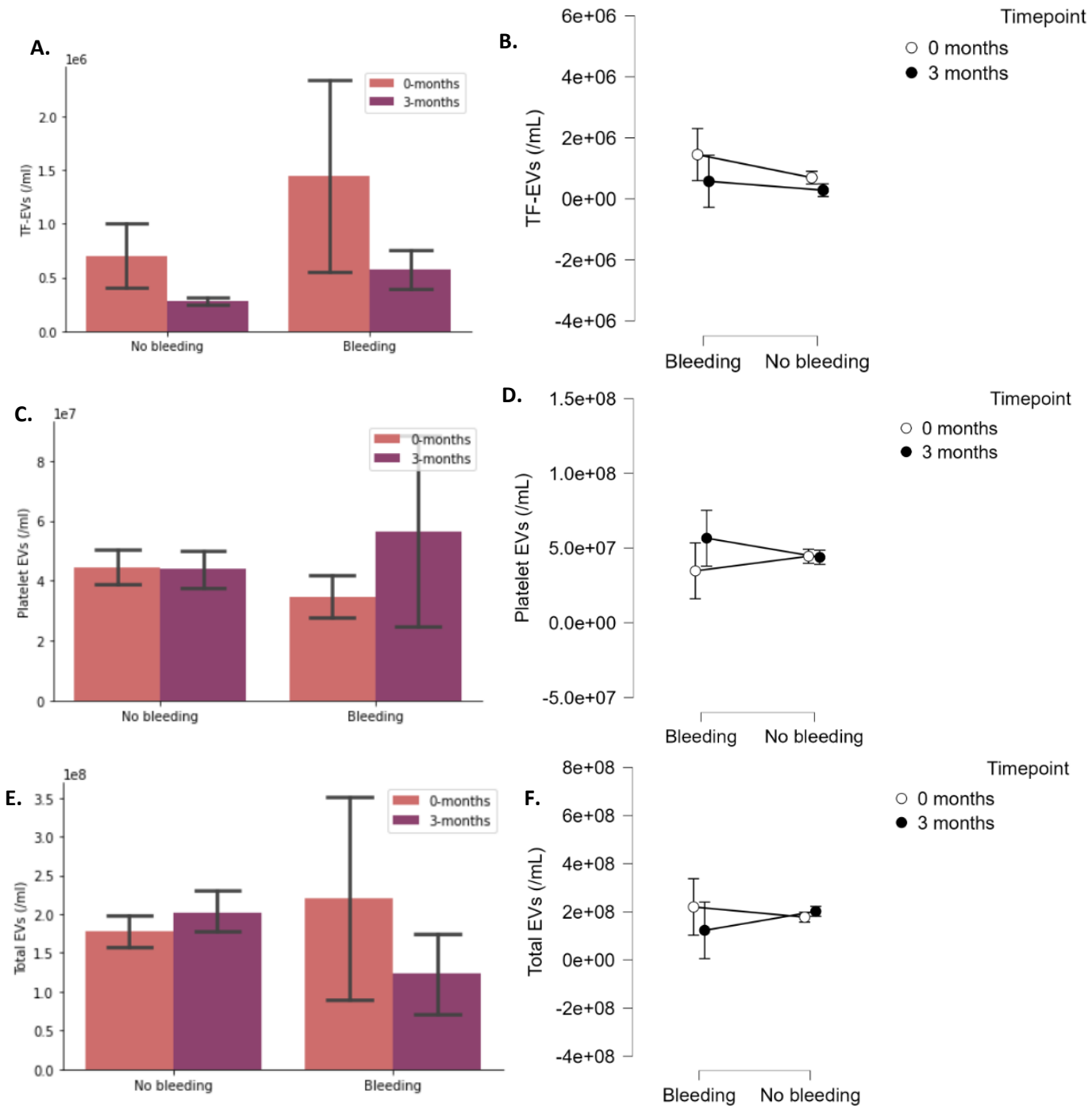




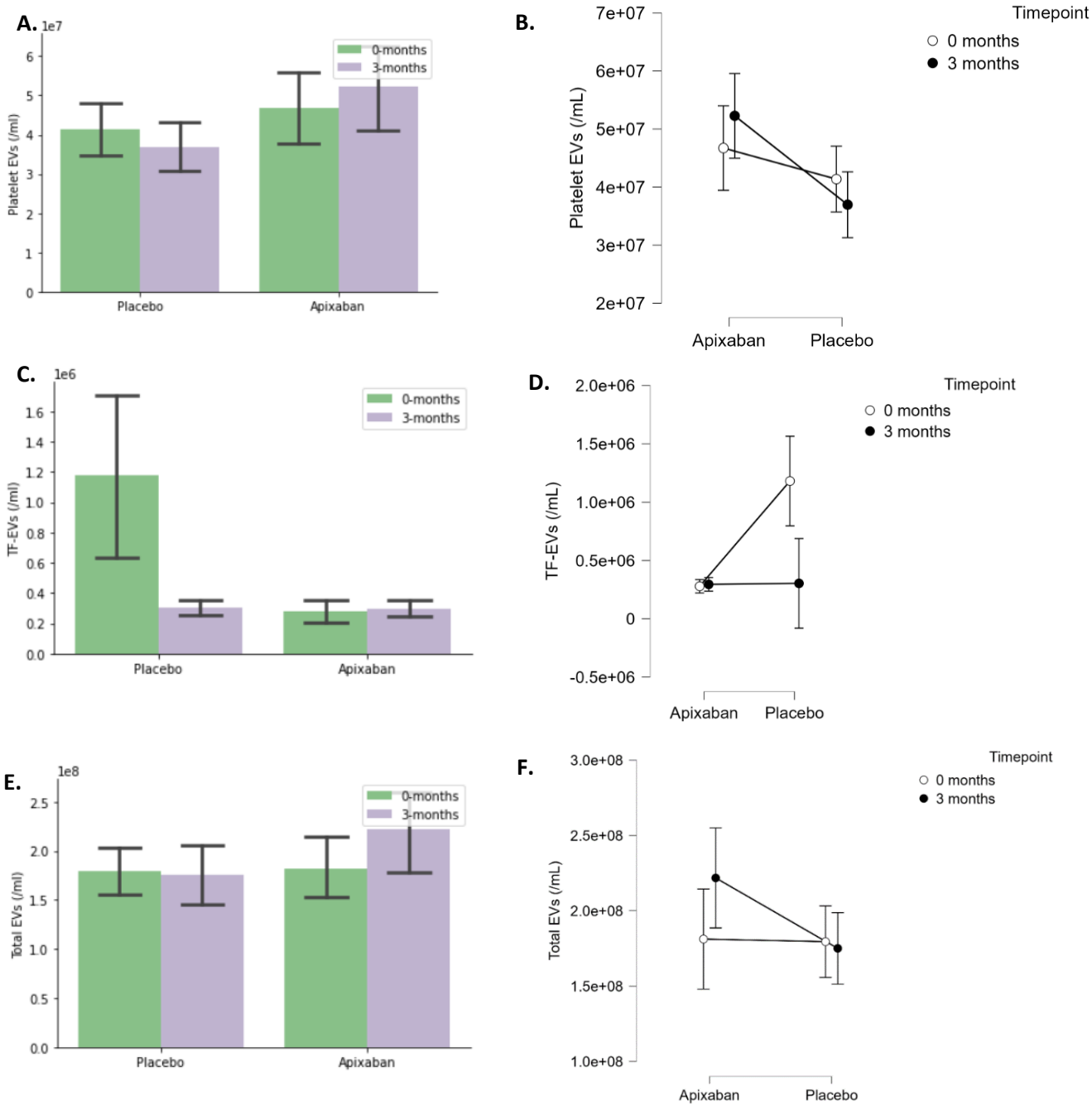
Supplementary Figure 3.7S3. Circulating EVs over time in new and recurrent cancers were measured by flow cytometry. A, B. MP-activity concentration (nM); C, D. TF-EVs (/mL); E, F. platelet EVs (/mL); and G, H. total EVs (/mL) at 0-months and 3-months. Data presented as Mean +/- SEM; Repeated measures ANOVA with Bonferroni post hoc test.



Supplementary Figure 3.7S4. Circulating EVs over time in patients with or without VTE were measured by A, B. TF-EVs (/mL); C, D. platelet EVs (/mL); and E, F. total EVs (/mL); at 0-months and 3-months. Data presented as Mean +/- SEM; Repeated measures ANOVA with Bonferroni post hoc test.



Supplementary Figure 3.7S5. Circulating EVs over time in patients with or without bleeding were measured by A, B. TF-EVs (/mL); C, D. platelet EVs (/mL); and E, F. total EVs (/mL); at 0-months and 3-months. Data presented as Mean +/- SEM; Repeated measures ANOVA with Bonferroni post hoc test.



Supplementary Figure 3.7S6. Circulating EVs over time in patients receiving treatment (apixaban or placebo) was measured by flow cytometry A, B. platelet EVs (/mL); C, D. TF-EVs (/mL); and E, F. total EVs (/mL) at 0-months and 3-months. Data presented as Mean +/- SEM. Repeated measures ANOVA with Bonferroni post hoc test.

Supplementary Tables

Supplementary Table 1. Dunn's multiple post hoc comparisons test for pro-coagulant EV-activity (MP-activity concentration (nM)) at baseline in paired patient samples across the different cancer types. *p<0.05, **p<0.01, ***p<0.001.

Comparison	z	W_i	W_j	p
Bladder - Brain	0.832	162.000	103.000	0.203
Bladder - Breast	-0.623	162.000	207.417	0.267
Bladder - Colon	-0.497	162.000	201.700	0.309
Bladder - Gynecologic	0.661	162.000	116.889	0.254
Bladder - Lung	-0.501	162.000	196.917	0.308
Bladder - Lymphoma	-0.639	162.000	205.587	0.261
Bladder - Myeloma	0.541	162.000	122.769	0.294
Bladder - Pancreas	-0.041	162.000	164.838	0.484
Bladder - Stomach	-0.044	162.000	165.156	0.482
Bladder - Other	-0.881	162.000	225.536	0.189
Brain - Breast	-2.968	103.000	207.417	0.001**
Brain - Colon	-2.058	103.000	201.700	0.020*
Brain - Gynecologic	-0.577	103.000	116.889	0.282
Brain - Lung	-3.357	103.000	196.917	< .001**
Brain - Lymphoma	-4.267	103.000	205.587	< .001**
Brain - Myeloma	-0.576	103.000	122.769	0.282
Brain - Pancreas	-2.296	103.000	164.838	0.011*
Brain - Stomach	-1.920	103.000	165.156	0.027*

Comparison	z	W_i	W_j	p
Brain - Other	-3.646	103.000	225.536	< .001**
Breast - Colon	0.113	207.417	201.700	0.455
Breast - Gynecologic	3.088	207.417	116.889	0.001**
Breast - Lung	0.322	207.417	196.917	0.374
Breast - Lymphoma	0.062	207.417	205.587	0.475
Breast - Myeloma	2.216	207.417	122.769	0.013*
Breast - Pancreas	1.343	207.417	164.838	0.090
Breast - Stomach	1.160	207.417	165.156	0.123
Breast - Other	-0.483	207.417	225.536	0.315
Colon - Gynecologic	1.935	201.700	116.889	0.027*
Colon - Lung	0.104	201.700	196.917	0.459
Colon - Lymphoma	-0.089	201.700	205.587	0.465
Colon - Myeloma	1.572	201.700	122.769	0.058
Colon - Pancreas	0.811	201.700	164.838	0.209
Colon - Stomach	0.748	201.700	165.156	0.227
Colon - Other	-0.480	201.700	225.536	0.316
Gynecologic - Lung	-3.979	116.889	196.917	< .001**
Gynecologic - Lymphoma	-6.271	116.889	205.587	< .001**
Gynecologic - Myeloma	-0.208	116.889	122.769	0.418
Gynecologic - Pancreas	-2.573	116.889	164.838	0.005**
Gynecologic - Stomach	-1.865	116.889	165.156	0.031*
Gynecologic - Other	-	116.88	225.53	< .00**

Comparison	z	W_i	W_j	p
	3.964	9	6	1*
Lung - Lymphoma	-0.432	196.917	205.587	0.333
Lung - Myeloma	2.341	196.917	122.769	0.010**
Lung - Pancreas	1.369	196.917	164.838	0.086
Lung - Stomach	1.075	196.917	165.156	0.141
Lung - Other	-0.927	196.917	225.536	0.177
Lymphoma - Myeloma	2.930	205.587	122.769	0.002**
Lymphoma - Pancreas	2.194	205.587	164.838	0.014*
Lymphoma - Stomach	1.564	205.587	165.156	0.059
Lymphoma - Other	-0.729	205.587	225.536	0.233
Myeloma - Pancreas	-1.368	122.769	164.838	0.086
Myeloma - Stomach	-1.190	122.769	165.156	0.117
Myeloma - Other	-2.797	122.769	225.536	0.003**
Pancreas - Stomach	-0.011	164.838	165.156	0.496
Pancreas - Other	-2.028	164.838	225.536	0.021*
Stomach - Other	-1.729	165.156	225.536	0.042*

Supplementary Table 2. Dunn's multiple post hoc comparisons test for platelet EVs at baseline in paired patient samples across the different cancer types. *p<0.05, **p<0.01, ***p<0.001

Comparison	z	W_i	W_j	p
Brain - Breast	0.296	27.833	23.500	0.383
Brain - Gynecologic	0.388	27.833	23.941	0.349
Brain - Lung	0.170	27.83	25.75	0.432

Comparison	z	W_i	W_j	p
		3	0	
Brain - Lymphoma	-1.103	27.833	39.154	0.135
Brain - Myeloma	1.311	27.833	12.500	0.095
Brain - Other	0.758	27.833	16.750	0.224
Brain - Pancreas	0.217	27.833	25.300	0.414
Brain - Stomach	-1.158	27.833	42.000	0.123
Breast - Gynecologic	-0.037	23.500	23.941	0.485
Breast - Lung	-0.162	23.500	25.750	0.436
Breast - Lymphoma	-1.286	23.500	39.154	0.099
Breast - Myeloma	0.821	23.500	12.500	0.206
Breast - Other	0.421	23.500	16.750	0.337
Breast - Pancreas	-0.134	23.500	25.300	0.447
Breast - Stomach	-1.333	23.500	42.000	0.091
Gynecologic - Lung	-0.203	23.941	25.750	0.419
Gynecologic - Lymphoma	-2.577	23.941	39.154	0.005**
Gynecologic - Myeloma	1.404	23.941	12.500	0.080
Gynecologic - Other	0.600	23.941	16.750	0.274
Gynecologic - Pancreas	-0.167	23.941	25.300	0.434
Gynecologic - Stomach	-2.028	23.941	42.000	0.021*
Lung - Lymphoma	-1.463	25.750	39.154	0.072
Lung - Myeloma	1.233	25.750	12.500	0.109

Comparison	z	W_i	W_j	p
Lung - Other	0.649	25.750	16.750	0.258
Lung - Pancreas	0.042	25.750	25.300	0.483
Lung - Stomach	-1.434	25.750	42.000	0.076
Lymphoma - Myeloma	3.162	39.154	12.500	< .001**
Lymphoma - Other	1.841	39.154	16.750	0.033*
Lymphoma - Pancreas	1.643	39.154	25.300	0.050
Lymphoma - Stomach	-0.311	39.154	42.000	0.378
Myeloma - Other	-0.317	12.500	16.750	0.376
Myeloma - Pancreas	-1.263	12.500	25.300	0.103
Myeloma - Stomach	-2.745	12.500	42.000	0.003**
Other - Pancreas	-0.638	16.750	25.300	0.262
Other - Stomach	-1.820	16.750	42.000	0.034*
Pancreas - Stomach	-1.554	25.300	42.000	0.060

Supplementary Table 3. Dunn's multiple post hoc comparisons test for total AnnV+ EVs at baseline in paired samples across the different cancer types. *p<0.05, **p<0.01, ***p<0.001

Comparison	z	W_i	W_j	p
Brain - Breast	0.194	27.333	24.500	0.423
Brain - Gynecologic	0.499	27.333	22.324	0.309
Brain - Lung	0.293	27.333	23.750	0.385
Brain - Lymphoma	-1.216	27.333	39.808	0.112

Comparison	z	W_i	W_j	p
Brain - Myeloma	1.165	27.333	13.700	0.122
Brain - Other	-0.422	27.333	33.500	0.337
Brain - Pancreas	0.328	27.333	23.500	0.372
Brain - Stomach	-1.117	27.333	41.000	0.132
Breast - Gynecologic	0.182	24.500	22.324	0.428
Breast - Lung	0.054	24.500	23.750	0.478
Breast - Lymphoma	-1.258	24.500	39.808	0.104
Breast - Myeloma	0.806	24.500	13.700	0.210
Breast - Other	-0.562	24.500	33.500	0.287
Breast - Pancreas	0.075	24.500	23.500	0.470
Breast - Stomach	-1.189	24.500	41.000	0.117
Gynecologic - Lung	-0.160	22.324	23.750	0.436
Gynecologic - Lymphoma	-2.962	22.324	39.808	0.002**
Gynecologic - Myeloma	1.058	22.324	13.700	0.145
Gynecologic - Other	-0.933	22.324	33.500	0.175
Gynecologic - Pancreas	-0.144	22.324	23.500	0.443
Gynecologic - Stomach	-2.098	22.324	41.000	0.018*
Lung - Lymphoma	-1.753	23.750	39.808	0.040*
Lung - Myeloma	0.935	23.750	13.700	0.175
Lung - Other	-0.703	23.750	33.500	0.241
Lung - Pancreas	0.023	23.75	23.50	0.491

Comparison	z	W_i	W_j	p
		0	0	
Lung - Stomach	-1.523	23.750	41.000	0.064
Lymphoma - Myeloma	3.097	39.808	13.700	< .001**
Lymphoma - Other	0.518	39.808	33.500	0.302
Lymphoma - Pancreas	1.934	39.808	23.500	0.027*
Lymphoma - Stomach	-0.130	39.808	41.000	0.448
Myeloma - Other	-1.477	13.700	33.500	0.070
Myeloma - Pancreas	-0.967	13.700	23.500	0.167
Myeloma - Stomach	-2.540	13.700	41.000	0.006**
Other - Pancreas	0.746	33.500	23.500	0.228
Other - Stomach	-0.541	33.500	41.000	0.294
Pancreas - Stomach	-1.628	23.500	41.000	0.052

Supplementary Table 4. Post hoc comparisons with Bonferroni correction for EV-activity (MP-Activity concentration (nM)) at 0 months (baseline) and 3-months in each cancer type.

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
Bladder, 0 months	Brain, 0 months	3.673	9.478	0.388	1.000
	Breast, 0 months	-4.598	9.737	-0.472	1.000
	Colon, 0 months	-5.935	10.667	-0.556	1.000
	Gynecologic, 0 months	1.774	9.115	0.195	1.000
	Lung, 0 months	-9.066	9.311	-0.974	1.000
	Lymphoma, 0 months	-7.332	9.112	-0.805	1.000
	Myeloma, 0 months	-0.009	9.684	-9.135e-4	1.000

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
	Pancreas, 0 months	-3.163	9.255	-0.342	1.000
	Stomach, 0 months	-4.436	9.562	-0.464	1.000
	Other, 0 months	-6.683	9.637	-0.693	1.000
	Bladder, 3 months	3.740	9.881	0.378	1.000
	Brain, 3 months	4.762	9.478	0.502	1.000
	Breast, 3 months	-2.982	9.737	-0.306	1.000
	Colon, 3 months	-2.457	10.667	-0.230	1.000
	Gynecologic, 3 months	0.974	9.115	0.107	1.000
	Lung, 3 months	-4.604	9.311	-0.495	1.000
	Lymphoma, 3 months	-2.941	9.112	-0.323	1.000
	Myeloma, 3 months	-2.711	9.684	-0.280	1.000
	Pancreas, 3 months	-5.746	9.255	-0.621	1.000
	Stomach, 3 months	0.354	9.562	0.037	1.000
	Other, 3 months	0.608	9.637	0.063	1.000
Brain, 0 months	Breast, 0 months	-8.271	4.701	-1.759	1.000
	Colon, 0 months	-9.608	6.408	-1.499	1.000
	Gynecologic, 0 months	-1.899	3.219	-0.590	1.000
	Lung, 0 months	-12.739	3.738	-3.408	0.162
	Lymphoma, 0 months	-11.005	3.213	-3.425	0.152
	Myeloma, 0 months	-3.682	4.589	-0.802	1.000
	Pancreas, 0 months	-6.836	3.598	-1.900	1.000
	Stomach, 0 months	-8.109	4.326	-1.875	1.000
	Other, 0 months	-10.356	4.491	-2.306	1.000
	Bladder, 3 months	0.067	9.478	0.007	1.000
	Brain, 3 months	1.089	3.206	0.340	1.000
	Breast, 3 months	-6.655	4.701	-1.416	1.000
	Colon, 3 months	-6.130	6.408	-0.957	1.000
	Gynecologic, 3 months	-2.699	3.219	-0.838	1.000
	Lung, 3 months	-8.277	3.738	-2.214	1.000
	Lymphoma, 3 months	-6.614	3.213	-2.059	1.000
	Myeloma, 3 months	-6.384	4.589	-1.391	1.000
	Pancreas, 3 months	-9.419	3.598	-2.618	1.000
	Stomach, 3 months	-3.319	4.326	-0.767	1.000
	Other, 3 months	-3.065	4.491	-0.683	1.000
Breast, 0 months	Colon, 0 months	-1.337	6.786	-0.197	1.000

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
	Gynecologic, 0 months	6.372	3.918	1.626	1.000
	Lung, 0 months	-4.468	4.355	-1.026	1.000
	Lymphoma, 0 months	-2.734	3.913	-0.699	1.000
	Myeloma, 0 months	4.589	5.104	0.899	1.000
	Pancreas, 0 months	1.435	4.235	0.339	1.000
	Stomach, 0 months	0.162	4.869	0.033	1.000
	Other, 0 months	-2.085	5.015	-0.416	1.000
	Bladder, 3 months	8.338	9.737	0.856	1.000
	Brain, 3 months	9.360	4.701	1.991	1.000
	Breast, 3 months	1.617	4.034	0.401	1.000
	Colon, 3 months	2.141	6.786	0.316	1.000
	Gynecologic, 3 months	5.572	3.918	1.422	1.000
	Lung, 3 months	-0.006	4.355	-0.001	1.000
	Lymphoma, 3 months	1.657	3.913	0.424	1.000
	Myeloma, 3 months	1.887	5.104	0.370	1.000
	Pancreas, 3 months	-1.148	4.235	-0.271	1.000
	Stomach, 3 months	4.952	4.869	1.017	1.000
	Other, 3 months	5.206	5.015	1.038	1.000
Colon, 0 months	Gynecologic, 0 months	7.709	5.858	1.316	1.000
	Lung, 0 months	-3.131	6.158	-0.508	1.000
	Lymphoma, 0 months	-1.397	5.854	-0.239	1.000
	Myeloma, 0 months	5.926	6.709	0.883	1.000
	Pancreas, 0 months	2.772	6.075	0.456	1.000
	Stomach, 0 months	1.499	6.532	0.229	1.000
	Other, 0 months	-0.748	6.642	-0.113	1.000
	Bladder, 3 months	9.675	10.667	0.907	1.000
	Brain, 3 months	10.697	6.408	1.669	1.000
	Breast, 3 months	2.953	6.786	0.435	1.000
	Colon, 3 months	3.478	6.249	0.557	1.000
	Gynecologic, 3 months	6.909	5.858	1.179	1.000
	Lung, 3 months	1.331	6.158	0.216	1.000
	Lymphoma, 3 months	2.994	5.854	0.511	1.000
	Myeloma, 3 months	3.224	6.709	0.481	1.000
	Pancreas, 3 months	0.189	6.075	0.031	1.000

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
	Stomach, 3 months	6.289	6.532	0.963	1.000
	Other, 3 months	6.543	6.642	0.985	1.000
Gynecologic, 0 months	Lung, 0 months	-10.840	2.688	-4.033	0.015 *
	Lymphoma, 0 months	-9.106	1.890	-4.818	< .001 **
	Myeloma, 0 months	-1.783	3.783	-0.471	1.000
	Pancreas, 0 months	-4.937	2.490	-1.983	1.000
	Stomach, 0 months	-6.210	3.459	-1.795	1.000
	Other, 0 months	-8.457	3.663	-2.309	1.000
	Bladder, 3 months	1.966	9.115	0.216	1.000
	Brain, 3 months	2.988	3.219	0.928	1.000
	Breast, 3 months	-4.756	3.918	-1.214	1.000
	Colon, 3 months	-4.231	5.858	-0.722	1.000
	Gynecologic, 3 months	-0.800	1.473	-0.543	1.000
	Lung, 3 months	-6.379	2.688	-2.373	1.000
	Lymphoma, 3 months	-4.715	1.890	-2.495	1.000
	Myeloma, 3 months	-4.485	3.783	-1.186	1.000
	Pancreas, 3 months	-7.520	2.490	-3.020	0.610
	Stomach, 3 months	-1.420	3.459	-0.411	1.000
	Other, 3 months	-1.166	3.663	-0.318	1.000
Lung, 0 months	Lymphoma, 0 months	1.734	2.680	0.647	1.000
	Myeloma, 0 months	9.057	4.233	2.140	1.000
	Pancreas, 0 months	5.903	3.132	1.885	1.000
	Stomach, 0 months	4.630	3.947	1.173	1.000
	Other, 0 months	2.383	4.127	0.578	1.000
	Bladder, 3 months	12.806	9.311	1.375	1.000
	Brain, 3 months	13.828	3.738	3.699	0.055
	Breast, 3 months	6.085	4.355	1.397	1.000
	Colon, 3 months	6.609	6.158	1.073	1.000
	Gynecologic, 3 months	10.040	2.688	3.736	0.048 *
	Lung, 3 months	4.462	2.551	1.749	1.000
	Lymphoma, 3 months	6.125	2.680	2.285	1.000
	Myeloma, 3 months	6.355	4.233	1.501	1.000
	Pancreas, 3 months	3.320	3.132	1.060	1.000
	Stomach, 3 months	9.420	3.947	2.387	1.000
	Other, 3 months	9.674	4.127	2.344	1.000

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
Lymphoma, 0 months	Myeloma, 0 months	7.323	3.778	1.939	1.000
	Pancreas, 0 months	4.169	2.482	1.680	1.000
	Stomach, 0 months	2.896	3.453	0.839	1.000
	Other, 0 months	0.649	3.657	0.178	1.000
	Bladder, 3 months	11.072	9.112	1.215	1.000
	Brain, 3 months	12.094	3.213	3.764	0.043*
	Breast, 3 months	4.350	3.913	1.112	1.000
	Colon, 3 months	4.875	5.854	0.833	1.000
	Gynecologic, 3 months	8.306	1.890	4.394	0.003**
	Lung, 3 months	2.728	2.680	1.018	1.000
	Lymphoma, 3 months	4.391	1.457	3.014	0.643
	Myeloma, 3 months	4.621	3.778	1.223	1.000
	Pancreas, 3 months	1.586	2.482	0.639	1.000
	Stomach, 3 months	7.686	3.453	2.226	1.000
	Other, 3 months	7.940	3.657	2.171	1.000
Myeloma, 0 months	Pancreas, 0 months	-3.154	4.110	-0.767	1.000
	Stomach, 0 months	-4.427	4.760	-0.930	1.000
	Other, 0 months	-6.674	4.911	-1.359	1.000
	Bladder, 3 months	3.749	9.684	0.387	1.000
	Brain, 3 months	4.771	4.589	1.040	1.000
	Breast, 3 months	-2.973	5.104	-0.582	1.000
	Colon, 3 months	-2.448	6.709	-0.365	1.000
	Gynecologic, 3 months	0.983	3.783	0.260	1.000
	Lung, 3 months	-4.596	4.233	-1.086	1.000
	Lymphoma, 3 months	-2.932	3.778	-0.776	1.000
	Myeloma, 3 months	-2.702	3.876	-0.697	1.000
	Pancreas, 3 months	-5.737	4.110	-1.396	1.000
	Stomach, 3 months	0.363	4.760	0.076	1.000
	Other, 3 months	0.617	4.911	0.126	1.000
Pancreas, 0 months	Stomach, 0 months	-1.273	3.815	-0.334	1.000
	Other, 0 months	-3.520	4.000	-0.880	1.000
	Bladder, 3 months	6.903	9.255	0.746	1.000
	Brain, 3 months	7.925	3.598	2.202	1.000
	Breast, 3 months	0.181	4.235	0.043	1.000
	Colon, 3 months	0.706	6.075	0.116	1.000
	Gynecologic, 3 months	4.137	2.490	1.662	1.000

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
	Lung, 3 months	-1.442	3.132	-0.460	1.000
	Lymphoma, 3 months	0.222	2.482	0.089	1.000
	Myeloma, 3 months	0.452	4.110	0.110	1.000
	Pancreas, 3 months	-2.583	2.297	-1.124	1.000
	Stomach, 3 months	3.517	3.815	0.922	1.000
	Other, 3 months	3.771	4.000	0.943	1.000
Stomach, 0 months	Other, 0 months	-2.247	4.666	-0.482	1.000
	Bladder, 3 months	8.176	9.562	0.855	1.000
	Brain, 3 months	9.198	4.326	2.126	1.000
	Breast, 3 months	1.455	4.869	0.299	1.000
	Colon, 3 months	1.979	6.532	0.303	1.000
	Gynecologic, 3 months	5.410	3.459	1.564	1.000
	Lung, 3 months	-0.168	3.947	-0.043	1.000
	Lymphoma, 3 months	1.495	3.453	0.433	1.000
	Myeloma, 3 months	1.725	4.760	0.362	1.000
	Pancreas, 3 months	-1.310	3.815	-0.343	1.000
	Stomach, 3 months	4.790	3.494	1.371	1.000
	Other, 3 months	5.044	4.666	1.081	1.000
Other, 0 months	Bladder, 3 months	10.423	9.637	1.081	1.000
	Brain, 3 months	11.445	4.491	2.549	1.000
	Breast, 3 months	3.701	5.015	0.738	1.000
	Colon, 3 months	4.226	6.642	0.636	1.000
	Gynecologic, 3 months	7.657	3.663	2.090	1.000
	Lung, 3 months	2.078	4.127	0.504	1.000
	Lymphoma, 3 months	3.742	3.657	1.023	1.000
	Myeloma, 3 months	3.972	4.911	0.809	1.000
	Pancreas, 3 months	0.937	4.000	0.234	1.000
	Stomach, 3 months	7.037	4.666	1.508	1.000
	Other, 3 months	7.291	3.735	1.952	1.000
Bladder, 3 months	Brain, 3 months	1.022	9.478	0.108	1.000
	Breast, 3 months	-6.722	9.737	-0.690	1.000
	Colon, 3 months	-6.197	10.667	-0.581	1.000
	Gynecologic, 3 months	-2.766	9.115	-0.303	1.000
	Lung, 3 months	-8.344	9.311	-0.896	1.000
	Lymphoma, 3 months	-6.681	9.112	-0.733	1.000

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
	Myeloma, 3 months	-6.451	9.684	-0.666	1.000
	Pancreas, 3 months	-9.486	9.255	-1.025	1.000
	Stomach, 3 months	-3.386	9.562	-0.354	1.000
	Other, 3 months	-3.132	9.637	-0.325	1.000
Brain, 3 months	Breast, 3 months	-7.743	4.701	-1.647	1.000
	Colon, 3 months	-7.219	6.408	-1.127	1.000
	Gynecologic, 3 months	-3.788	3.219	-1.177	1.000
	Lung, 3 months	-9.366	3.738	-2.506	1.000
	Lymphoma, 3 months	-7.703	3.213	-2.398	1.000
	Myeloma, 3 months	-7.473	4.589	-1.628	1.000
	Pancreas, 3 months	-10.508	3.598	-2.920	0.841
	Stomach, 3 months	-4.408	4.326	-1.019	1.000
	Other, 3 months	-4.154	4.491	-0.925	1.000
Breast, 3 months	Colon, 3 months	0.525	6.786	0.077	1.000
	Gynecologic, 3 months	3.956	3.918	1.010	1.000
	Lung, 3 months	-1.623	4.355	-0.373	1.000
	Lymphoma, 3 months	0.041	3.913	0.010	1.000
	Myeloma, 3 months	0.271	5.104	0.053	1.000
	Pancreas, 3 months	-2.764	4.235	-0.653	1.000
	Stomach, 3 months	3.335	4.869	0.685	1.000
	Other, 3 months	3.590	5.015	0.716	1.000
Colon, 3 months	Gynecologic, 3 months	3.431	5.858	0.586	1.000
	Lung, 3 months	-2.147	6.158	-0.349	1.000
	Lymphoma, 3 months	-0.484	5.854	-0.083	1.000
	Myeloma, 3 months	-0.254	6.709	-0.038	1.000
	Pancreas, 3 months	-3.289	6.075	-0.541	1.000
	Stomach, 3 months	2.811	6.532	0.430	1.000
	Other, 3 months	3.065	6.642	0.461	1.000
Gynecologic, 3 months	Lung, 3 months	-5.578	2.688	-2.075	1.000
	Lymphoma, 3 months	-3.915	1.890	-2.071	1.000
	Myeloma, 3 months	-3.685	3.783	-0.974	1.000
	Pancreas, 3 months	-6.720	2.490	-2.699	1.000
	Stomach, 3 months	-0.620	3.459	-0.179	1.000
	Other, 3 months	-0.366	3.663	-0.100	1.000
Lung, 3 months	Lymphoma, 3 months	1.663	2.680	0.621	1.000

Post Hoc Comparisons - Primary_CA ✻ Timepoint

		Mean Difference	SE	t	p_{bonf}
	Myeloma, 3 months	1.893	4.233	0.447	1.000
	Pancreas, 3 months	-1.141	3.132	-0.364	1.000
	Stomach, 3 months	4.958	3.947	1.256	1.000
	Other, 3 months	5.212	4.127	1.263	1.000
Lymphoma, 3 months	Myeloma, 3 months	0.230	3.778	0.061	1.000
	Pancreas, 3 months	-2.805	2.482	-1.130	1.000
	Stomach, 3 months	3.295	3.453	0.954	1.000
	Other, 3 months	3.549	3.657	0.970	1.000
Myeloma, 3 months	Pancreas, 3 months	-3.035	4.110	-0.738	1.000
	Stomach, 3 months	3.065	4.760	0.644	1.000
	Other, 3 months	3.319	4.911	0.676	1.000
Pancreas, 3 months	Stomach, 3 months	6.100	3.815	1.599	1.000
	Other, 3 months	6.354	4.000	1.588	1.000
Stomach, 3 months	Other, 3 months	0.254	4.666	0.054	1.000

* p < .05, ** p < .01, *** p < .001

Note. P-value adjusted for comparing a family of 231

Supplementary Table 5. Summary of all statistical analyses conducted on JASP.

Supplementary Table 5: Statistical Analyses														
			Main Effect					Post hoc						
Brief Description	n	DESCRIPTIVE STATS	Test	Factor	F value	P value	Significant?	Test	Description	t value	q value	P value	Significant?	Mark
MP-Activity concentration at baseline; sexes (male vs. female)	139, 191	Mean ± SEM	Mann-whitney U	Sexes		0.01	Yes							
MP-Activity concentration at baseline; aging	330	Regression	Linear regression	Aging	7.417	0.007	Yes							
MP-Activity concentration at baseline; aging (<55 vs. >55 years)	249, 81	Mean ± SEM	Mann-whitney U	Aging		0.044	Yes							
MP-Activity concentration at baseline; cancer type	13, 19, 14, 92, 30, 37, 16, 90, 12, 5, 2	Mean ± SEM	One-way ANOVA	Cancer type	3.641	<0.001	Yes	See supplementary table 1						
MP-Activity concentration at baseline; new vs. recurrent cancers	52, 278	Mean ± SEM	Mann-whitney U	New vs. recurrent		0.040	Yes							
MP-Activity concentration at baseline; VTE events	312, 18	Mean ± SEM	Mann-whitney U	VTE		0.188	No							
MP-Activity concentration at baseline; Bleeding events	303, 27	Mean ± SEM	Mann-whitney U	Bleeding		0.559	No							
Platelet EVs at baseline; cancer type	5, 2, 4, 13, 17, 5, 2, 1, 3, 4, 1	Mean ± SEM	One-way ANOVA (Kruskal-Wallis)	Cancer type	1.921	0.036	Yes	See Supplementary Table 2						
TF-EVs at baseline; aging	51	Regression	Linear regression	Aging	4.293	0.044	Yes	N/A						
TF-EVs at baseline; aging	36, 15	Mean ± SEM	Mann-whitney U	Aging		0.362	No							

(<55 vs. >55 years)														
Total EVs at baseline; cancer type	5, 2, 4, 13, 17, 5, 2, 1, 3, 4, 1	Mean ± SEM	One-way ANOVA (Kruskal-Wallis)	Cancer type		0.032	Yes	See Supplementary Table 3						
MP-Activity concentration over time; all patients	330, 330	Mean ± SEM	Wilcoxon signed-rank paired t-test	Timepoint		0.024	Yes	N/A						
TF-EVs over time; all patients	51, 51	Mean ± SEM	Wilcoxon signed-rank paired t-test	Timepoint		0.899	No	N/A						
Platelet EVs over time; all patients	57, 57	Mean ± SEM	Wilcoxon signed-rank paired t-test	Timepoint		0.927	No	N/A						
Total EVs over time; all patients	57, 57	Mean ± SEM	Wilcoxon signed-rank paired t-test	Timepoint		0.520	No	N/A						
MP-Activity concentration over time; sexes (male vs. female)	139, 139, 191, 191	Mean ± SEM	RM ANOVA	Interaction	0.507	0.477	No	Bonferroni	M, 0 mo. vs.F, 0 mo.	3.940		0.04	Yes	*
									M, 0 mo. vs. M, 3 mo.	2.402		0.273	No	
				Sexes	7.731	0.006	Yes		M, 0 mo. vs. F, 3 mo.	5.222		0.002	Yes	**
									F, 0 mo. vs. F, 3 mo.	1.283		1.000	No	
				Timepoint	5.493	0.02	Yes		F, 0 mo. vs. M, 3 mo.	-1.538		1.000	No	
									F, 3 mo. vs. M, 3 mo.	-2.821		0.311	No	
MP-Activity concentration over time; aging (>55 vs. <55 years)	249, 249, 81, 81	Mean ± SEM	RM ANOVA	Interaction	1.745	0.187	No	Bonferroni	<55, 0 mo. vs. >55, 0 mo.	-1.614		0.642	No	
									<55, 0 mo. vs. <55, 3 mo.	2.269		0.143	No	
				Aging	7.691	0.006	Yes		<55, 0 mo. vs. >55, 3 mo.	-0.909		1.000	No	
									>55, 0 mo. vs. <55, 3 mo.	3.751		0.001	Yes	**

				Timepoint	6.871	0.009	Yes		>55, 0 mo. vs. >55, 3 mo.	1.312		1.000	No	
									<55, 3 mo. vs. >55, 3 mo.	-3.046		0.015	Yes	*
TF-EVs over time; aging (>55 vs. <55 years)	25, 25, 32, 32	Mean ± SEM	RM ANOVA	Interaction	3.308	0.075	No	Bonferroni	<55, 0 mo. vs. >55, 0 mo.	2.319		0.135	No	
									<55, 0 mo. vs. <55, 3 mo.	2.385		0.126	No	
				Aging	2.128	0.151	No		<55, 0 mo. vs. >55, 3 mo.	2.582		0.068	No	
									>55, 0 mo. vs. <55, 3 mo.	0.529		1.000	No	
				Timepoint	4.794	0.033	Yes		>55, 0 mo. vs. >55, 3 mo.	0.342		1.000	No	
									<55, 3 mo. vs. >55, 3 mo.	-0.265		1.000	No	
Platelet EVs over time; aging (>55 vs. <55 years)		Mean ± SEM	RM ANOVA	Interaction	5.167	0.027	Yes	Bonferroni	<55, 0 mo. vs. >55, 0 mo.	1.089		1.000	No	
									<55, 0 mo. vs. <55, 3 mo.	1.862		0.408	No	
				Aging	0.014	0.906	No		<55, 0 mo. vs. >55, 3 mo.	0.342		1.000	No	
									>55, 0 mo. vs. <55, 3 mo.	0.544		1.000	No	
				Timepoint	0.717	0.401	No		>55, 0 mo. vs. >55, 3 mo.	-1.306		1.000	No	
									<55, 3 mo. vs. >55, 3 mo.	-1.291		1.000	No	
Total EVs over time; aging (>55 vs. <55 years)		Mean ± SEM	RM ANOVA	Interaction	5.725	0.025	Yes	Bonferroni	<55, 0 mo. vs. >55, 0 mo.	1.27		1.000	No	
									<55, 0 mo. vs. <55, 3 mo.	1.653		0.625	No	
				Aging	0.039	0.844	No		<55, 0 mo. vs. >55, 3 mo.	0.066		1.000	No	
									>55, 0 mo. vs. <55, 3 mo.	0.383		1.000	No	
				Timepoint	0.141	0.708	No		>55, 0 mo. vs. >55, 3 mo.	-1.846		0.421	No	
									<55, 3 mo. vs. >55, 3 mo.	-1.587		0.694	No	
MP-Activity concentration	(A) 2, 19, 12, 14, 5, 90, 30, 92, 13, 37, 16	Mean ± SEM	RM ANOVA	Interaction	1.541	0.124	No	Bonferroni	See Supplementary Table 4					

over time; cancer type														
				Cancer Type	3.207	<0.001	Yes							
				Timepoint	2.783	0.096	No							
MP-Activity concentration over time; VTE events	312, 312, 18, 18	Mean ± SEM	RM ANOVA	Interaction	0.234	0.629	No	N/A						
				VTE	0.034	0.853	No							
				Timepoint	2.122	0.146	No							
MP-Activity concentration over time; Bleeding events	303, 303, 27, 27	Mean ± SEM	RM ANOVA	Interaction	0.729	0.394	No	N/A						
				Bleeding	1.015	0.314	No							
				Timepoint	3.815	0.052	No							
MP-Activity concentration over time; Treatment	161, 161, 169, 169	Mean ± SEM	RM ANOVA	Interaction	0.096	0.757	No	Bonferroni	Apixaban, 0 mo. vs. placebo, 0 mo.	0.629		1.000	No	
									Apixaban, 0 mo. vs. apixaban, 3 mo.	1.400		0.974	No	
				Treatment	0.893	0.345	No		Apixaban, 0 mo. vs. placebo, 3 mo.	2.017		0.265	No	
									Placebo, 0 mo. vs. apixaban, 3 mo.	0.426		1.000	No	
				Timepoint	5.131	0.024	Yes		Placebo, 0 mo. vs. placebo, 3 mo.	1.799		0.438	No	
									Apixaban, 3 mo. vs. placebo, 3 mo.	0.963		1.000	No	
Platelet EVs at baseline; sexes (male vs. female)	25, 32	Mean ± SEM	Mann-whitney U	Sexes		0.624	No							
Platelet EVs at baseline; aging	56	Regression	Linear regression	Aging	3.483	0.067	No							
Platelet EVs at baseline; aging (<55 vs. >55 years)	40, 17	Mean ± SEM	Mann-whitney U	Aging		0.148	No							
Platelet EVs at baseline, new	46, 11	Mean ± SEM	Mann-whitney U	New vs. recurrent		0.245	No							

vs. recurrent cancers														
Platelet EVs at baseline; VTE events	55, 2	Mean ± SEM	Mann-whitney U	VTE		0.680	No							
Platelet EVs at baseline; Bleeding events	54, 3	Mean ± SEM	Mann-whitney U	Bleeding		0.900	No							
TF-EVs at baseline; sexes (male vs. female)	22, 29	Mean ± SEM	Mann-whitney U	Sexes		0.158	No							
TF-EVs at baseline; cancer type	4, 13, 1, 15, 2, 1, 3, 3, 4, 4, 1	Mean ± SEM	One-way ANOVA	Cancer type	0.224	0.977	No	N/A						
TF-EVs at baseline, new vs. recurrent cancers	43, 8	Mean ± SEM	Mann-whitney U	New vs. recurrent		0.638	No							
TF-EVs at baseline; VTE events	49, 2	Mean ± SEM	Mann-whitney U	VTE		0.904	No							
TF-EVs at baseline; Bleeding events	48, 3	Mean ± SEM	Mann-whitney U	Bleeding		0.129	No							
Total EVs at baseline; sexes (male vs. female)	25, 32	Mean ± SEM	Mann-whitney U	Sexes		0.296	No							
Total EVs at baseline; aging	56	Regression	Linear regression	Aging	1.97	0.166	No							
Total EVs at baseline; aging (<55 vs. >55 years)	40, 17	Mean ± SEM	Mann-whitney U	Aging		0.168	No							
Total EVs at baseline, new vs. recurrent cancers	46, 11	Mean ± SEM	Mann-whitney U	New vs. recurrent		0.322	No							
Total EVs at baseline; VTE events	55, 2	Mean ± SEM	Mann-whitney U	VTE		0.501	No							
Total EVs at baseline; Bleeding events	54, 3	Mean ± SEM	Mann-whitney U	Bleeding		0.900	No							
TF-EVs over time; sexes	22, 22, 29, 29	Mean ± SEM	RM ANOVA	Interaction	0.389	0.536	No	N/A						

				Sexes	0.039	0.844	No							
				Timepoint	2.062	0.157	No							
Platelet EVs over time; sexes	25, 25, 32, 32	Mean ± SEM	RM ANOVA	Interaction	0.141	0.709	No	N/A						
				Sexes	3.571	0.064	No							
				Timepoint	0.014	905	No							
Total EVs over time; sexes	25, 25, 32, 32	Mean ± SEM	RM ANOVA	Interaction	0.002	0.963	No	N/A						
				Sexes	3.999	0.05	No							
				Timepoint	0.377	0.542	No							
TF-EVs over time; cancer type	(A) 4, 13, 1, 15, 2, 1, 3, 3, 4, 4, 1	Mean ± SEM	RM ANOVA	Interaction	0.185	0.996	No	N/A						
				Cancer Type	0.187	0.996	No							
				Timepoint	0.056	0.815	No							
Platelet EVs over time; cancer type	(A) 5, 2, 4, 13, 17, 5, 2, 1, 3, 4, 1	Mean ± SEM	RM ANOVA	Interaction	0.497	0.883	No	N/A						
				Cancer Type	1.603	0.136	No							
				Timepoint	0.013	0.908	No							
Total EVs over time; cancer type	(A) 5, 2, 4, 13, 17, 5, 2, 1, 3, 4, 1	Mean ± SEM	RM ANOVA	Interaction	0.282	0.982	No	N/A						
				Cancer Type	1.638	0.126	No							
				Timepoint	0.798	0.376	No							
MP-Activity concentration over time; new vs. recurrent cancers	278, 278, 52, 52	Mean ± SEM	RM ANOVA	Interaction	1.946	0.164	No	N/A						
				New vs. recurrent	2.394	0.123	No							
				Timepoint	0.483	0.488	No							
TF-EVs over time; New and recurrent cancers	8, 8, 43, 43	Mean ± SEM	RM ANOVA	Interaction	0.900	0.347	No	N/A						
				New vs. recurrent	0.256	0.615	No							
				Timepoint	0.223	0.639	No							
Platelet EVs over time; New and recurrent cancers	11, 11, 46, 46	Mean ± SEM	RM ANOVA	Interaction	0.295	0.589	No	N/A						
				New vs. recurrent	1.378	0.245	No							
				Timepoint	0.153	0.697	No							

Total EVs over time; New and recurrent cancers	11, 11, 46, 46	Mean ± SEM	RM ANOVA	Interaction	0.147	0.703	No	N/A						
				New vs. recurrent	0.331	0.567	No							
				Timepoint	0.517	0.475	No							
TF-EVs over time; VTE events	49, 49, 2, 2	Mean ± SEM	RM ANOVA	Interaction	0.115	0.736	No	N/A						
				VTE	0.009	0.924	No							
				Timepoint	0.079	0.779	No							
Platelet EVs over time; VTE events	55, 55, 2, 2	Mean ± SEM	RM ANOVA	Interaction	0.008	0.929	No	N/A						
				VTE	0.421	0.519	No							
				Timepoint	0.012	0.912	No							
Total EVs over time; VTE events	55, 55, 2, 2	Mean ± SEM	RM ANOVA	Interaction	0.027	0.869	No	N/A						
				VTE	0.518	0.475	No							
				Timepoint	0.144	0.706	No							
TF-EVs over time; Bleeding events	48,48,3,3	Mean ± SEM	RM ANOVA	Interaction	0.145	0.705	No	N/A						
				Bleeding	0.778	0.382	No							
				Timepoint	1.119	0.295	No							
Platelet EVs over time; Bleeding events	54,54,3,3	Mean ± SEM	RM ANOVA	Interaction	0.602	0.441	No	N/A						
				Bleeding	0.004	0.947	No							
				Timepoint	0.529	0.47	No							
Total EVs over time; Bleeding events	54,54,3,3	Mean ± SEM	RM ANOVA	Interaction	0.893	0.349	No	N/A						
				Bleeding	0.049	0.825	No							
				Timepoint	0.324	0.571	No							
Platelet EVs over time; Treatment (apixaban vs. placebo)	29, 29, 28, 28	Mean ± SEM	RM ANOVA	Interaction	0.588	0.447	No	N/A						
				Treatment	1.053	0.309	No							
				Timepoint	0.008	0.93	No							
TF-EVs over time;	26, 26, 25, 25	Mean ± SEM	RM ANOVA	Interaction	2.544	0.117	No	N/A						

Treatment (apixaban vs. placebo)														
				Treatment	2.765	0.103	No							
				Timepoint	2.364	0.131	No							
Total EVs over time; Treatment (apixaban vs. placebo)	29, 29, 28, 28	Mean ± SEM	RM ANOVA	Interaction	0.616	0.436	No	N/A						
				Treatment	0.434	0.513	No							
				Timepoint	0.397	0.531	No							