Published in final edited form as:

Transl Res. 2020 November ; 225: 33–53. doi:10.1016/j.trsl.2020.06.012.

Mechanisms and biomarkers of cancer-associated thrombosis

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Abstract

Cancer-associated thrombosis is a leading cause of non-cancer death in cancer patients and is comprised of both arterial and venous thromboembolism. There are multiple risk factors for developing VTE, including cancer type, stage, treatment, and other medical comorbidities, which suggests that the etiology of thrombosis is multifactorial. While cancer-associated thrombosis can be treated with anticoagulation, benefits of therapy must be balanced with the increased bleeding risks seen in patients with cancer. Although risk models exist for primary and recurrent VTE, additional predictors are needed to improve model performance and discrimination of high-risk patients. This review will outline the diverse mechanisms driving thrombosis in cancer patients, as well as provide an overview of biomarkers studied in thrombosis risk and important considerations when selecting candidate biomarkers.

Introduction

Cancer-associated thrombosis (CAT), which includes both arterial and venous thromboembolism (VTE), is a significant complication and leading cause of non-cancer death in ambulatory cancer patients receiving systemic therapy.¹ VTE has been more widely studied than arterial thromboembolism (ATE), with cancer patients having 4–7 times the risk of VTE compared to the general populace, generally concentrated in the first few months after cancer diagnosis.^{2,3} Interestingly, the timing of ATE risk appears to be more uniform, with a cumulative incidence of 1–5% at 6-months after cancer diagnosis.^{4,5} More than half of deaths from pulmonary embolism are potentially preventable through appropriate prophylaxis, but risks of VTE must be balanced with increased bleeding risks in cancer patients while on anticoagulation.^{6,7} Known risk factors for VTE include cancer type, stage, treatment regimen, and comorbid disease with rates being highest in pancreatic, brain, and stomach cancer.⁸ This variety of risk factors likely reflects the multifactorial etiology of thrombosis in cancer. New advances in research have deepened the mechanistic knowledge driving CAT and have identified new targets for study.

Management of CAT is an evolving field, especially given the availability of new direct oral anticoagulants, and there is continued research on what are the best practices for improving CAT outcomes. Risk assessment models can guide clinical decision-making and are available for predicting primary VTE and recurrent VTE and include the Khorana, Vienna

CATS, PROTECHT, CONKO, ONKOTEV, COMPASS-CAT, TiC-Onco, CATS nomogram, and Ottawa scores.^{9–17} However, these models have mixed performances and require further refinement to improve discrimination and identification of high-risk patients.

Biomarkers are measurable, biologic parameters that can be used to improve prediction, diagnostic, and prognostic models of disease. In CAT, they have been extensively studied for prediction of primary VTE risk (Table 1), with fewer studies on recurrent VTE (Table 2) and primary ATE (Table 3). No studies have assessed biomarker use in predicting risk of recurrent ATE.

While many potential biomarkers exist, selection of candidate biomarkers require consideration of scalability and practicality as their use transitions from research to clinical practice. Ideal biomarkers are noninvasive, low cost, simple to perform, accurate, and discriminative.¹⁸ During clinical practice, physicians may avoid ordering invasive or costly tests to reduce physical and fiscal patient burdens. Complex tests that rely heavily on skilled laboratory personnel or expensive technology can limit accuracy of biomarker measurement and reduce testing availability. Finally, biomarkers should be able to adequately separate patients with different risks. Biomarkers and their associated models must balance both simplicity, which encourages clinical use, with complexity and enhanced model performance to improve clinical outcomes.¹⁹

This review will cover key mechanisms underlying CAT and relevant biomarkers for risk assessment, along with associated challenges in biomarker assays (Table 4). Where appropriate, both mechanisms and biomarker roles will be described, but some topics will be limited to mechanistic or biomarker research. Detailed descriptions of CAT epidemiology, risk factors, and treatment have been previously described elsewhere and are beyond this scope of this review.^{8,20–24}

Extracellular Vesicles

Mechanism—Extracellular vesicles (EVs) are defined by the International Society for Extracellular Vesicles (ISEV) as “the generic term for naturally released particles from the cell that are delimited by a lipid bilayer and cannot replicate”.²⁵ Historically, the literature has used the terms microvesicles or microparticles to generally refer to EVs that are 1000 nm in size and derived from plasma membranes of cells. However, this classification is controversial and we will refer to these particles as EVs in this review, in accordance with ISEV guidelines. Cancer cells are capable of shedding EVs which can contain cell-specific surface proteins and cargo such as RNAs, DNAs, proteins, and lipid rafts.^{26–28}

Intrinsic procoagulant activity of EVs stem from their negatively charged plasma membrane which serves as a catalytic surface for the vitamin K-dependent clotting factors: VII, IX, X, and prothrombin. This negative charge results from the asymmetric externalization of aminophospholipids, such as phosphatidylserine, from the inner plasma membrane leaflet during the formation of EVs by calcium-dependent scramblases, such as TMEM16F.^{29–31} Phosphatidylserine on EVs binds clotting factors through their positively charged γ -carboxyglutamic acid domains and increases catalytic efficiency of phospholipid-dependent

coagulation reactions such as the tenase and prothrombinase complexes by 3 orders of magnitude.³²

EVs also contain differential procoagulant activity depending on their mechanism of formation and cargo contents. Prothrombotic surface proteins and cargo include tissue factor (TF), inflammatory molecules, and podoplanin. A simplified model of key mechanisms occurring in blood are presented in Figure 1.

Biomarker Use in Primary VTE Risk—Studies on VTE risk with total EV quantities are limited. In a prospective study of newly diagnosed and progressive disease cancer patients, procoagulant activity of EVs due to the exposure of negatively charged phospholipids, as quantified by a prothrombinase assay, was not significantly associated with VTE risk (HR [hazard ratio] 0.95, 95% CI [confidence interval] 0.55–1.64).³³

Testing of EVs falls broadly into phenotypic or functional assays and include flow cytometry, electron microscopy, confocal microscopy, and atomic force microscopy in addition to function-specific assays. Phenotypic assays allow for quantification and assessment of morphology and cellular origin of EVs. Flow cytometry is the most common assay for EV quantification and assessment of cellular origin. Electron microscopy can study membrane morphology and composition, while confocal microscopy can evaluate cellular origin. Atomic force microscopy measures interaction forces between a cantilever tip and the test sample, allowing for three-dimensional topography construction as well as quantification.³⁴ Functional assays commonly require extraction of EVs by capture techniques or ultracentrifugation prior to study.

While research on EVs as biomarkers has rapidly grown, reproducible and reliable testing of EVs has been hampered by both pre-analytical and analytical obstacles. Differences in pre-analytical steps, such as blood collection, sample processing, and handling and storage can severely impact the testing results. For example, traumatic blood collection can activate platelets and falsely increase EV quantity, while different centrifugation speeds will impact extraction efficiency of EVs.^{34,35}

Analytical challenges are intrinsic to the assay itself, such as older flow cytometers having limited capability to detect smaller EVs.³⁵ These obstacles, combined with the sheer diversity of assay options makes EV testing for biomarkers candidacy difficult as they invoke problems of assay accuracy and limited comparability of results depending on assay selection. Given the complicated landscape of EV experimentation, ISEV has published guidelines for studying EVs with the most recent update in 2018 and protocols for biofluid collection and EV isolation/enrichment are available through the Extracellular RNA Communication Consortium (ERCC).^{25,36}

Tissue Factor/Tissue Factor-Positive Extracellular Vesicles

Mechanism—TF is a procoagulant protein that initiates the extrinsic pathway of the coagulation cascade, leading to thrombin and fibrin clot formation. TF is expressed on perivascular cells such as fibroblasts and vascular smooth muscle cells and binds factor VII to stabilize its catalytic domain for downstream activation of factors IX and X. TF

expression can also be induced in monocytes and endothelial cells upon activation with ligands such as lipopolysaccharide and released in TF extracellular vesicles (TF⁺ EVs), although small subsets of resting monocytes have also been shown to express TF.^{37–41} In contrast, malignant tissues can constitutively express TF and release TF⁺ EVs.^{42–48}

Clinical studies of cancer patient plasma have shown elevated TF and TF⁺ EV expression and activity, including in breast, colorectal, prostate, gastric, brain, and pancreatic cancers.^{49–56} However, the source of TF⁺ EVs in cancer patients is unclear and likely results from multiple etiologies, including host and malignant cells.^{51,52,57} Studies in nonmalignant settings have shown generation of TF⁺ EVs from endothelial cells, monocytes, and macrophages.⁵⁸ Platelet TF expression and TF⁺ EV generation is controversial with discrepant results of expression and function being reported.^{59–66} Some of these conflicting results may be attributed to the use of non-specific anti-TF antibodies and Xa functional assays.⁶⁷ This issue is further complicated by monocyte-derived TF which can be transferred to platelets via EVs and can represent a contaminating source of TF if not adequately removed from the samples of interest.^{67–71} Together, these findings suggest that while platelets may not have intrinsic expression of TF, they can acquire TF through external sources. Notably, although TF may be present on platelet-derived EVs, the thrombogenicity of these EVs appears to be driven primarily by exposed phosphatidylserine, rather than TF.⁷² In contrast, thrombin formation from EVs derived from lipopolysaccharide-stimulated monocytes was effectively inhibited by anti-TF antibodies, suggesting that TF on monocyte-derived EVs plays a greater thrombogenic role than on platelet-derived EVs. Similar to platelets, granulocytes have been shown to take up external TF from monocytes.^{73,74}

Despite elevated expression and activity in malignancy, consistent association of TF or TF⁺ EVs with VTE have only clearly been shown in pancreatic cancer.^{75,76} While studies of thrombotic risk in cancer patients with TF or TF⁺ EVs have primarily focused on VTE, a recent study has shown that TF⁺ EVs were elevated in patients with cancer-related stroke compared to those with cancer-unrelated stroke.⁷⁷ However, TF⁺ EVs did not mediate an association between cancer-cell derived EVs and D-dimer levels by statistical path analysis; effects of TF⁺ EVs on cancer-related stroke were not modeled.

In pancreatic cancer mouse models, platelet-dependent and independent pathways have been proposed for TF⁺ EV induced thrombosis. In platelet-dependent thrombosis, TF⁺ EVs localize at thrombus sites by binding markers such as P-selectin to activate and aggregate platelets via a TF-dependent pathway.^{45,47} Recently, a platelet- and leukocyte-independent mechanism was shown that requires host TF to initiate thrombus formation for propagation by phosphatidylethanolamine-dependent TF⁺ EVs.⁷⁸

Biomarker Use in Primary VTE Risk—In prospective studies of mixed cancer and multiple myeloma patients, TF⁺ EV activity as measured by a TF-dependent factor Xa generation assay did not significantly correlate with VTE risk.^{55,79} Similarly, a prospective study of mixed cancer patients using a fibrin generation assay for TF⁺ EV activity showed no association with increased VTE risk (sHR [subdistribution hazard ratio] 1.94, 95% CI 0.99–3.80).⁸⁰ However, a retrospective study showed that pancreaticobiliary patients had 4.78 times the risk of developing VTE (95% CI 1.64–13.98) with TF⁺ EV activity 2.5

pg/ml, as measured by TF-dependent factor Xa generation.⁵⁶ In a prospective study of pancreatic cancer patients, TF⁺ EV activity 2.37 pg/ml by TF-dependent factor Xa generation increased VTE risk by 10.5-fold, although precision of the estimated risk was low (95% CI 1.5–72.4).⁸¹ A meta-analysis of cancer patients showed an increased 76% risk of VTE with elevated TF⁺ EV quantity or function (95% CI 1.21–2.56).⁸² Subgroup analyses revealed that the increased risk was driven by elevated TF⁺ EV levels on flow cytometry assays (OR [odds ratio] 2.97, 95% CI 1.81–4.86) rather than activity assays (OR 1.25, 95% CI 0.95–1.65).⁸²

TF⁺ EV assays are often based on factor Xa generation using a chromogenic substrate after incubation with factors VII and X in the presence of anti-TF antibodies. These assays can incorporate exogenous addition of negatively charged phospholipids, which may increase total factor Xa generation in a TF-independent manner.⁸³ Another TF⁺ EV functional assay is the fibrin generation test which measures fibrin formation using optical densitometry in the presence of anti-factor VIIa antibodies.⁸⁴

When studying specific sources of TF⁺ EV, a serious pre-analytical challenge includes monocytes which can be a major source of contaminating TF and care should be taken to purify samples, such as by using microbeads bound to anti-CD14 antibodies.^{67,71} In regards to analytical challenges, flow cytometry assays are not recommended as they suffer from poor specificity of anti-TF antibodies, which bind non-specifically at high concentrations and may cross-react with phosphatidylserine on cell membranes.^{67,85,86} Enzyme-linked immunosorbent assays (ELISA) suffer from similar poor antibody specificity and selectivity.⁶⁷ Functional assays of TF⁺ EV activity have variable specificity depending on the methods used, as TF-independent mechanisms can also generate factor Xa. One method to limit TF-independent Xa generation in these assays is to add prothrombin, FVa, and phospholipids and reduce incubation time to 3–4 minutes, rather than the standard minimum incubation time of 60 minutes.^{67,87} An alternative method avoids adding exogenous phospholipids, but is time consuming with high interassay variability and therefore not appropriate for clinical use.^{83,88}

Neutrophils and Neutrophil Extracellular Traps

Mechanism

Activated neutrophils can form neutrophil extracellular traps (NETs), which are extracellular fibers containing histones, DNA, and proteins derived from neutrophilic granules.⁸⁹ While they were originally discovered in the role of host defense, neutrophils and NET formation, also known as NETosis, have since been proposed to be a mediator of CAT. During thrombus formation, neutrophils are the first leukocyte to arrive at the site of vessel injury, only secondary to TF.^{90,91} Stimulation of neutrophils by multiple factors including activated endothelial cells, activated platelets, interleukin-8 (IL-8), tumor necrosis factor- α (TNF- α), and granulocyte-colony stimulating factor (G-CSF) can induce NETosis, which serves as a scaffold for platelets, red blood cells, and other procoagulants.^{92–94} During NETosis, neutrophils swell and lose their nuclear membrane integrity leading to mixing of chromatin, histones, and granule-derived proteins.⁹⁵ Peptidylarginine deiminase 4 is a required nuclear

enzyme for NETosis that citrullinates arginine residues of histones H3 and H4 and causes dissociation of chromatin from histones due to the loss of electrostatic force.⁹⁶

Histones present in NETs activate platelets through Toll-like receptors 2 and 4, leading to thrombin generation.⁹⁷ Furthermore, histones induce endothelial cells to release von Willebrand factor (vWF) which binds glycoprotein Ib (GPIb) on platelets, leading to vessel wall adhesion and clot initiation.^{98,99} Histone-independent activation of platelets can also occur via direct binding of platelets to cell free DNA (cfDNA), as well as cathepsin G activity.¹⁰⁰ Cathepsin G and neutrophil elastase are present on NETs and inhibit TF pathway inhibitor to enhance TF and factor XII-dependent fibrin and thrombus formation.^{101,102} Neutrophils have also been shown to both release TF and EVs.^{103–106} Neutrophil- and tumor-derived EVs scaffold to NETs through phosphatidylserine-histone interactions and contribute to thrombosis.^{106–108}

In mouse studies of cancer, tumor-bearing mice were associated with increased neutrophil counts, G-CSF, cfDNA, citrullinated histone H3 (H3Cit), and NET formation.^{94,109} Thrombi from a mouse model of human pancreatic cancer also contained elevated levels of H3Cit, cfDNA, and the neutrophil marker Ly6G, and were reduced in size with neutrophil depletion and DNase treatment.¹⁰⁹ Tumor-derived EVs also increased NET formation from mouse neutrophils treated with G-CSF and injection of these EVs increased venous thrombus formation in vivo.¹⁰⁸

NET markers such as nucleosomes, cfDNA, and H3Cit are also elevated in cancer patients.^{110–112} In a case-control study of patients with ischemic stroke, elevated post-stroke levels of H3Cit were associated with positive cancer status, elevated thrombin-antithrombin complexes, and soluble P-selectin levels.¹¹¹

Biomarker Use in Primary VTE Risk—Research on NET-related biomarkers in CAT has just begun with a prospective study of cancer patients showing that each 100 ng/ml increase in plasma H3Cit was associated with an 11% increase in VTE (95% CI 1.03–1.20).¹¹² Upon subgroup analysis by cancer type, only pancreatic and lung cancers showed a significantly increased risk of VTE with elevated H3Cit levels. However, these results should be cautiously interpreted as the study was underpowered for subgroup analysis. Elevated nucleosome and cfDNA levels were not significantly correlated with VTE. Studies on neutrophil-to-lymphocyte ratios (NLR) have also been explored in cancer patients but have failed to show significance.^{113–115} Plasma H3Cit can be measured by ELISA, but clinical access to this assay is not yet widely available.¹¹⁶

Inflammatory Molecules

Mechanism—Cancer cells synthesize and secrete a variety of inflammatory molecules that have diverse procoagulant capabilities, including TNF- α , IL-1 β , IL-6, IL-8, and vascular endothelial growth factor (VEGF). TNF- α , IL-1 β , and IL-6 can induce TF expression and activity from endothelial cells.^{117,118} TNF- α has also been shown to increase EV release from cancer cells, with elevated TF activity seen on EVs derived from lung, pancreatic, and colon cancer.¹¹⁹ TNF- α and IL-1 β can also upregulate plasminogen activator inhibitor-1 (PAI-1), although fibrinolysis was not completely abolished in cancer patients after TNF- α

infusion.^{120,121} IL-1 β has been shown to be synthesized by activated platelets from pre-messenger RNA stored in polysomes which are spliced to messenger RNA (mRNA) upon activation and can be released in platelet EVs to interact with other inflammatory cells.¹²² Platelets can also express IL-1 receptor and respond to IL-1 β in an autocrine loop to amplify platelet activation.¹²³ IL-8 expression can be induced by fibrin and has been shown to induce granulocyte chemotaxis and NET formation.^{124–126} Finally, VEGF can upregulate TF expression in endothelial cells via the transcription factor early growth response 1.¹²⁷ TF has also been shown to induce VEGF expression in tumor cells and may represent a potential positive procoagulant feedback loop.¹²⁸

Biomarker Use in Primary VTE Risk—Data on the use of inflammatory molecules for VTE risk have been mixed. A retrospective study demonstrated ovarian cancer patients with plasma IL-6 levels ≥ 20 pg/ml had 8.9 times the risk of developing VTE, although precision of the increased VTE risk is low (95% CI 1.04–76.0).¹²⁹ In contrast, a prospective study on diffuse large B cell lymphoma patients showed no association VTE risk with IL-6, IL-10, or TNF- α .¹³⁰ Similarly, a prospective study of solid tumor patients showed no association of IL-1 β , IL-3, IL-4, IL-6, IL-8, IL-10, IL-11, or chemokine ligand 3 (CCL3) with VTE.¹³¹ In glioma patients, for each two-fold increase in pg/ml of CCL3, VTE risk was reduced approximately 60–80%, depending on other variables in the Cox regression model.¹³² In the same study, IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-11, TNF- α , and VEGF were not found to be significantly associated with VTE.¹³² However in a study of metastatic colorectal cancer patients, low pre-chemotherapy serum TNF- α levels (<6.6 pg/ml) decreased VTE risk by 83% (95% CI 0.04–0.75), but the estimated effect is imprecise due to the wide CI.¹³³ Another study showed that for every 10 pg/ml increase in soluble VEGF, VTE risk increased by 4% (95% CI 1.00–1.09) in cancer patients.¹³⁴

Inflammatory markers are common candidates for biomarkers and can be assayed with ELISA, enzyme-linked immune spot assays (ELISPOT), polymerase chain reaction (PCR), and multiplexed bead- or protein-based arrays.¹³⁵ These markers are advantageous as quantitative assays are readily available in clinical practice, and many markers can be assayed at once through multiplexing. However, cytokines have short half-lives, diurnal variation, and are prone to degradation with multiple freeze-thaw cycles. Furthermore, sample preparation, such as collecting serum or plasma and anticoagulation choice can affect cytokine synthesis and release after blood collection.¹³⁶ When considering these markers for risk models in clinical practice, blood collection procedures should be developed in accordance with the assays used. EDTA-treated tubes provide consistent results for most cytokine immunoassays, although citrate can also be considered. If using bioassays, blood should be collected in tubes for serum preparation or low-level preservative free-heparin tubes for plasma preparation. Blood should be processed immediately within two hours after collection and samples divided into small aliquots to minimize need for repeated freezing and thawing. Freeze-thaw cycles should be limited to no more than 3 cycles and stored samples should be used within approximately 4 years, depending on the specific cytokines of interest.^{136,137}

Podoplanin and Isocitrate Dehydrogenase 1

Mechanism—Podoplanin is a transmembrane glycoprotein that is normally present in a variety of normal tissue, including lymphatic endothelial cells, kidney podocytes, type 1 alveolar lung cells, and lymph node fibroblastic reticular cells, but not vascular endothelial cells.^{138,139} Expression is also seen in cancer cells such as squamous cell carcinomas, germinal tumors, brain tumors, mesotheliomas and acute promyelocytic leukemia.^{138–140} Podoplanin binds to the C-type lectin-like receptor 2 (CLEC-2) on platelets to induce platelet activation and aggregation.^{141,142}

Elevated expression of podoplanin has been observed in primary brain cancer tissue as well as in podoplanin-containing EVs in the blood of pancreatic cancer patients.^{143,144} A mouse melanoma (B16F10) model has shown podoplanin-dependent thrombus formation through CLEC-2-podoplanin interaction.¹⁴⁵ While podoplanin can arise from cancer cells, it can also be abnormally induced by thromboinflammation in host vascular cells. In a mouse model of inferior vena cava stenosis, podoplanin was expressed in the inferior vena cava vessel wall after 48 hours of stenosis.¹⁴⁶

Isocitrate dehydrogenase 1 (IDH1) is commonly mutated in gliomas and the mutant form has been associated with decreased VTE in glioma patients compared to the IDH1 wild-type.¹⁴⁷ Mutated IDH1 can produce D-2-hydroxyglutarate to reduce platelet aggregation and clotting activity in vitro.¹⁴⁷ IDH1 mutants are also associated with increased *F3* promoter hypermethylation and decreased *F3* mRNA transcripts, leading to decreased TF expression in vitro, which could be reversed with a demethylating agent.^{147,148} Notably, IDH1 mutations are associated with hypermethylation in human gliomas, but not in acute myeloid leukemia, cholangiocarcinoma, or melanoma, suggesting that the protective effects of IDH1 mutation are specific to the brain cancer setting.¹⁴⁹

Biomarker Use in Primary VTE Risk—Studies using brain cancer patients from the Vienna CATS cohort have shown increased risk of VTE with >70% podoplanin expression in tumor tissue (HR 5.71, 95% CI 1.52–21.36) and a score ≥ 3 based on a combined scoring system of podoplanin tissue expression and IDH1 mutant status (3 points: HR 8.4, 95% CI 1.04–67.58; 4 points: HR 13.28, 95% CI 1.65–106.97).^{143,150} However, the reported CIs are wide and demonstrate low precision of estimated VTE risk. Furthermore, because the studies do not report the timing of VTE events relative to acquisition of tumor sample for podoplanin expression and IDH1 genotyping, causative conclusions about the effect of podoplanin and IDH1 on VTE risk cannot be made. In a separate study of newly diagnosed glioma patients, podoplanin tissue positivity (≥ 30% expression) and IDH1 mutant status were significantly correlated with subsequent VTE risk with low precision of estimated risk (OR 3.423, 95% CI 1.083–10.814; OR 0.101, 95% CI 0.010–0.975, respectively).¹⁵¹

Podoplanin and IDH1 tissue expression are assessed histologically after immunostaining of tumor tissue. While these assays can be completed in the clinical setting, the invasive nature of tissue collection, especially in the setting of brain cancer, can delay and limit use of risk models.

Extracellular microRNAs

Mechanism—MicroRNAs (miRNAs) are small noncoding RNAs that are about 20–22 nucleotides in length and function as inhibitors of gene expression. miRNAs bind to complementary sequences on target mRNAs at 3' untranslated regions and form miRNA-induced silencing complexes with Argonaute proteins leading to mRNA degradation.¹⁵² miRNAs can be released into plasma bound to Argonaute proteins or in vesicles, but the relative proportions of protein- and vesicle-bound miRNAs are unclear.¹⁵³ Interestingly, miRNAs have also been found in anucleate platelets; these can be shed in EVs and transferred to other cells for gene regulation, suggesting a potential role for intercellular signaling.^{154–156}

Dysregulation of miRNAs are associated with increased procoagulant phenotypes. In patients with systemic lupus erythematosus and antiphospholipid syndrome, miR19-b and miR-20a, which inhibit TF, were significantly decreased compared to healthy controls and may contribute to their hypercoagulable states.¹⁵⁷ Case-control studies of acute, non-cancerous deep vein thrombosis and recurrent VTE patients also showed distinct miRNA profiles in cases compared to controls.^{158,159} Similarly, in a case-control study of colorectal cancer patients, 9 miRNAs were differentially expressed in patients who developed VTE compared to controls.¹⁶⁰

Biomarker Use in Primary VTE Risk—Studies assessing miRNA biomarkers for cancer VTE risk are limited. Recently, a study on newly diagnosed pancreatic and cholangiocarcinoma patients assessed the predictive capabilities of miRNAs for primary VTE. Study subjects were split into screening (n = 10) and confirmatory groups (n = 32) and 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p and miR-103a-3p) were identified for inclusion in a predictive model. Model testing in the confirmatory group demonstrated a receiver operating characteristic (ROC) curve area under the curve of 0.95 (95% CI 0.87–1).¹⁶¹ However, the number of events included in this study is small for the number of predictors included in regression modeling and patients from the screening cohort were also included in the confirmatory group, leading to a highly overfit model and results. Nevertheless, miRNAs are a growing field of study in CAT and further studies are needed.

New advances in miRNA assays are appearing rapidly, but quantification is most commonly done through quantitative reverse transcription polymerase chain reaction (qRT-PCR), hybridization assays such as microarrays, and next-generation sequencing. Hybridization platforms such as microarrays are often low-cost and have fast turnaround times with high-throughput but have the lowest specificity. qRT-PCR performs similarly with improved specificity. Next-generation sequencing is the most costly, but is the only assay that can identify novel miRNAs, and with continued technological advances, cost has dropped dramatically over time.¹⁶²

While miRNAs are exciting candidate biomarkers, there are considerable challenges for research and clinical use, similar to EVs. Though miRNAs are quite stable both at room temperature and upon multiple freeze-thaw cycles, study of miRNAs remain challenging due to their multiple transport mechanisms (RNA-binding proteins, EVs), technical difficulties in

isolation and data analysis, and lack of widely used standards.¹⁶³ Prior studies have shown there are significant biases in RNA isolation and library preparation kits that can impact results.^{164,165} Furthermore, data analysis is hampered by the lack of endogenous normalization controls which has generated controversy on the most appropriate normalization methods.¹⁶⁶ The ERCC was formed to combat these challenges and create guidelines for standardization of testing.^{36,167}

Polyphosphate and the Contact Pathway

Mechanism—Polyphosphates (polyPs) are linear polymers of anionic phosphate units that promote length-dependent mechanisms of thrombosis. On microbes, polyPs are found in a long chain form ranging from tens to thousands of units which activates the contact pathway through generation of factor XIIa and kallikrein.^{168–170} Short chain forms of polyP are released by platelets from dense granules which function to accelerate factor XI and V activation for enhanced thrombin generation.^{171–175} Short chain polyP also inhibits TF pathway inhibitor and remodels fibrin clots to increase resistance to fibrinolysis.^{169,174,176,177} Activated platelets can also express membrane-associated polyPs as spherical nanoparticles which potentially activate factor XII and the contact pathway.¹⁷⁸

The contact pathway, or contact activation system, is a proteolytic pathway including factors XII, XI, prekallikrein (PK), and high molecular weight kininogen (HK) that is activated upon contact of factor XII with a negatively charged surface, such as polyP or cfDNA, leading to cleavage of PK into kallikrein, which generates bradykinin from HK.¹⁷⁹ Factor XII-independent contact activation can also occur via HK-PK complexes on endothelial cells leading to PK activation and cleavage.¹⁷⁹ The principal inhibitor of the pathway is C1 esterase inhibitor which inhibits factor XIIa and kallikrein.¹⁸⁰

Human studies investigating the role of polyP in cancer and CAT have not yet been reported. However, prostasomes or prostate-cancer derived EVs express long chain polyP that potentially induces pulmonary embolism in mouse models through factor XII-dependent mechanisms, suggesting a potential role in prostate CAT.¹⁸¹

Current studies of contact pathway function in cancer patients are limited, but suggest that activation of the contact pathway leads to consumption and decrease of factor XII in gastrointestinal, colorectal, and lung cancer patients.^{182–184} In prostate cancer, patient-derived exosomes showed significantly more thrombin generation potential and factor XIIa activity compared to controls.¹⁸¹

Plasminogen Activator Inhibitor-1

Mechanism—PAI-1 is a serine protease that inhibits tissue plasminogen activator and urokinase, which are activators of plasminogen and fibrinolysis. Elevated levels of PAI-1 have been found in glioma, non-small cell lung, multiple myeloma, and pancreatic cancer patients.^{185–188} However, studies of PAI-1 in CAT are limited and even in nonmalignant settings, there has been limited evidence to support PAI-1-associated VTE.¹⁸⁹ An in vitro study of human umbilical vein endothelial cells treated with PAI-1 lead to increased EV release, which were associated with increased anionic phospholipid expression and thrombin

generation.¹⁹⁰ Interestingly, a mouse lung cancer model showed increased expression of PAI-1 in tumor tissue and plasma after treatment with bevacizumab, an antiangiogenic VEGF antibody that is associated with increased VTE in cancer patients, suggesting that PAI-1 may play a more significant procoagulant role in antiangiogenic therapy-based VTE.^{191,192}

Platelet Activators

Mechanism—Platelets are key actors in thrombus formation and can interact with cancer cells for enhanced activation and aggregation. Resting platelets adhere to and are activated at sites of endothelial damage upon binding of exposed vWF through GPIb, leading to rise of intracellular calcium which drives shape change and degranulation of adenosine diphosphate (ADP), fibrinogen, GPIb, and glycoprotein IIb/IIIa (GPIIb/IIIa). Further activation is driven by newly released ADP, thromboxane A₂ (TxA₂), and CD40 ligand (CD40L). ADP is stored in dense granules and upon release binds receptors P2Y₁ and P2Y₁₂ on platelets for activation. TxA₂ is synthesized by thromboxane synthase after platelet activation and diffuses readily across the plasma membrane for binding of TxA₂ receptor on platelets. CD40 is expressed on activated platelets and binds soluble CD40L which is released after platelet activation. Thrombin, a serine protease, can also activate platelets by cleaving the extracellular domains of protease-activated receptors 1 and 4 (PAR1/PAR4) on platelet membranes. After activation, platelets aggregate through crosslinking of fibrinogen and vWF with GPIIb/IIIa, forming the hemostatic plug.^{193,194}

Cancer cells are capable of secreting these platelet activating factors and in vitro studies have shown release of tumor-derived ADP, TxA₂, and CD40L, leading to tumor cell-induced platelet aggregation.^{195–198} Thromboxane synthase overexpression with increased TxA₂ metabolites have also been demonstrated in cancer cell lines and human tissue.^{199–203} Additionally, large multimers of vWF have been found in the plasma of patients with disseminated cancer and vWF expression has been reported in both patient-derived glioblastoma and glioma and osteosarcoma cell lines.^{204,205} Platelet-tumor cell interactions using these activating factors have well-established roles in metastasis, where platelets coat tumor cells to provide a protective covering against host immune cells and allow tumor cell adhesion to endothelium for extravasation at distal tissue sites.^{206–208} These same interactions may also play a role in CAT.

Blood Count

Biomarker Use in Primary VTE Risk—Blood counts such as leukocyte and platelet count have been widely studied as biomarkers of primary VTE and are used broadly across many risk models. In prospective studies of cancer patients, both elevated leukocyte ($>11 \times 10^9/l$) and platelet ($>350 \times 10^9/l$) counts increased risk of initial VTE.^{9,14,209} This elevated risk remained when assessing leukocyte counts per 2-fold increase and elevated platelet counts as $443 \times 10^9/l$.²¹⁰ Leukocyte and platelet counts feature in the Khorana, Vienna CATS, PROTECHT, CONKO, and COMPASS-CAT scores.^{9–12,14} Leukocyte and platelet counts are assayed through the complete blood count (CBC) and are ubiquitously available in clinical practice. Furthermore, CBC tests are routinely acquired during typical management of patients, which reduces testing burden of patients during risk modeling.

D-dimer

Biomarker Use in Primary VTE Risk—D-dimer is a degradation product of plasmin-induced fibrinolysis and is one of the most common biomarkers studied for primary VTE development in cancer patients. Prospective studies of newly diagnosed cancer patients have shown that elevated pre-treatment levels of D-dimer increase the risk of primary VTE in colorectal, gynecologic, and mixed cancer populations.^{211–214} Another prospective study on newly diagnosed cancer patients and those with disease progression also found increased levels of D-dimer with elevated risk of primary VTE (HR 1.8, 95% CI 1.0–3.2).²¹⁵ In a retrospective study of lung cancer patients who were categorized as intermediate risk by the Khorana score, elevated prechemotherapy D-dimer levels were associated with increased risk for VTE (HR 11.0, 95% CI 2.62–46.2), suggesting that D-dimer levels may help discriminate between intermediate Khorana score patients.²¹⁶ However, due to the wide CI, the precision of estimated VTE risk is low. Borderline associations of D-dimer levels with VTE risk have also been demonstrated.^{81,112} D-dimer has been incorporated into the Vienna CATS and CATS nomogram risk models.^{10,16}

Assays for D-dimer quantification include ELISA, enzyme-linked immunofluorescence assays (ELFA), latex-based assays, and whole-blood assays. While ELISA-based values represent the reference assay for D-dimer, ELFA and latex-based assays provide faster turnaround times and are fully automated. Whole-blood is often used for point-of-care testing and suffers from low sensitivity.²¹⁷

A major obstacle with D-dimer quantification is the lack of transparency on unit measurement in laboratory reports, which can be expressed as purified D-dimer units (DDUs) or fibrinogen equivalent units (FEUs), with 1 FEU being equivalent to approximately 2 DDUs.^{217,218} This discrepancy makes development of standard D-dimer cut-offs difficult, as chosen cut-off limits in different studies may not be comparable. In clinical practice, physicians may input incorrect units of D-dimer into risk models and reduce validity of their results. We recommend that D-dimer biomarker research clearly state which assay and D-dimer unit type is used and that risk calculators specify D-dimer unit type when allowing users to input data. Furthermore, D-dimer levels increase with age, contributing to the difficulty in developing standard reference levels.²¹⁹ Finally, levels of D-dimer may be elevated in a variety of inflammatory settings, not directly linked to activation of the coagulation system.²²⁰

P-selectin

Biomarker Use in Primary VTE Risk—P-selectin is a cell adhesion molecule found on activated endothelial cells and platelets and can be released into plasma, including on EVs. Multiple prospective studies derived from the Vienna CATS cohort have shown that increased soluble P-selectin levels are correlated with VTE risk, with one study estimating that a soluble P-selectin level 53.1 ng/ml increased the risk of VTE by 2.6-fold (95% CI 1.4–4.9).^{132,134,214,221} However, in another study based on the Vienna CATS cohort, soluble P-selectin levels were not significantly correlated with increased risk.¹¹² Soluble P-selectin is measured by ELISA, and while assays are available for research purposes, clinical assays are not widely available. Although the Vienna CATS risk model may improve VTE

prediction, the lack of access to soluble P-selectin assays limit its proposed clinical function.
10

Biomarkers in Recurrent VTE Risk

The Ottawa score is a risk model for recurrent VTE and includes predictors for sex, primary tumor site (lung or breast), stage, and prior VTE.¹⁷ However, subsequent validation studies have shown mixed results on its discriminative capability, suggesting need for additional predictors.^{222–225} A study of cancer patients treated with either tinzaparin or warfarin demonstrated that elevated pre-anticoagulant TF levels (>64.6 pg/ml) and C-reactive protein (CRP; >75 mg/l) increased risk of recurrent VTE (sHR, 3.4; 95% CI, 2.1–5.5; sHR, 2.3; 95% CI, 1.2–4.4).²²⁵ Another study of cancer patients on low molecular weight heparin for 6 months showed that pre-anticoagulant soluble P-selectin (>136 ng/ml) but not D-dimer (>4.0 μ g/ml) levels increased risk of recurrent VTE (sHR, 4.4, 95% CI 1.3–16; sHR 2.5, 95% CI 0.76–7.9).²²⁶ Elevated CRP (>4.5 mg/l) 21-days after 3 months of low molecular weight heparin treatment was also associated with increased risk for recurrent VTE (sHR 9.8; 95% CI 1.86–51.7).²²⁷ Similarly, elevated D-dimer, as determined by ROC curve analysis (>600 ng/ml) and age adjustment ($>\text{age} \times 10$ ng/ml) at 21 days post-anticoagulation increased risk of recurrent VTE (sHR 5.81, 95% CI 1.06–31.72; sHR 5.11, 95% CI 1.032–25.3).²²⁷ However, precision of estimated effects of CRP and D-dimer with VTE risk are low.

Biomarkers in Primary ATE Risk

Currently, no risk models are available for primary arterial thromboembolism and biomarker studies are sparse. Elevated neutrophil counts ($>4.9 \times 10^9$ /l) and soluble P-selectin levels (>46.3 ng/ml) have been shown to increase arterial thromboembolic risk (sHR 2.4, 95% CI 1.3–4.6; sHR 2.1, 95% CI 1.2–3.8).²²⁸ However, D-dimer, NLR, platelet-to-lymphocyte ratio, H3Cit, cfDNA, and nucleosome levels failed to show significance.^{114,228,229}

Future Directions and Considerations

Improvements in understanding CAT and its mechanisms have opened up a variety of potential biomarker targets for improving risk models and clinical decision making. While TF and TF⁺ EV have been extensively studied, research on NET-related proteins, polyphosphate, podoplanin, and miRNAs has just begun. Currently, risk models exist for predicting primary and recurrent VTE, but few have studied risk models and biomarkers for arterial thromboembolism—the other component of CAT. Furthermore, the role of biomarkers in guiding duration of treatment and predicting bleeding complications from anticoagulation has yet to be assessed. However, selection of candidate biomarkers must also balance predictive potential with clinical feasibility to ensure usage of the biomarker in practice. Awareness of these challenges can advance biomarker study to generate improved risk tools for CAT.

Acknowledgements

This work was supported by the U01 HL143402 research grant to KRM and AAK from the National Heart, Lung and Blood Institute CLOT Consortium, as well as the North American Society on Thrombosis and Hemostasis/Hemostasis and Thrombosis Research Society research fellowship to ASK. AAK additionally acknowledges

research support from the Sondra and Stephen Hardis Chair in Oncology Research. All authors have read and approve the journal's authorship agreement and policy on disclosure of potential conflicts of interest. The authors declare no conflicts of interest. No sources of editorial support were used in preparation of this manuscript.

Abbreviations

ADP	adenosine diphosphate
ATE	arterial thromboembolism
CAT	cancer-associated thrombosis
CBC	complete blood count
CCL3	chemokine ligand 3
CD40L	CD40 ligand
cfDNA	cell free DNA
CI	confidence interval
CLEC-2	C-type lectin-like receptor 2
CRP	C-reactive protein
DDU	D-dimer unit
ELFA	enzyme-linked immunofluorescence assay
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme linked immune spot assays
ERCC	Extracellular RNA Communication Consortium
EV	extracellular vesicle
FEU	fibrinogen equivalent unit
G-CSF	granulocyte-colony stimulating factor
GPIb	glycoprotein Ib
GPIIb/IIIa	glycoprotein IIb/IIIa
H3Cit	citrullinated histone H3
HK	high molecular weight kininogen
HR	hazard ratio
IDH1	isocitrate dehydrogenase 1
IL	interleukin
ISEV	International Society for Extracellular Vesicles

miRNA	microRNA
mRNA	messenger RNA
NET	neutrophil extracellular trap
NLR	neutrophil-to-lymphocyte ratio
OR	odds ratio
PAI-1	plasminogen activator inhibitor-1
PAR1	protease-activated receptor 1
PAR4	protease-activated receptor 4
PCR	polymerase chain reaction
PK	prekallikrein
polyP	polyphosphate
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ROC	receiver operating characteristic
sHR	subdistribution hazard ratio
TF	tissue factor
TF⁺ EV	tissue factor-positive extracellular vesicle
TNF-α	tumor necrosis factor- α
TxA₂	thromboxane A ₂
VEGF	vascular endothelial growth factor
VTE	venous thromboembolism
vWF	von Willebrand factor

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Figure 1. Graphical summary of key mechanisms driving cancer-associated thrombosis. Interleukin-8 (IL-8), tumor necrosis factor- α (TNF- α), and granulocyte-colony stimulating factor (G-CSF) stimulate neutrophils to form neutrophil extracellular traps (NETs) containing cell free DNA (cfDNA) and citrullinated histone H3 (H3Cit). Extracellular vesicles (EVs), derived from tumor or host cells, express negatively charged phosphatidylserine (PS) which bind histones in NETs. EVs also express tissue factor (TF) which binds factor VIIa (FVIIa) to initiate the extrinsic pathway of coagulation and generate thrombin. Tumor cells can also secrete exosomes expressing polyphosphate (polyP) to activate the contact pathway. Similarly, platelets can express polyP to initiate contact pathway activation, as well as interact with cfDNA and histones present in NETs for thrombin and thrombus formation. Podoplanin-expressing activated endothelial cells can bind C-type lectin-like receptor 2 (CLEC-2) on platelets to induce platelet activation and aggregation. Inhibitory microRNAs (miRNAs) can be secreted extracellularly in a protein- or EV-bound form. Platelets and other host cells can take up miRNAs which regulate gene expression of coagulation factors and re-secrete miRNAs into plasma. FXIIa, factor XIIa; RBC, red blood cell.

Table 1.

Biomarker studies assessing primary venous thromboembolism risk in cancer patients

Biomarker	Study Design	Cancer Type	Number of patients	Biomarker Cutoff or Numeric Variable	Statistic	95% CI	Reference
Leukocyte count							
	Prospective	Mixed	2701	$>11 \times 10^9/l$	OR 2.2	1.2–4	9
	Prospective	Mixed	4405	$>11 \times 10^9/l$	HR 2.10	1.30–3.40	209
	Prospective	Solid tumor	665	Per 2-fold increase	HR 1.15	0.63–2.11	210
Platelet count							
	Prospective	Mixed	2701	$350 \times 10^9/l$	OR 1.8	1.1–3.2	9
	Prospective	Mixed	4405	$350 \times 10^9/l$	HR 1.83	1.19–2.83	209
	Prospective	Solid tumor	665	$443 \times 10^9/l$	HR 3.50	1.52–8.06	210
	Prospective	Mixed	1023	$350 \times 10^9/l$	OR 2.53	1.35–4.74	14
D-dimer							
	Prospective	Colorectal	176	$>0.3 \mu g/ml$	HR 6.53	1.58–27.0	211
	Prospective	Gynecologic	267	$>5 \mu g/ml$	OR 1.19	1.04–1.37	212
	Prospective	Mixed	124	$>0.65 \mu g/ml$	HR 4.04	1.22–13.3	213
	Prospective	Mixed	112	Per 2-fold increase	HR 1.76 *	1.32–2.35	214
	Prospective	Mixed	821	$1.44 \mu g/ml$	HR 1.8	1.0–3.2	215
	Retrospective	Lung	108	$>1.50 \mu g/ml$	HR 11.0	2.62–46.2	216
	Prospective	Pancreatic	140	$2.16 \mu g/ml$	HR 4.9	1.0–23.1	81
	Prospective	Mixed	946	Per 10 $\mu g/ml$ increase	sHR 1.31	1.00–1.73	112
Soluble P-selectin							
	Prospective	Mixed	112	Per 2-fold increase	HR 2.44 *	1.31–4.53	214
	Prospective	Glioma	76	n/a	HR 1.068	1.017–1.122	132
	Prospective	Mixed	797	Per 10 ng/ml increase	HR 1.19	1.07–1.33	134
	Prospective	Mixed	687	53.1 ng/ml	HR 2.6	1.4–4.9	221
	Prospective	Mixed	946	Per 10 ng/ml increase	sHR 1.05	1.00–1.73	112
Extracellular vesicles							
	Prospective	Mixed	728	4.62 nM PS	HR 0.95	0.55–1.64	33
Tissue factor-positive extracellular vesicles							
	Prospective	Pancreas	60	Per 2-fold increase	HR 1.5	1.0–2.4	55
		Brain	119		HR 0.9	0.7–1.3	
		Stomach	43		n/a	n/a	
		Colorectal	126		HR 0.9	0.6–1.6	
	Prospective	Multiple myeloma	122	$>11.8 \text{ fM Xa/min}$	OR 1.4	0.4–4.7	79
	Prospective	Mixed	648	13%	sHR 1.0	0.99–3.8	80

Biomarker	Study Design	Cancer Type	Number of patients	Biomarker Cutoff or Numeric Variable	Statistic	95% CI	Reference
	Retrospective	Pancreaticobiliary	117	2.5 pg/ml	OR 4.78	1.64–13.98	56
	Prospective	Pancreatic	140	2.37 pg/ml	HR 10.5	1.5–72.4	81
	Meta-analysis	Solid tumor	n/a	n/a	OR 1.76	1.21–2.56	82
Neutrophil extracellular traps							
<i>H3Cit</i>	Prospective	Mixed	946	Per 100 ng/ml increase	sHR 1.11	1.03–1.20	112
<i>Cell free DNA</i>	Prospective	Mixed	946	Per 100 ng/ml increase	sHR 1.03 *	0.96–1.10	112
<i>Nucleosome</i>	Prospective	Mixed	946	Per 1 unit increase	sHR 0.95 *	0.89–1.02	112
<i>Neutrophil-to-lymphocyte ratio</i>	Prospective	Solid tumor	810	>3	HR 1.37	0.52–3.57	113
	Prospective	Solid tumor	1469	Per 2-fold increase	sHR 1.2	1.0–1.4	114
	Retrospective	Gastric	112	>3	HR 0.8 *	0.3–2.5	6
Inflammatory molecules							
<i>IL-1β</i>	Prospective	Mixed	726	Per 2-fold increase	HR 0.98 *	0.80–1.20	131
	Prospective	Glioma	76	Per 2-fold increase	HR 1.046 *	0.732–1.495	132
<i>IL-3</i>	Prospective	Mixed	726	Per 2-fold increase	HR 0.99 *	0.84–1.18	131
<i>IL-4</i>	Prospective	Mixed	726	Per 2-fold increase	HR 1.11 *	0.86–1.42	131
	Prospective	Glioma	76	Per 2-fold increase	HR 0.907 *	0.634–1.298	132
<i>IL-6</i>	Retrospective	Ovarian	200	5–19.9 pg/ml	HR 7.98	0.99–64.0	129
				20 pg/ml	HR 8.90	1.04–76.0	
	Prospective	Diffuse large B cell lymphoma	322	>Detection limit	sHR 1.07	0.56–2.06	130
	Prospective	Mixed	726	Per 2-fold increase	HR 1.08 *	0.98–1.20	131
	Prospective	Glioma	76	Per 2-fold increase	HR 0.894 *	0.602–1.328	132
<i>IL-8</i>	Prospective	Mixed	726	Per 2-fold increase	HR 1.06 *	0.94–1.20	131
	Prospective	Glioma	76	Per 2-fold increase	HR 0.918 *	0.585–0.585	132
<i>IL-10</i>	Prospective	Diffuse large B cell lymphoma	322	Median value	sHR 1.66	0.84–3.26	130
	Prospective	Mixed	726	Per 2-fold increase	HR 1.01 *	0.83–1.22	131
	Prospective	Glioma	76	Per 2-fold increase	HR 0.822 *	0.505–1.340	132
<i>IL-11</i>	Prospective	Mixed	726	Per 2-fold increase	HR 0.90 *	0.76–1.06	131
	Prospective	Glioma	76	Per 2-fold increase	HR 0.621 *	0.284–1.357	132
<i>CCL3</i>	Prospective	Mixed	726	Per 2-fold increase	HR 0.99 *	0.90–1.10	131
	Prospective	Glioma	76	Per 2-fold increase	HR 0.348	0.146–0.830	132
<i>TNF-α</i>	Prospective	Diffuse large B cell lymphoma	322	0.285 pg/ml	sHR 0.77 *	0.46–1.29	130
	Retrospective	Metastatic colorectal	45	<6.6 pg/ml	HR 0.17	0.04–0.75	133
	Prospective	Glioma	76	Per doubling	HR 1.097 *	0.697–1.727	132

Biomarker	Study Design	Cancer Type	Number of patients	Biomarker Cutoff or Numeric Variable	Statistic	95% CI	Reference
<i>Soluble VEGF</i>	Prospective	Mixed	797	Per 10 pg/ml increase	HR 1.04	1.00–1.09	134
	Prospective	Glioma	76	Per 2-fold increase	HR 0.995*	0.640–1.548	132
Podoplanin (tissue)	Prospective	Brain	213	<50% expression	HR 2.44	0.73–8.17	143
				50–70% expression	HR 3.28	0.91–11.75	
				>70% expression	HR 5.71	1.52–21.36	
	Retrospective	Glioma	165	30% expression	OR 3.423	1.083–10.814	151
Isocitrate dehydrogenase 1	Prospective	Brain	213	IDH1 R132H mutation	HR 0.11	0.01–0.83	150
	Retrospective	Glioma	165	IDH1 R132H mutation	OR 0.101	0.010–0.975	151

* Denotes a univariable analysis. All other statistics are given for multivariable analyses. CCL3, chemokine ligand 3; CI, confidence interval; HR, hazard ratio; IL, interleukin; OR, odds ratio; PS, phosphatidylserine; sHR, subdistribution hazard ratio; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor.

Table 2.

Biomarker studies assessing recurrent venous thromboembolism risk in cancer patients

Biomarker	Study Design	Cancer Type	Number of patients	Biomarker Cutoff or Numeric Variable	Statistic	95% CI	Reference
Tissue factor							
	Prospective	Mixed	805	>64.6 pg/ml	sHR 3.4	2.1–5.5	225
C-reactive protein							
	Prospective	Mixed	482	>75 mg/l	sHR 2.3	1.2–4.4	225
	Prospective	Mixed	99	>4.5 mg/l	sHR 9.82	1.86–51.7	227
Soluble P-selectin							
	Prospective	Mixed	117	>136 ng/ml	sHR 4.4	1.3–16	226
D-dimer							
	Prospective	Mixed	117	>4.0 µg/ml	sHR 2.5 *	0.76–7.9	226
	Prospective	Mixed	111	>600 ng/ml	sHR 5.81	1.06–31.72	227
				>age × 10 ng/ml	sHR 5.11	1.032–25.3	

* Denotes a univariable analysis. All other statistics are given for multivariable analyses. CI, confidence interval; sHR, subdistribution hazard ratio.

Table 3.

Biomarker studies assessing primary arterial thromboembolism risk in cancer patients

Biomarker	Study Design	Cancer Type	Number of patients	Biomarker Cutoff or Numeric Variable	Statistic	95% CI	Reference
Neutrophil count	Prospective	Mixed	1883	$>4.9 \times 10^9/l$	sHR 2.4	1.3-4.6	228
Soluble P-selectin	Prospective	Mixed	1883	>46.3 ng/ml	sHR 2.1	1.2-3.8	228
D-dimer	Prospective	Mixed	1883	Per 2-fold increase	sHR 1.1	0.9-1.4	228
Neutrophil-to-lymphocyte ratio	Prospective	Mixed	1469	Per 2-fold increase	sHR 1.2	0.9-1.6	114
Platelet-to-lymphocyte ratio	Prospective	Mixed	1469	Per 2-fold increase	sHR 1.1	0.7-1.5	114
Citrullinated histone H3	Prospective	Mixed	957	Per 100 ng/ml increase	sHR 1.0 [*]	0.7-1.4	229
Cell free DNA	Prospective	Mixed	957	Per 100 ng/ml increase	sHR 1.0 [*]	0.9-1.2	229
Nucleosome	Prospective	Mixed	957	Per unit increase	sHR 1.1 [*]	1.0-1.2	229

^{*} Denotes a univariable analysis. All other statistics are given for multivariable analyses. CI, confidence interval; sHR, subdistribution hazard ratio.

Table 4.

Comparison of biomarker assays and challenges for clinical use

Biomarker	Assay	Clinical Assay	Comments	Reference
Leukocyte count	CBC	Yes	Routinely acquired in clinical practice	
Platelet count	CBC	Yes	Routinely acquired in clinical practice	
D-dimer	ELISA	Yes		217
	ELFA	Yes	Reference units not standardized	
	Latex-based assay	Yes	(1 FEU ~ 2 DDU)	
	Whole blood assay	Yes		
Soluble P-selectin	ELISA	No		
EVs	Flow cytometry	No		
	Electron microscopy	No	Subject to varied pre-analytical challenges (blood collection trauma, centrifugation protocol)	25,34,36
	Confocal microscopy	No		
	Atomic force microscopy	No	See ISEV and ERCC for more details and protocols	
TF ⁺ EV	Flow cytometry	No	Monocytes may be contaminating TF source	67,71,85,86
	ELISA	No	Poor anti-TF antibody specificity Monocytes may be contaminating TF source	
	Factor Xa generation	No	Variable specificity depending on exact methods May be labor intensive with high interassay variability	67,71,83
	Fibrin generation	No	Monocytes may be contaminating TF source	67,71,84
H3Cit	ELISA	No		116
Inflammatory markers	ELISA	Yes		
	ELISPOT	Yes		
	PCR	Yes	Subject to pre-analytical challenges (blood collection tubes, processing time, freeze-thaw use, storage)	136
	Multiplexed assay	Yes		
Podoplanin (tissue)	Immunohistochemistry	Yes	Requires invasive tissue collection	
IDH1 (tissue)	Immunohistochemistry	Yes	Requires invasive tissue collection	
miRNAs	qRT-PCR	Yes	Biased RNA isolation and library kits can change results	36,164–166
	Hybridization assay	Yes		
	NGS	Yes	Lack of endogenous normalization controls See ERCC for recommended protocols	

CBC, complete blood count; DDU, D-dimer unit; ELFA, enzyme-linked immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immune spot assay; EV, extracellular vesicle; FEU, fibrinogen equivalent unit; IDH1, isocitrate dehydrogenase 1; miRNA, micro RNA; NGS, next-generation sequencing; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TF⁺ EV, tissue factor-positive extracellular vesicles