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Escuela de Doctorado

TESIS DOCTORAL

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New thrombotic biomarkers in gastrointestinal tumors

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RESUMEN

RESUMEN

En los pacientes con cáncer, la trombosis es una de las comorbilidades más frecuentes. De hecho, es una complicación que disminuye la supervivencia global de los pacientes. Tanto las trombosis arteriales como venosas se incluyen en este contexto, aunque las segundas son mucho más frecuentes que las primeras. Entre los tipos de trombosis que ocurren en el sistema venoso, el más frecuente es el tromboembolismo venoso, considerado como la segunda causa de muerte en los pacientes con cáncer, siendo la primera la progresión tumoral. Existen diferentes factores de riesgo que pueden aumentar la incidencia de la trombosis asociada al cáncer. Estos factores pueden estar relacionados con las características de los pacientes, con el tratamiento antitumoral o con la biología del propio tumor. Así, el envejecimiento, la obesidad o la inmovilización asociada al ingreso hospitalario son factores relativos al paciente que pueden incrementar el riesgo de trombosis. Por otro lado, la quimioterapia, los factores antiangiogénicos o la terapia hormonal son tratamientos que pueden inducir daños endoteliales y, como consecuencia, generar un ambiente hipercoagulante. Por último, la localización primaria del tumor y la capacidad de las células para expresar y liberar factores procoagulantes son también características que condicionan el riesgo de alteraciones hemostáticas. En este último contexto, las células tumorales pueden inducir un escenario hipercoagulante a partir de diferentes mecanismos. Uno de ellos es su capacidad para producir sus propios factores de la coagulación, como es el caso del factor tisular, uno de los iniciadores de la cascada de la coagulación. Otro mecanismo consiste en la liberación de factores que inhiben la degradación fisiológica del coágulo, como el inhibidor del activador del plasminógeno, haciendo que el trombo permanezca más tiempo en la luz del vaso. En los pacientes con cáncer, también se han descrito alteraciones moleculares frecuentes, como el factor V Leiden, o las que reducen la expresión o la actividad de agentes anticoagulantes. Además de estas interferencias en la hemostasia secundaria, los tumores también pueden afectar directamente a células de la sangre. Así, pueden, por ejemplo, inducir la activación de los neutrófilos para que liberen sus trampas extracelulares (formadas por DNA e histonas principalmente, con actividad procoagulante), o promover la activación y agregación plaquetaria. La creciente incidencia de eventos trombóticos en los pacientes con cáncer y su impacto negativo en el pronóstico han llevado a la indicación a lo largo de los años de diferentes tipos de tratamiento o profilaxis anticoagulante. Uno de estos primeros agentes antitrombóticos fueron los antagonistas de la vitamina-K, como la warfarina. Con el tiempo, la eficacia y la forma de administración de este tratamiento fueron superadas por las heparinas de bajo peso molecular, como la enoxaparina o nadroparina. Finalmente, en la última década, en base a su eficacia similar o superior y a la administración por vía oral, los anticoagulantes orales directos, como rivaroxabán o apixaban, han ido sustituyendo poco a poco a las heparinas. Pese a los demostrados efectos positivos de estos agentes anticoagulantes, lo cierto es que su uso aún es controvertido y, de hecho, hay muchos casos en los que, pese a que los pacientes desarrollen un cáncer especialmente trombogénico, la tromboprofilaxis primaria se descarta. Las principales razones detrás de este escenario son: la baja eficacia de la tromboprofilaxis generalizada, puesto que es necesario tratar a un elevado número de pacientes para que uno de ellos se beneficie del tratamiento, la carga que supone un tratamiento diario que muchas veces se administra por vía parenteral y el aumento del riesgo de sangrado asociado a este tipo de

tratamientos. Esta última limitación, responsable de un impacto negativo en el pronóstico, se acentúa en los tumores gastrointestinales. Este tipo de neoplasias están asociadas a un riesgo de trombosis mucho mayor que muchos otros tipos de tumores. Así, el cáncer gástrico, pancreático, biliar y hepático son 4 tumores presentes entre los 5 más trombogénicos a nivel global. Al igual que ocurre con el resto de neoplasias, la trombosis en los pacientes con un tumor digestivo reduce considerablemente su supervivencia, y por ello, la profilaxis antitrombótica podría mejorar su pronóstico significativamente. Sin embargo, como se ha descrito anteriormente, las limitaciones de la anticoagulación llevan muchas veces a que se descarte, dejando a pacientes con una neoplasia digestiva expuestos a un alto riesgo de trombosis. Los tumores gastrointestinales presentan una mayor tendencia al sangrado que el resto de tumores sólidos, y el uso de agentes antitrombóticos acentúa aún más esta tendencia. En este contexto, a lo largo de los años, se han estudiado diferentes variables clínicas, biomarcadores y modelos predictivos con el fin de seleccionar a los pacientes con mayor riesgo de trombosis, sobre los cuales administrar un tratamiento anticoagulante a pesar del riesgo de sangrado. Sin embargo, hasta el momento, no existen marcadores que predigan un riesgo trombótico suficientemente alto como para justificar la tromboprofilaxis en los pacientes con tumores digestivos. Por ello, es preciso seguir buscando herramientas que complementen las limitaciones de los modelos predictivos de trombosis existentes. Así, esta tesis se centra en dicha necesidad dentro de los pacientes con cáncer colorrectal y cáncer gástrico avanzado, dos neoplasias digestivas asociadas a un alto riesgo de trombosis donde el uso de anticoagulantes aún es controvertido.

En el caso del cáncer colorrectal, es el segundo tumor más frecuentemente diagnosticado a nivel global, y el cuarto que más fallecimientos provoca. Aunque su incidencia trombótica no es tan elevada como la del cáncer pancreático o gástrico, el riesgo de eventos tromboembólicos sigue siendo elevado. Con la intención de buscar biomarcadores trombóticos que ayudasen a predecir un riesgo suficientemente elevado para justificar la anticoagulación en pacientes con cáncer colorrectal, nos centramos en el estudio de la hepsina, una serín-proteasa transmembrana de tipo II. Una de las principales razones para centrarnos en esta proteína fue su demostrada capacidad para activar al factor VII de la coagulación, que formando un complejo con el factor tisular, inicia la vía extrínseca de la coagulación. La otra razón está basada en la implicación de la hepsina en la invasión tumoral y la consecuente metástasis, estudiada en diferentes tipos de tumores sólidos. Esta última justificación relacionaría la expresión de la hepsina con la trombosis teniendo en cuenta que, en cáncer colorrectal, la ocurrencia de eventos tromboembólicos aumenta con el estadío tumoral. Así, lo primero que hicimos fue un estudio retrospectivo sobre 169 pacientes con tumor localizado y 118 pacientes metastásicos en los que asociamos el nivel expresión de hepsina en el tumor primario (medida según una tinción inmunohistoquímica específica) con la incidencia de eventos trombóticos a lo largo del seguimiento desde el diagnóstico, así como con otras variables como la supervivencia global, la recaída metastásica, la progresión de la enfermedad, etc. Como resultados significativos, destacamos que en el tumor primario de los pacientes localizados, el aumento de la intensidad de tinción de hepsina incrementaba, de forma independiente, el

riesgo de sufrir un evento trombótico y la recaída metastásica, pero estas asociaciones no se dieron en los pacientes metastásicos. Para comprender los mecanismos subyacentes a estas asociaciones, hicimos una serie de estudios *in vitro* e *in vivo* basados en células de cáncer colorrectal con expresión basal y sobreexpresión (basada en una transfección estable) de hepsina. Comprobamos el efecto de la sobreexpresión de esta proteína en ensayos de migración, invasión, proliferación, expresión de proteínas oncogénicas y generación de trombina. Como resultados significativos *in vitro*, la hepsina alteró algunos de los parámetros de la generación de trombina hacia un escenario hipercoagulante, incrementó la expresión de las proteínas ERK1/2 y STAT3 fosforiladas, y promovió el fenotipo invasivo de las células tumorales. Este último efecto se corroboró *in vivo* en un modelo de pez cebra. Una vez entendidos los mecanismos por los que la hepsina podría incrementar el riesgo de trombosis y metástasis en los pacientes con cáncer colorrectal localizado, nos propusimos identificar fármacos aprobados por la Administración de Alimentos y Medicamentos (FDA) que pudiesen inhibir la hepsina y, en consecuencia, suprimir sus efectos protumorales y procoagulantes. A partir del "virtual screening", "docking molecular" y ensayos *in vitro* de inhibición de la actividad proteolítica de hepsina, identificamos a Venetoclax y Suramin como dos nuevos inhibidores de esta proteína. Además, observamos que en las células de cáncer colorrectal con expresión basal y sobreexpresión de hepsina, ambos fármacos redujeron significativamente su fenotipo invasivo y la generación de trombina. Sintetizando los resultados derivados de estos trabajos en cáncer colorrectal, concluimos que la hepsina es un potencial biomarcador de trombosis y metástasis en pacientes con cáncer localizado, probablemente debido a su capacidad para promover el fenotipo invasivo de las células tumorales y para aumentar la generación de trombina. Como diana terapéutica, la hepsina puede ser inhibida por Suramin y Venetoclax, dos fármacos capaces de reducir sus efectos protumorales y procoagulantes. Todos estos resultados impulsarán en el futuro la validación de la hepsina como biomarcador de trombosis y metástasis en cohortes más grandes de pacientes con cáncer colorrectal localizado. Esta validación podría suponer el uso de la hepsina como biomarcador para seleccionar a aquellos pacientes con mayor riesgo trombótico y de metástasis así como para complementar las limitaciones de los modelos actuales de predicción de la trombosis asociada al cáncer colorrectal. Además, estos resultados apoyarían el uso de Venetoclax y Suramin como terapias moleculares dirigidas para prevenir las complicaciones derivadas de la expresión de hepsina.

Respecto al cáncer gástrico avanzado, este tumor está presente entre las 5 neoplasias más comunes y más mortales a nivel global. Además, es uno de los tumores más trombogénicos que existen, una complicación que empeora significativamente el pronóstico de los pacientes. Por ello, el objetivo de esta tesis relativo al cáncer gástrico avanzado consistió en identificar nuevos biomarcadores que ayudasen a seleccionar a los pacientes con mayor riesgo de tromboembolismo venoso. En este contexto, primero quisimos descubrir genes cuya expresión en el tumor primario se asociase a la ocurrencia de tromboembolismo venoso. Para ello, reclutamos una cohorte de 48 pacientes con cáncer gástrico avanzado que sufrieron tromboembolismo venoso a lo largo del seguimiento y 49 controles con el mismo tumor, pero sin dicha comorbilidad. Ambos

grupos de pacientes estaban apareados por "*propensity score matching*" en 38 variables clínico-histopatológicas, para poder asociar la trombosis a las diferencias de expresión génica con mayor fiabilidad. A partir del ARN del tumor primario, hicimos un análisis comparativo de la expresión génica entre ambos grupos mediante un *array* de expresión, buscando genes con una expresión diferencial significativa. En este análisis comparativo, seleccionamos solo aquellos genes que estuviesen diferencialmente expresados en los pacientes con tromboembolismo venoso tanto en el subtipo intestinal como difuso de nuestra cohorte, puesto que en ambos subtipos existe riesgo de trombosis asociada al cáncer. Estos subtipos constituyen las dos entidades diferentes de cáncer gástrico según la clasificación histopatológica de Lauren. Como resultados, obtuvimos 15 genes cuya expresión era significativamente diferente entre los pacientes con y sin tromboembolismo venoso, tanto en el subtipo difuso como intestinal de nuestra cohorte. Una vez identificados estos genes cuya expresión se asociaba a la ocurrencia de tromboembolismo venoso, buscamos validar su uso como predictores de este tipo de trombosis en una nueva cohorte de pacientes con cáncer gástrico avanzado. En esta ocasión, reclutamos 44 pacientes con tromboembolismo venoso y 39 sin trombosis, aunque esta vez, no se aparearon en las variables clínicas, con el fin de ajustarnos a una cohorte más próxima a la realidad clínica ("*real world cohort*"). A partir del ARN del tumor primario, analizamos la expresión absoluta de los 15 genes anteriores mediante PCR digital, y asociamos los datos experimentales con el riesgo de tromboembolismo venoso a lo largo del seguimiento. En base a los genes cuya expresión seguía la misma tendencia respecto a la ocurrencia de tromboembolismo venoso que en el primer estudio, y que además estratificaba dos grupos de pacientes con un riesgo significativamente diferente, concluimos que la sobreexpresión de *PRKD3* o *EPS8* y la infraexpresión de *SAA1* predecían un mayor riesgo de tromboembolismo venoso en la nueva cohorte de cáncer gástrico avanzado. Además, observamos que un modelo basado en la combinación de la sobreexpresión de *EPS8* y la infraexpresión de *SAA1* estratificaba a los pacientes con mayor riesgo mejor que el análisis de los genes por separado o que cualquier otra combinación. En el futuro, la validación de estos genes como predictores de tromboembolismo venoso en cohortes más grandes de cáncer gástrico avanzado podría suponer su uso como herramienta que ayuden a mejorar las limitaciones de los modelos predictores actuales.

ABSTRACT

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In cancer patients, thrombosis is one of the most frequent comorbidities. In fact, it is a complication that decreases the overall survival of patients. Both arterial and venous thromboses are included in this context, although the latter are much more common than the former. Among the types of thrombosis occurring in the venous system, venous thromboembolism is the most frequent, considered the second leading cause of death in cancer patients, with tumor progression being the first. Various risk factors can increase the incidence of cancer-associated thrombosis. These factors may be related to patient characteristics, cancer treatment, or the tumor's biology. Aging, obesity, or immobilization associated with hospitalization, are patient-related factors that may increase the risk of thrombosis. On the other hand, chemotherapy, anti-angiogenic factors, or hormone therapy, are treatments that can induce endothelial damage and, consequently, create a hypercoagulant environment. Lastly, the tumor's primary location and the ability of the cells to express and release procoagulant factors also influence the risk of hemostatic alterations. In this context, tumor cells can induce a hypercoagulable state through various mechanisms. One of these is their ability to produce their own clotting factors, such as tissue factor, which initiates the coagulation cascade. Another mechanism involves releasing factors that inhibit the physiological breackdown of clots, like plasminogen activator inhibitor, prolonging the presence of the thrombus within the blood vessel. In cancer patients, common molecular abnormalities, such as factor V Leiden or others that reduce the expression or activity of anticoagulant agents have also been described. In addition to these disruptions in secondary haemostasis, tumors can directly affect blood cells. For instance, they may induce neutrophil activation to release extracellular traps (mainly composed of DNA and histones, with procoagulant activity) or promote platelet activation and aggregation. The increasing incidence of thrombotic events in cancer patients and their negative impact on prognosis have led to the recommendation of various types of anticoagulant treatment or prophylaxis over the years. One of the first antithrombotic agents used was vitamin-K antagonists, like warfarin. Over time, the efficacy and administration route of this treatment were surpassed by low molecular weight heparins, such as enoxaparin or nadroparin. Finally, in the last decade, due to their equal or superior efficacy and oral administration, direct oral anticoagulants, such as rivaroxaban or apixaban, have gradually replaced heparins. Despite the proven positive effects of these anticoagulant agents, their use is still controversial, and in many cases, even in patients with particularly prothrombogenic cancers, primary thromboprophylaxis is often ruled out. The main reasons for this scenario are low efficiency of widespread thromboprophylaxis (as many patients must be treated for one to benefit), the burden of daily treatment, often administered parenterally, and the increased risk of bleeding associated with these treatments. This last limitation, which negatively impacts prognosis, is particularly pronounced in gastrointestinal tumors. These types of cancers carry a much higher risk of thrombosis than many other types of tumors. Gastric, pancreatic, biliary and liver cancer are among the five most thrombogenic tumors worldwide. As with other neoplasms, thrombosis in patients with digestive tumors significantly reduces survival, and antithrombotic prophylaxis could improve their prognosis. However, as previously mentioned, the limitations of anticoagulants often lead to its rejection, leaving patients with digestive tumors at high

risk of thrombosis. Gastrointestinal tumors have a greater tendency to bleed than other solid tumors, and the use of antithrombotic agents exacerbates this tendency. In this context, various clinical variables, biomarkers and predictive models have been studied over the years to select patients at the highest risk of thrombosis, who might benefit anticoagulant treatment despite the risk of bleeding. However, so far, there are no markers that predict a sufficiently high thrombotic risk to justify thromboprophylaxis in patients with digestive tumors. Therefore, further research is needed to find tools that complement the limitations of existing predictive models of thrombosis. Thus, this thesis focuses on this need in patients with colorectal cancer and advanced gastric cancer, two digestive cancers associated with a high risk of thrombosis where the use of anticoagulants remains controversial.

In the case of colorectal cancer, it is the second most frequently diagnosed tumor globally, and the fourth most deadly neoplasm. Although its thrombotic incidence is not as high as pancreatic or gastric cancer, the risk of thromboembolism remains significant. In an effort to find thrombotic biomarkers that could help predict a sufficiently high risk to justify anticoagulation in patients with colorectal cancer, we focused on the study of hepsin, a type II transmembrane serine protease. One of the main reasons for focusing on this protein was its proven ability to activate coagulation factor VII, which, by forming a complex with tissue factor, initiates the extrinsic coagulation pathway. Another reason was the implication of hepsin in tumor invasion and metastasis, studied in different types of solid tumors. This latter justification connects hepsin expression with thrombosis, considering that thromboembolic events increase with tumour stage in colorectal cancer. Thus, we first conducted a retrospective study on 169 patients with localized tumors and 118 metastatic patients, correlating hepsin expression levels in the primary tumor (measured through specific immunohistochemical staining) with the incidence of thrombotic events during follow-up since diagnosis, as well as other variables such as overall survival, metastatic relapse, disease progression, etc. Significant results showed that in patients with localized tumors, increased hepsin staining intensity independently raised the risk of thrombotic events and metastatic relapse, but these associations were not found in metastatic patients. To understand the underlying mechanisms, we performed a series of *in vitro* and *in vivo* studies based on colorectal cancer cells with both basal expression and overexpression (*via* stable transfection) of hepsin. We tested the effects of hepsin overexpression in migration, invasion, proliferation, oncogenic protein expression and thrombin generation assays. Significant *in vitro* results included hepsin altering thrombin generation parameters towards a hypercoagulable state, increasing the expression of phosphorylated ERK1/2 and STAT3 proteins, and promoting an invasive tumor cell phenotype. This last effect was confirmed *in vivo* using a zebrafish model. Once we understood the mechanisms by which hepsin might increase the risk of thrombosis and metastasis in patients with localized colorectal cancer, we aimed to identify Food and Drug Administration (FDA)-approved drugs that could inhibit hepsin and thereby suppress its pro-tumour and procoagulant effects. Through virtual screening, molecular docking and *in vitro* inhibition assays of hepsin's proteolytic activity, we identified Venetoclax and Suramin as two new hepsin inhibitors. Furthermore, we

observed that in colorectal cancer cells with basal and overexpressed hepsin, these drugs significantly reduced their invasive phenotype and thrombin generation. Summarizing the results derived from these studies in colorectal cancer, we concluded that hepsin is a potential biomarker for thrombosis and metastasis in localized cancer patients, likely due to its ability to promote an invasive tumor cell phenotype and increase thrombin generation. As a therapeutic target, hepsin can be inhibited by Suramin and Venetoclax, two drugs capable of reducing its pro-tumor and procoagulant effects. These findings will drive future validation of hepsin as a biomarker for thrombosis and metastasis in larger cohorts of patients with localized colorectal cancer. This validation could lead to the use of hepsin as a biomarker for selecting patients at higher thrombotic and metastatic risk, as well as to complement the limitations of current thrombosis prediction models in colorectal cancer. Additionally, these results support the use of Venetoclax and Suramin as targeted molecular therapies to prevent complications arising from hepsin expression.

Regarding advanced gastric cancer, this tumor is among the five most common and deadliest cancers worldwide. Additionally, it is also one of the most thrombogenic tumors, a complication that significantly worsens the prognosis of patients. Therefore, the aim of this thesis related to advanced gastric cancer was to identify new biomarkers that could help select patients at higher risk of venous thromboembolism. In this context, we first sought to discover genes whose expression in the primary tumour was associated with the occurrence of venous thromboembolism. To this end, we recruited a cohort of 48 patients with advanced gastric cancer who developed venous thromboembolism during follow-up and 49 controls with the same tumor, but without this comorbidity. Both patient groups were matched using propensity score matching on 38 clinicopathological variables to reliably associate thrombosis with differences in gene expression. From the RNA of the primary tumor, we performed a comparative gene expression analysis between the two groups using an expression array, identifying genes with significant differential expression. We selected only those genes that were differentially expressed in patients with venous thromboembolism in both the intestinal and diffuse subtypes of our cohort, since both subtypes carry a risk of cancer-associated thrombosis. These subtypes represent the two distinst entities of gastric cancer according to Lauren's histopathological classification. As results, we obtained 15 genes whose expression was significantly different between patients with and without venous thromboembolism in both the diffuse and intestinal subtypes of our cohort. Once these genes were identified, we sought to validate their use as predictors of venous thromboembolism in a new cohort of advanced gastric cancer patients. This time, we recruited 44 subjects with venous thromboembolism and 39 without thrombosis, though they were not matched on clinical variables to reflect a more real-world clinical cohort. Using RNA from the primary tumor, we analyzed the absolute expression of the 15 previously identified genes through digital PCR, and associated the experimental data with the risk of venous thromboembolism during follow-up. Based on the genes whose expression followed the same trend regarding venous thromboembolism occurrence as in the first study, and that also stratified two groups of patients with significantly different risk, we concluded that the overexpression of *PRKD3* or *EPS8* and underexpression of *SAA1* predicted a higher risk of venous

thromboembolism in the new cohort of advanced gastric cancer. Furthermore, we observed that a model based on the combination of *EPS8* and *SAA1* stratified patients with higher risk more effectively than analyzing the genes separately or any other combination. In the future, validating these genes as predictors of venous thromboembolism in larger cohorts of advanced gastric cancer could lead to their use as a tool to help overcome the limitations of current predictive models.

ABBREVIATIONS

ABBREVIATIONS

ADP: Adenosine diphosphate

AGC: Advanced gastric cancer

ATEE: Arterial thromboembolic events

AT: Arterial thrombosis

AUC: Area under the receiver operating characteristic curve

AVERT: A very early rehabilitation trial after stroke

CAT: Cancer-associated thrombosis

CI: Confidence interval

CRC: Colorectal cancer

DNA: Deoxyribonucleic acid

DOAC: Direct oral anticoagulants

DVT: Deep venous thrombosis

EV: Extracellular vesicles

G-CSF: Granulocyte colony stimulating factor

GC: Gastric cancer

GI: Gastrointestinal

GM-CSF: Granulocyte-macrophage colony stimulating factor

HPN: Hepsin

IS: Ischemic stroke

KRAS: Kirsten rat sarcoma viral oncogene homolog

LMWH: Low molecular weight heparins

MI: Myocardial infarction

NET: Neutrophil extracellular traps

PAD-4: Peptidyl-arginine deiminase-4

PAI: Plasminogen activator inhibitors

PDI: Protein disulfide isomerase

PE: Pulmonary embolism

ABBREVIATIONS

PROTECHT: Prophylaxis of thromboembolism during chemotherapy

PTP: Primary thromboprophylaxis

P2Y12: Purinergic receptor P2Y, G-protein coupled, 12

P2Y1: Purinergic receptor P2Y, G-protein coupled, 1

TF: Tissue factor

VTE: Venous thromboembolism

VT: Venous thrombosis

GENERAL INTRODUCTION

1. CANCER-ASSOCIATED THROMBOSIS

1.1. EPIDEMIOLOGY AND IMPACT ON PROGNOSIS

The first data on the association between thrombosis and cancer date back to the 19th century. They were reported by Jean-Baptiste Bouillaud, in 1823, and Armand Trousseau, in 1865, who described a link between gastric cancer (GC) and venous thrombosis (VT) (1). Since then, increasing attention has been paid to the interaction between malignant neoplasms and thrombotic phenomena. To date, compared to the general population, cancer patients present a higher risk of both arterial and venous thrombotic events, with the latter being much more frequent (2).

Regarding cancer-associated arterial thrombosis (AT), one study in 300,000 oncology patients reported that the 6-month cumulative incidence of AT (arterial thromboembolic events [ATEE], myocardial infaction [MI], and ischemic stroke [IS]) was 4.70%, in contrast to 2.20% for patients without cancer (3). Other studies have reported similar data. For example, in one study of 66,000 cancer patients, followed from 1995 to 2002, Khorana's group recorded the incidence of ATEE, MI and IS as 1.72%, 0.87% and 0.64%, respectively. In another study of 5,717 oncology patients, followed from 2009 to 2014, Brenner and colleagues reported incidences of ATEE, MI and IS as 1.10%, 0.26% and 0.73%, respectively. Navi's group, in a study involving 280,000 cancer patients between 2002 and 2011, reported that, at 6 months post-tumor diagnosis, the risk of ATEE, MI and IS was, respectively, 2.20, 2.90 and 1.20 times higher than in control patients without cancer (4). Regarding mortality, AT may increase risk of death up to three times. One article from 2018, involving 1,880 cancer patients, described that ATEE increased the risk of death by 3.20 times (5). In Navi's aforementioned study, the hazard ratio of death for ATEE vs non-ATEE cancer patients was 3.10 (95% confidence interval [CI], 3.00 - 3.10) (6). Another study involving 66,106 oncology patients found that ATEE increased in-hospital mortality with an odd ratio of 5.04 (95% CI, 4.38 - 5.79) (7).

With respect to VT, it is much more common than AT in oncology patients. In fact, 20 - 30% of primary venous thrombotic events are associated to malignant neoplasms (8). One study carried out in 1,041 patients with various types of solid tumors, with a median follow-up of 26 months, reported an absolute risk of VT of 7.8% (9). The Cancer and Thrombosis Study, which involved 840 cancer patients, found that 1-year absolute risk of VT following a cancer diagnosis was 8% (10). The relative risk of cancer-associated VT varies depending on the study consulted. Thus, in a cohort of Olmsted, composed of 625 cancer patients and 625 controls, VT risk was 4.10 times higher among oncology patients. In the Multiple Environmental and Genetic Assessment study, which included 2,131 tumor patients and 3,220 controls, the risk of VT was 6.70 times higher in the cancer group. According to United Kingdom databases, VT risk was 4.70 times higher in 82,000 cancer patients compared to 577,000 healthy controls (8). Among the different types of cancer-associated VT, the most common is venous thromboembolism (VTE), which includes deep venous thrombosis (DVT) and pulmonary embolism (PE) (11). Over time, the cumulative incidence of VTE in cancer patients has increased, rising from 1% in 1993

to 3.40% in 2017 (12). This trend, along with a comparison of VTE incidence between oncology patients and controls, is shown in Figure 1 (13).

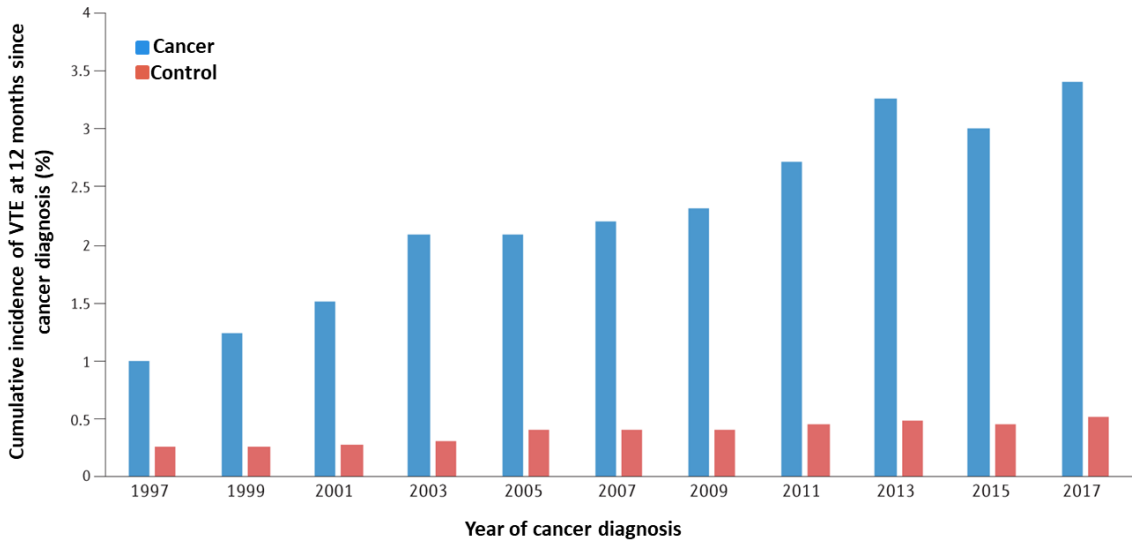

Figure 1. Cumulative incidence of venous thromboembolism in cancer patients and controls over years. Controls are subjects without cancer who were paired with oncology patients diagnosed in each year of the X axis. *VTE*: venous thromboembolism. Figure adapted from reference *13. Khorana AA, Mackman N, Falanga A, Pabinger I, Noble S, Ageno W, Moik F, Lee AYY. 2022. Cancer-associated venous thromboembolism. Nat Rev Dis Primers. 8(1):11*.

VTE is a comorbidity which affects quality of life, treatment and prognosis of tumor patients. Regarding mortality, as shown in Figure 2, VTE is the second-leading cause of death among cancer patients (14). Furthermore, it is estimated that 1-year overall survival rate following a cancer diagnosis for patients who experienced a VTE during follow-up is one-third of the rate for oncology patients without VTE (15).

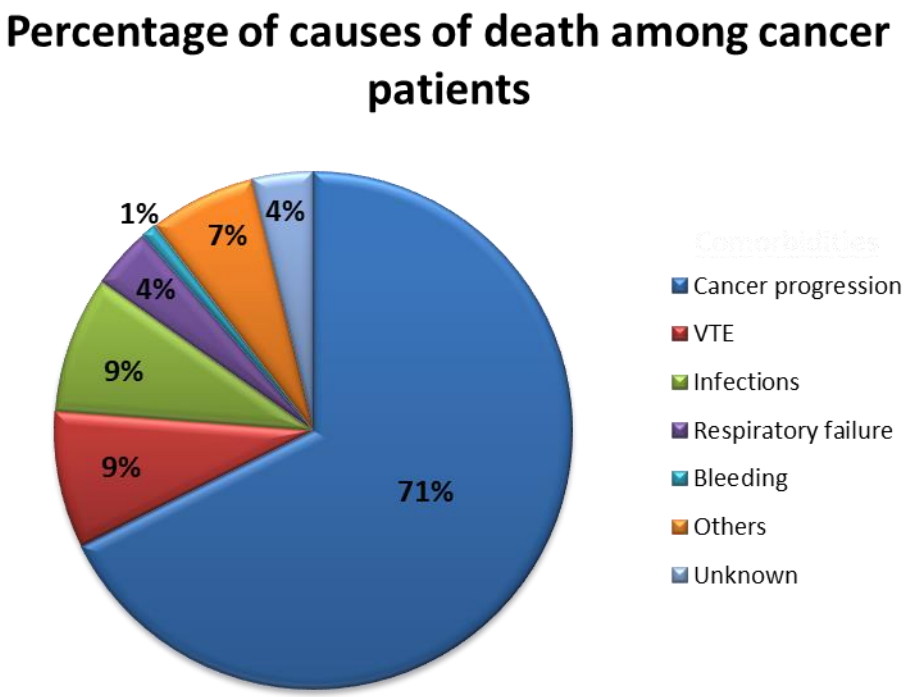

Figure 2. Percentage distribution of causes of death in 4,466 oncology patients under chemotherapy treatment. Percentages exceed 100% due to rounding. Figure adapted from reference *14. Khorana AA. 2010. Venous thromboembolism and prognosis in cancer. Thromb Res. 125(6):490-493.*

1.2. RISK FACTORS

Cancer-associated thrombosis (CAT) depends on various factors that can increase patient's susceptibility. In the case of AT, variables such as male sex, age, hypertension, smoking and lung and kidney tumors raise its incidence (5). Other studies indicate that blood cancers, such as polycythemia vera and multiple myeloma, may promote AT more than other types of cancer. Among these, a study conducted on a large Swedish cohort found that risk of AT at 1 and 10 years after diagnosis of multiple myeloma was 1.90 and 1.50 times higher, respectively, compared to non-cancer patients (16). Certain cancer treatments also increase AT incidence, with radiotherapy being particularly significant. In one study in breast cancer, each gray of radiation increased the risk of coronary disease by 7.40%. For head and neck tumors, the cumulative incidence of IS 15 years after radiotherapy was 12% (16). The literature also highlights high tumor burden as a risk factor of AT, as advanced cancer stages are more prone to this comorbidity (17).

Since VT, particularly VTE, is much more common than AT in cancer, the risk factors of VT have been studied more extensively. Typically, these VTE risk factors can be classified into three categories: patient-related, treatment-related and cancer-related factors (Table 1) (18).

Table 1. Risk factors of venous thromboembolism in oncology patients. *TF*: tissue factor; *VTE*: venous thromboembolism. Data obtained from reference *18. Ikushima S,*

Ono R, Fukuda K, Sakayori M, Awano N, Kondo K. 2016. Trousseau's syndrome: cancer-associated thrombosis. Jpn J Clin Oncol. 46(3):204-208.

<table>
<tr><th colspan="3">Factors that increase risk of cancer-associated VTE</th></tr>
<tr><th>Patient-related</th><th>Treatment-related</th><th>Cancer-related</th></tr>
<tr><td>Older age</td><td>Chemotherapy</td><td>Primary site</td></tr>
<tr><td>Prolonged immobility</td><td>Hormonal agents</td><td>Stage</td></tr>
<tr><td>Prior history of thrombosis</td><td>Growth factors</td><td>Lymphovascular invasion</td></tr>
<tr><td>Elevated leukocyte and platelet count</td><td>Antiangiogenic agents</td><td>Mucin from adenocarcinoma</td></tr>
<tr><td>Acute infection</td><td>Surgery</td><td rowspan="3">Expression of procoagulant factors such as TF</td></tr>
<tr><td>Comorbidities such as heart disease</td><td rowspan="2">Central venous catheter</td></tr>
<tr><td>Obesity</td></tr>
</table>

One study in 2021 carried out in a cohort of Danish patients diagnosed with cancer between 1997 and 2017, reported many of these risk factors (19). One notable variable is cancer stage, as tumor size, growth and invasion increase VTE risk. Another important factor is the type of anti-tumor treatment, particularly chemotherapy or anti-angiogenesis therapies, which considerably elevate VTE risk. Cancer type also impacts VTE incidence. Some examples of highly thrombogenic tumors include pancreatic, ovarian, and liver cancer, as well as multiple myeloma and Hodking-lymphoma, while melanoma, prostate or breast cancer have a much lower VTE risk. The relative risk of VTE associated with all these risk factors is displayed in Table 2 (19).

Table 2. Risk factors of venous thromboembolism in a Danish cohort composed of nearly 500,000 patients diagnosed with cancer between 1997 and 2017. For each variable, the calculation of hazard ratios is based on a reference level. *CI*: confidence interval; *VEGF*: vascular endothelial growth factor; *VTE*: venous thromboembolism. Data obtained from reference *19. Mulder FI, Horváth-Puhó E, van Es N, van Laarhoven HWM, Pedersen L, Moik F, Ay C, Büller HR, Sørensen HT. 2021. Venous thromboembolism in cancer patients: a population-based cohort study. Blood. 137(14):1959-1969.*

Risk factor	Adjusted hazard ratio of VTE (95% CI)	%Cumulative incidence of VTE at 6 months since cancer diagnosis (95% CI)
Cancer stage at diagnosis		
Localized	Reference	0.80 (0.75 - 0.84)
Regional	2.29 (2.14 - 2.45)	1.93 (1.85 - 2.01)
Metastasis	3.15 (2.94 - 3.37)	3.14 (3.03 - 3.25)
Anti-tumor treatment		
No treatment	Reference	1.05 (0.98 - 1.13)

Surgery	2.20 (2.02 - 2.39)	1.84 (1.79 - 1.90)
Radiotherapy	2.16 (1.94 - 2.39)	2.07 (1.96 - 2.18)
Chemotherapy	3.35 (3.06 - 3.66)	3.50 (3.39 - 3.61)
VEGF inhibitors	4.29 (3.54 - 5.19)	6.13 (5.35 - 6.98)
Immunotherapy	3.56 (2.75 - 4.59)	4.08 (3.21 - 5.10)
Cancer type		
Melanoma	Reference	0.36 (0.30 - 0.43)
Breast	1.53 (1.25 - 1.88)	0.64 (0.59 - 0.70)
Prostate	1.86 (1.51 - 2.29)	0.80 (0.73 - 0.87)
Leukemia	2.79 (2.19 - 3.55)	1.27 (1.10 - 1.47)
Endometrium	3.64 (2.88 - 4.60)	1.43 (1.24 - 1.65)
Bladder	3.62 (2.89 - 4.53)	1.66 (1.47 - 1.87)
Rectum	4.07 (3.31 - 5.01)	2.07 (1.90 - 2.25)
Kidney	4.11 (3.29 - 5.14)	2.17 (1.92 - 2.44)
Colon	4.06 (3.33 - 4.96)	2.21 (2.09 - 2.34)
Brain	7.49 (5.91 - 9.48)	2.18 (1.88 - 2.51)
Hodking-lymphoma	5.70 (4.23 - 7.70)	2.88 (2.27 - 3.61)
Gastric	4.27 (3.40 - 5.36)	2.48 (2.19 - 2.80)
Non-small cell lung	4.03 (3.31 - 4.91)	2.60 (2.48 - 2.73)
Ovarian	5.25 (4.22 - 6.54)	3.10 (2.78 - 3.44)
Liver	4.50 (3.53 - 5.75)	2.82 (2.42 - 3.26)
Pancreas	6.38 (5.19 - 7.84)	4.43 (4.12 - 4.76)

Risk factors for cancer-associated VTE impact the three vertices of Virchow´s triad (20). According to this triad, hemostatic disorders may result, individually or in combination, from one of these 3 situations: blood stasis, endothelial damage and hypercoagulability. Among oncology patients, hospitalizations and immobility reduce blood flow. Vascular compression induced by tumor cells interferes with this flow, and vessel invasion may increase blood viscosity, promoting blood stasis (20). Regarding endothelial damage, anti-tumor treatments induce an endothelial stress. This includes anti-angiogenic agents such as anti-vascular endothelial growth factors, as well as anti-oncogene therapies like those based on the inhibition of V-Abl Abelson murine leukemia viral oncogene homolog 1 kinase. Finally, tumor cells can release procoagulant factors such as tissue factor (TF), platelet agonists or anti-inflammatory proteins (20). This last topic will be developed in the following section.

1.3. MECHANISMS OF CANCER-ASSOCIATED THROMBOSIS

The increase in thrombotic risk among patients with malignant neoplasms is due to several molecular mechanisms promoted by tumors that alter hemostatic balance. Over time, studies examining the pathways through which cancer interferes with coagulation have gained importance. Figure 3 summarizes some of these pathways, which are explained in the following paragraphs.

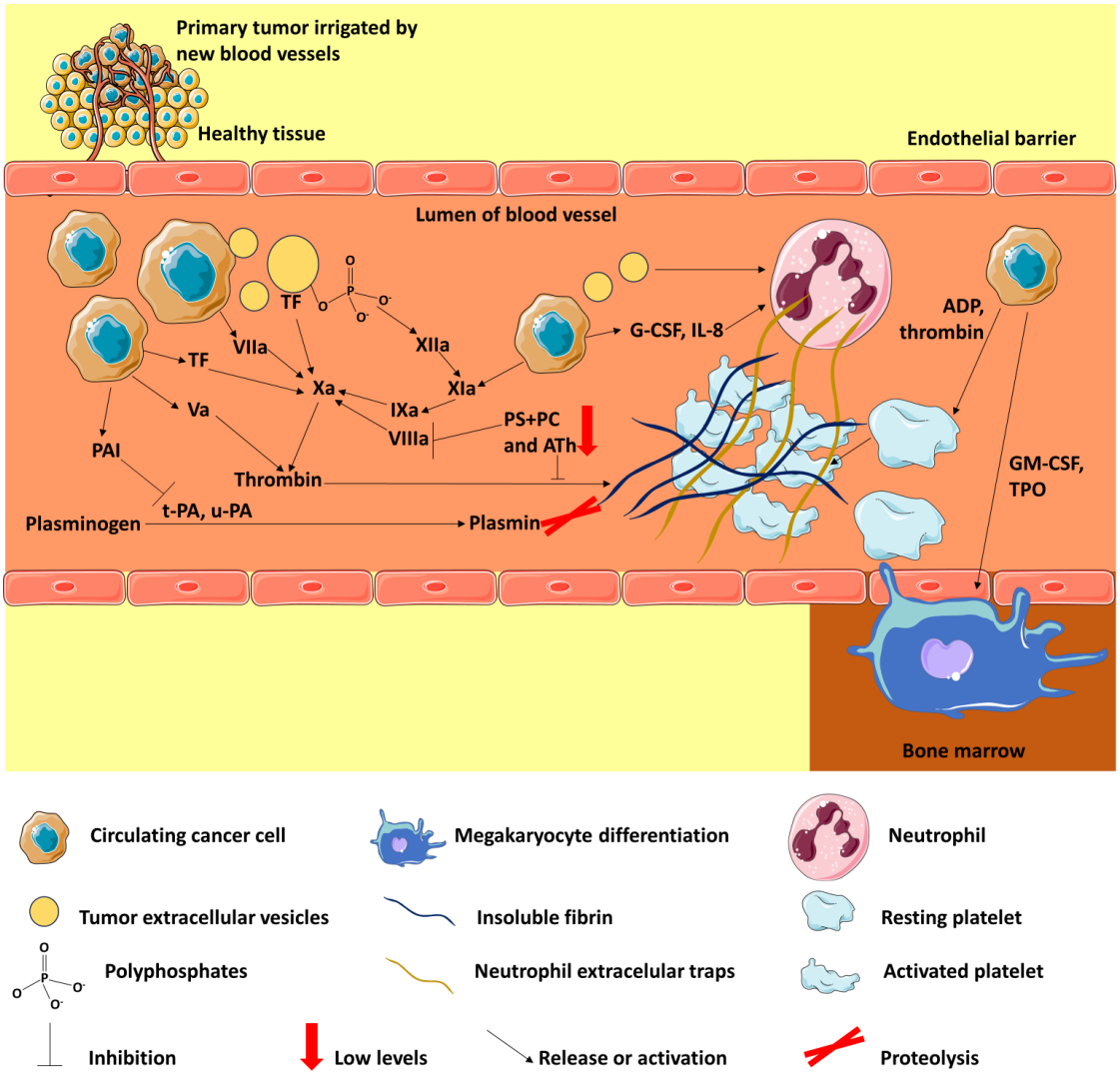

Figure 3. Mechanisms of cancer-associated thrombosis. Through progressive invasion and angiogenesis, the primary tumor reaches the bloodstream. There, circulating tumor cells may release extracellular vesicles that expose clotting factors and negatively-charged molecules, which activate extrinsic and intrinsic coagulation pathways, respectively. Cancer cells can also release their own clotting factors, as well as inhibitors of plasmin activators. Activation of coagulation pathways leads to insoluble fibrin formation, while the inhibition of plasmin activation interferes with fibrin proteolysis. In addition, reduced levels of natural anticoagulants are common among oncology patients. Circulating tumor cells and their extracellular vesicles can promote the release of neutrophil extracellular traps. Furthermore, cancer cells deliver megakaryopoietic factors that induce the differentiation of megakaryocytes in the bone marrow, promoting production of new platelets. These respond to platelet agonists released by tumor cells, becoming activated platelets which aggregate among themselves. In summary, cancer cells induce thrombus formation by promoting insoluble fibrin deposition, neutrophil extracellular traps and platelet aggregation. *ADP*: adenosine diphosphate; *ATh*:

antithrombin; *G-CSF*: granulocyte colony stimulating factor; *GM-CSF*: granulocyte-monocyte colony stimulating factor; *IL-8*: interleukin-8; *PAI*: plasminogen activator inhibitors; *PC*: protein C; *PS*: protein S; *TF*: tissue factor; *TPO*: thrombopoietin; *T-* and *u-PA*: tissue- and urokinase-plasminogen activators; *Va*: activated factor V; *VIIa*: activated factor VII; *VIIIa*: activated factor VIII; *IXa*: activated factor IX; *Xa*: activated factor X; *XIa*: activated factor XI; *XIIa*: activated factor XII. Figure created with *Power Point* and *Servier Medical ART*.

A well-known CAT mechanism is the delivery of extracellular vesicles (EV) from the membranes of tumor cells. On their surface, EV expose negatively-charged molecules, such as polyphosphates, which may promote the coagulation contact pathway by activating clotting factors like factor XII. This activation leads to factor XI activation, whose downstream signaling triggers thrombin activation and insoluble fibrin deposition (21). EV also expose phospholipids such as phosphatidylserine, which can induce the activation of factors VII, IX or X (22). In addition, EV membranes contain their own clotting factors, with TF being one of the most well-known. This procoagulant protein is constitutively expressed in various types of cancer cells, and can be exported in their EV. TF is the main activator of extrinsic coagulation pathway, where it forms a complex with factor VII, activating factor X, which in turn activates prothrombin to thrombin (22). Tumor-derived EV also interact with neutrophils to induce release of neutrophil extracellular traps (NET), which constitute a risk factor for thrombus formation in the bloodstream (23). In one in vitro study, after incubating neutrophils with breast cancer-derived exosomes (a type of EV), an increase in the release of deoxyribonucleic acid (DNA) fibers positive for citrullinated histones was observed, compared to non-incubated neutrophils (23). Cancer-derived EV also expose platelet agonists such as podoplanin, promoting platelet activation and subsequent aggregation (24).

Tumor cells express their own clotting factors. TF is overexpressed in several types of cancer tissues compared to healthy ones. This upregulation has been associated with a higher incidence of thromboembolic events and worse prognosis. In fact, highest levels of TF have been found in the most thrombogenic neoplasms, such as pancreatic, brain, lung, gastric and ovarian cancer (25). TF overexpression in cancer may result from molecular alterations affecting genes other than the TF gene itself. For example, in colorectal cancer (CRC) cells, higher levels of TF are a consequence of aberrant activation of the *Kirsten rat sarcoma viral oncogene homolog* (*KRAS*) gene and inactivation of the *Cellular tumor antigen p53* gene (26). In glioblastoma cells, hypoxia and downregulation of phosphatase and tensin homolog are also associated with TF overexpression (27). Apart from TF, tumor cells express other coagulation factors. In breast cancer tissues, it has been described the endogenous production of factor VII (28). In bladder cancer, overexpression of factor VIII compared to healthy tissues has been documented. The same study also showed the endogenous expression of this factor in hepatic, lung, ovarian, breast and colorectal tumors (29). Overexpression of coagulation factor X has been demonstrated in endometrial cancer compared to healthy uterine tissue (30). One study in GC reported endogenous expression of factors VII, IX, X and XI (31). Thus,

tumor cells synthesize components of coagulation pathways that lead to fibrin generation and subsequent thrombus formation.

On the other hand, there are also studies supporting the association between underexpression or resistance to anticoagulant factors and the development of neoplasms. For example, in multiple myeloma patients, protein C resistance and reduction of protein S levels are particularly common, both of which increase risk of thrombosis, as they are involved in the inhibition of factors V and VIII (32-34). The underlying mechanisms of tumor-induced low anticoagulant activity have been described. One example is the mutation responsible for factor V Leiden, a genetic alteration in which *factor V* gene codifies a protein that cannot be inhibited by activated protein C. This variant has been frequently described in breast cancer, with the incidence of factor V Leiden being much higher among VTE patients (18.50%) than in non-VTE ones (4.50%) (35). Regarding antithrombin, the main inhibitor of factor X and thrombin, many studies have described its downregulation in oncology patients compared to healthy controls, paricularly in colorrectal, ovarian and prostate cancer (36).

As previously explained, there are many ways by which cancer promote thrombus formation. Tumor cells are also known to interfere with clot degradation or fibrinolysis. In lymphoma patients, clot degradation time is significantly longer than that of healthy controls (37). In another study on GC, immunohistochemistry on adenocarcinoma biopsies from 37 patients supported an anti-fibrinolytic microenvironment, characterized by weak staining for plasminogen activators (urokinase- and tissue-plasminogen activators), which are zymogens of plasmin (the main protein responsible for clot lysis), and high levels of their inhibitors (plasminogen activator inhibitors [PAI]) (31). In brain cancer, associated hypofibrinolysis is a consequence of PAI-1 overexpression (38). One study involving 106 multiple myeloma patients and 100 healthy controls reported that cancer patients exhibited delayed clot degradation, a lower release rate of D-dimer (a product of thrombus lysis), and higher PAI-1 activity (39).

So far, we have explored the various mechanisms through which tumor cells alter hemostasis by affecting procoagulant, anticoagulant and fibrinolytic factors. However, several studies also suggest an impact on the cellular components of hemostasis, such as neutrophils and platelets. It has been shown that cancer cells from primary tumor attract circulating neutrophils by releasing chemokines such as C-X-C motif chemokine ligand-1,2,3 or tumor growth factor-beta. Once in tumor microenvironment, neutrophils may acquire a thrombogenic phenotype in response to cancer-related stimuli like interleukin-8, granulocyte colony stimulating factor (G-CSF) and peptidyl-arginine deiminase-4 (PAD-4), which induce NET release (40). NET are DNA fibers released from nucleus of neutrophils into the extracellular space, typically as a defense mechanism against pathogens (41). These fibers can trap platelets and promote their activation and aggregation, as well as binding clotting factors such as TF or fibrinogen. As a result, they act as scaffolds for various procoagulant components that interact to form the thrombus (40). Several studies have linked NET to CAT. For example, one study in 946 oncology patients found that 2-year cumulative incidence of VTE was 14.50% among those with

citrullinated histone-3 (marker of NET) levels above the first quartile, compared to 8.50% in the rest of the patients. However, the strongest associations were observed in lung and pancreatic cancers, while no relationship was found in breast cancer (42). In pancreatic cancer-bearing mice, neutrophils and extracellular DNA fibers were found to increase venous thrombus size (43). In CRC, neutrophils from patients released more NET than those from healthy controls, and this increase was accompanied by higher fibrin deposition. In GC, NET isolated from patients enhanced thrombin generation and fibrin deposition in plasma from healthy individuals (44). Regarding platelets, tumor cells interfere with their production and activation through various mechanisms. As the tumor grows, it releases circulating proteins like granulocyte-macrophage colony stimulating factor (GM-CSF) and thrombopoietin, which stimulate megakaryocytes differentiation in the bone marrow, leading to the production of new platelets (45). Over the years, a high number of circulating platelets (thrombocytosis) in cancer patients has been associated with the incidence of thromboembolic events (46). However, this association is not observed in all tumor types, being more frequent in gastric, endometrial, kidney, pancreatic and colorectal cancers (47-51). In terms of platelet activation, cancer can produce various types of agonists that, when recognized by platelet receptors, trigger intracellular signaling pathways leading to platelet aggregation and clot formation. An important agonist is adenosine diphosphate (ADP), which binds to purinergic receptor P2Y, G-protein coupled, 1 (P2Y1) and purinergic receptor P2Y, G-protein coupled, 12 (P2Y12) on the platelet membrane. Once activated, these receptors induce a conformational change in the platelets, thromboxane release and platelet aggregation. Tumor cells also produce thrombin, which, in addition to its direct role in fibrin deposition, is the most potent platelet agonist (45).

1.4. PREVENTION OF CANCER-ASSOCIATED THROMBOSIS, TREATMENT LIMITATIONS AND RISK MARKERS

Since thrombosis is the second-leading cause of death among cancer patients, its prevention is crucial. The treatment aimed at preventing thrombosis from the time of cancer diagnosis is known as primary thromboprophylaxis (PTP). The first data reporting clinical benefits from PTP date back to 1994, when Levene and colleagues found that, in 311 metastatic breast cancer patients, the 6-month incidence of VTE was 3.70% lower in subjects treated with warfarin (vitamin-K antagonist) compared to the placebo group (52). Over time, vitamin-K antagonists were replaced by low molecular weight heparins (LMWH), as they proved to be more effective and easier to administer (53). The “Prophylaxis of thromboembolism during chemotherapy” (PROTECHT) study demonstrated that, in 1,150 cancer patients, VTE risk in those receiving nadroparin was 2.00%, compared to 3.90% in the placebo group (52). A review carried out by Di Nisio and colleagues, which included nine trials, reported that relative risk ratio of VTE among cancer patients was 0.54 (95% CI, 0.38 – 0.75) when comparing patients treated with LMWH to those receiving placebo (54). As with vitamin-k antagonists, LMWH as eventually eclipsed by the emergence of direct oral anticoagulants (DOAC), as they were

easier to administer and did not require laboratory monitoring (55). Two well-known DOAC are rivaroxaban and apixaban, whose efficacy in preventing VTE was studied in the CASSINI and "A very early rehabilitation trial after stroke" (AVERT) trials, respectively. In the CASSINI trial, involving 841 cancer patients, the 6-month VTE risk for rivaroxaban-treated patients was 6.00%, compared to 8.80% in the placebo group. In the AVERT study, involving 563 oncology patients, the 6-month VTE risk for apixaban-treated patients was 4.20%, compared to 10.20% in the placebo group (56). Recent data on the efficacy of LMWH and DOAC in VTE prevention are shown in Table 3 (57), and in most studies, DOAC were more effective than LMWH.

Table 3. Comparative studies of cancer-associated venous thromboembolism risk between low molecular weight heparins- and direct oral anticoagulants-treated patients. Each study was cancer type-specific, and used a specific anticoagulant for each group. Study size refers to number of treated patients. Risk of venous thromboembolism refers to number of individuals who experienced the event relative to the total number of treated patients. *DOAC*: direct oral anticoagulants; *LMWH*: low molecular weight heparins; *VTE*: venous thromboembolism. Data obtained from reference *57. Zhou H, Chen TT, Ye LL, Ma JJ, Zhang JH. 2024. Efficacy and safety of direct oral anticoagulants versus low-molecular-weight heparin for thromboprophylaxis after cancer surgery: a systematic review and meta-analysis. World J Surg Oncol. 22(1):69.*

Name of study	Type of cancer	Study size		Type of anticoagulant		Risk of VTE	
		LMWH	DOAC	LMWH	DOAC	LMWH	DOAC
Guntupalli 2020	Gynecologic	196	204	Enoxaparin	Apixaban	1.50%	1.00%
Zhao 2023	Lung	203	200	Nadroparin	Rivaroxaban	17.70%	12.50%
Rashid 2018	Pancreatic	12	87	Enoxaparin	Dabigatran	0.00%	4.50%
Rich 2023	Bladder	250	124	Enoxaparin	Apixaban	3.20%	1.60%
Westerman 2022	Urological	79	84	Enoxaparin	Apixaban	1.70%	0.00%

Despite the clinical benefits of PTP in cancer patients, there are important limitations that need to be considered (Figure 4). First, in general cancer population, the number of patients that must be treated for one to benefit from thromboprophylaxis is high, ranging from 24 to 50 patients (52, 55, 56). Another major limitation is the risk of bleeding. Cancer itself can increase the bleeding risk because of tumor invasion or angiogenesis (58). In one study involving 1,075 patients with active cancer and 8,935 free-cancer controls, the hazard ratio for major bleeding was 3.80 (95% CI, 2.90 - 5.00) for oncology patients (59). This risk is often further elevated by anticoagulation therapy (58). In an article including 3,655 cancer patients, the odds ratio of hemorrhage for those treated with extended thromboprophylaxis (more than 2 weeks) was 2.11 (95% CI, 1.33 - 3.35), compared to patients treated with anticoagulants for 6 to 14 days (60). Moreover, the

increased risk of bleeding varies depending on anticoagulant therapy. Numerous studies have shown that DOAC are associated with a higher risk of bleeding compared to LMWH. In Zhao and colleagues' study involving 403 lung cancer patients, the risk of major bleeding was 9.70% for those treated with rivaroxaban, compared to 6.50% for those treated with nadroparin (61). Another study by Nagi and colleagues in 598 gynecologic cancer patients, reported a bleeding risk of 2.00% for rivaroxaban-treated patients versus 0.70% for those treated with LMWH (62). Similar trends were observed in studies by Swaroop in gynecologic neoplasm (63) and Rashid in pancreatic cancer (64). The PTP limitations are also related to administration strategies. This is particularly evident with LMWH, which requires daily subcutaneous injection, making it a burdensome treatment for the patient. Oral anticoagulants are less invasive but also need to be taken daily (56).

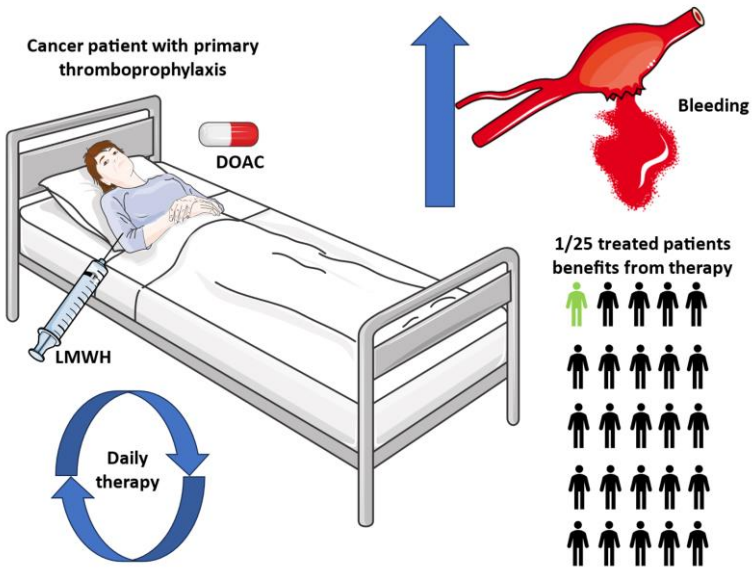

Figure 4. Main limitations of primary thromboprophylaxis in cancer patients. Cancer patients treated with low molecular weight heparins or direct oral anticoagulants for the VTE prevention face an increased risk of bleeding. This daily treatment imposes a significant burden on patients, and a small fraction benefit from it, compared to the total number of patients treated. *DOAC*: direct oral anticoagulants; *LMWH*: low molecular weight heparins. Figure created with *Power Point* and *Medical Server ART*.

Due to the limitations of PTP, research has been focused on identifying thrombotic markers that enable clinicians to select cancer patients at the highest VTE risk, for whom anticoagulation would be justified despite the potential adverse effects. These markers have been incorporated into clinical risk scores for thrombosis. A well-known example is the Khorana score (Table 4), which classifies patients according to their primary tumor site, thrombocytosis, hemoglobin levels, leukocytosis and body mass index, among other factors. Another model for assessing VTE risk is the PROTECHT score (Table 4), which, in addition to the variables from the Khorana model, takes into account the use of gemcitabine- or platinum-based therapy (65). Over time, clinical variables of VTE risk

assessment models have been complemented with thrombosis biomarkers. This is the case of the "Vienna Cancer and thrombosis study" score (Table 4), which adds D-dimer and soluble P-selectin levels to the Khorana score variables (65). Additionally, there are scores based purely on biomarkers, such as the 5-single nucleotide polymorphism score (Table 4) (66).

Table 4. Clinical scores of venous thromboembolism risk in cancer. Each assessment model is based on different clinical variables or biomarkers, which contribute cumulative points of thrombotic risk. *AB0*: blood group gene; *CATS*: cancer and thrombosis study; *FGG*: fibrinogen gamma chain gene; *log*: logarithm; *NA*: not applicable; *N*: number of affected alleles; *PROTECHT*: prophylaxis of thromboembolism during chemotherapy; *SNP*: single nucleotide polymorphism. Data obtained from references *65. van Es N, Di Nisio M, Cesarman G, Kleinjan A, Otten HM, Mahé I, Wilts IT, Twint DC, Porreca E, Arrieta O, Stépanian A, Smit K, De Tursi M, Bleker SM, Bossuyt PM, Nieuwland R, Kamphuisen PW, Büller HR. 2017. Comparison of risk prediction scores for venous thromboembolism in cancer patients: a prospective cohort study. Haematologica. 102(9):1494-1501* and *66. Guman NAM, van Geffen RJ, Mulder FI, van Haaps TF, Hovsepjan V, Labots M, Cirkel GA, Y F L de Vos F, Ten Tije AJ, Beerepoot LV, Tjan-Heijnen VCG, van Laarhoven HWM, Hamberg P, Vulink AJE, Los M, Zwinderman AH, Ferwerda B, Lolkema MPJK, Steeghs N, Büller HR, Kamphuisen PW, van Es N. 2021. Evaluation of the Khorana, PROTECHT, and 5-SNP scores for prediction of venous thromboembolism in patients with cancer. J Thromb Haemost. 19(12):2974-2983.*

Variable/Score	Khorana score (points)	PROTECHT score (points)	Vienna CATS score (points)	5-SNP score (points)
Tumor site: pancreas, gastric or brain	2	2	2	NA
Tumor site: lung, gynecologic, lymphoma, bladder, testicles, kidney	1	1	1	NA
Prechemotherapy platelet count: ≥350 x 10^9/L	1	1	1	NA
Prechemotherapy hemoglobin level: <6.2 mmol/L	1	1	1	NA
Prechemotherapy leukocyte count: >11 x 10^9/L	1	1	1	NA
Body mass index: ≥35 kg/m2	1	1	1	NA
D-dimer: >1.44 µg/L	NA	NA	1	NA
Soluble P-selectin: >53.10 ng/L	NA	NA	1	NA
Gemcitabine therapy	NA	1	NA	NA
Platinum-based therapy	NA	1	NA	NA
SNP in f*actor V*: rs6025	NA	NA	NA	N*log(3.79)
SNP in *AB0*: rs8176719	NA	NA	NA	N*log(1.85)
SNP in f*actor II*: rs1799963	NA	NA	NA	N*log(2.78)

SNP in *FGG*: rs2066865	NA	NA	NA	N*log(1.56)
SNP in f*actor XI*: rs2036914	NA	NA	NA	N*log(1.32)

All these scores aim to stratify patients with a significantly high risk of VTE. This stratification helps address the limitations of PTP. For example, according to PROTECHT score, in a group of high-risk VTE patients, 17 needed to be treated for one to benefit from PTP, compared to low and intermediate-risk groups, where the number needed was 77 (2). Another example is the stratification of VTE risk according to the primary tumor site. Many scores identify patients with pancreatic cancer as a high VTE risk group. In these patients, PTP is much more effective than in patients with tumors associated with a lower VTE risk. For both DOAC- and LMWH-based therapies, risk of VTE was significantly reduced compared to placebo in pancreatic cancer patients. The number needed to treat for one patient to benefit from anticoagulation ranged from 5 to 15 patients in most studies (67). Thus, selecting cancer patients with a high risk of thrombosis improves the benefit-to-treatment ratio and justifies PTP despite the risk of bleeding or the need for daily therapy. However, for many cancer types, there is still a lack of VTE markers that can stratify patients with a thrombosis risk high enough to justify the use of anticoagulants.

1.5. UNDERLYING MECHANISMS OF CANCER-ASSOCIATED THROMBOSIS BIOMARKERS AND DERIVED MOLECULAR THERAPY

As we have described in the previous section, prevention of VTE in cancer patients can be achieved through PTP. However, we have also highlighted that these anticoagulants increase risk of bleeding and are often inefficient across various contexts and tumor types. For this reason, many studies are focused on discovering new therapies that can complement existing anti-thrombotic strategies and help overcome their limitations. In recent years, advances in understanding specific thrombogenic mechanisms of known VTE biomarkers have led to new molecular targeted therapies.

Several components of CAT have been investigated as possible therapeutic targets (Figure 5). For example, NET are associated with VTE incidence in some cancer types, and studies involving leukemia, lung or breast tumor-bearing mice have demonstrated their role in thrombus formation (68). Since that the structure and procoagulant activity of NET are known to rely on extracellular DNA fibers, some studies have aimed to degrade these fibers by injecting DNase enzymes (Figure 5) into tumor-bearing mice. This therapy effectively reduced venous thrombus size in murine models (68). In addition, earlier studies on PAD-4 and its key role in NET release from neutrophils have promoted more recent research focusing on specific inhibitors of this protein (Figure 5). In one study involving breast cancer-bearing mice, the PAD-4 inhibitor GSK484 decreased NET formation and vascular occlusion (68).

Other studies are focused on TF-bearing microparticles, as many articles have linked these vesicles with hypercoagulation both in experimental models and cancer patients (69). In vivo cancer models with high levels of TF-bearing microparticles showed an increase in venous thrombus size. In a cohort of 96 cancer patients, the levels of these vesicles were significantly higher in individuals who experienced VTE compared to those without thrombotic symptoms. Insight into the mechanisms by which EV are released from cells have spurred studies aimed at disrupting these pathways in cancer (69). Statins, which inhibit the prenylation of proteins involved in regulating cytoskeleton dynamics and membrane blebbing (Figure 5), have been studied in this context. A study by Sapet and colleagues demonstrated that fluvastatin reduced endothelial EV formation following thrombin stimulation. As a result, statins have been tested in clinical trials to evaluate their effects on levels of TF-bearing microparticles in cancer patients (69). In one phase-II trial which involved women with breast cancer, administration of 40 mg rosuvastatin daily for 4 weeks significantly reduced the levels of these vesicles, proposing this statin as a potential therapy for VTE prevention.

Fibrin generation and platelet activation are important promoters of CAT. Studies on protein disulfide isomerase (PDI) have described it as an important enzyme for proper protein folding. PDI is present on the surface of platelets and activated endothelial cells, playing a key role in regulating fibrin deposition and platelet reactivity. In fact, its inhibition (Figure 5) has been shown to completely block platelet activation and fibrin formation in an in vivo model of laser-induced vascular injury (69). In a preliminary trial with healthy subjects, the flavonoid quercetin inhibited PDI activity in plasma, but also showed an inhibitory effect on platelet activation and thrombin generation. Further trials will be conducted in advanced cancer patients to assess the effect of this flavonoid on PDI activity and thrombotic diseases (69).

Regarding platelet aggregation, this hemostatic mechanism is mediated by platelet receptors activated by their specific ligands. Given that tumor cells exploit this aggregation process to induce thrombosis, some researchers have focused on targeting the platelet receptors responsible for the formation of platelet aggregates (Figure 5) (70). A study on P2Y12 and P2Y1 (ADP receptors on platelets) found that blocking activation of these receptors using ticagrelor reduced tumor cells-induced platelet aggregation in vitro, but also decreased levels of platelet aggregates in breast and CRC patients.

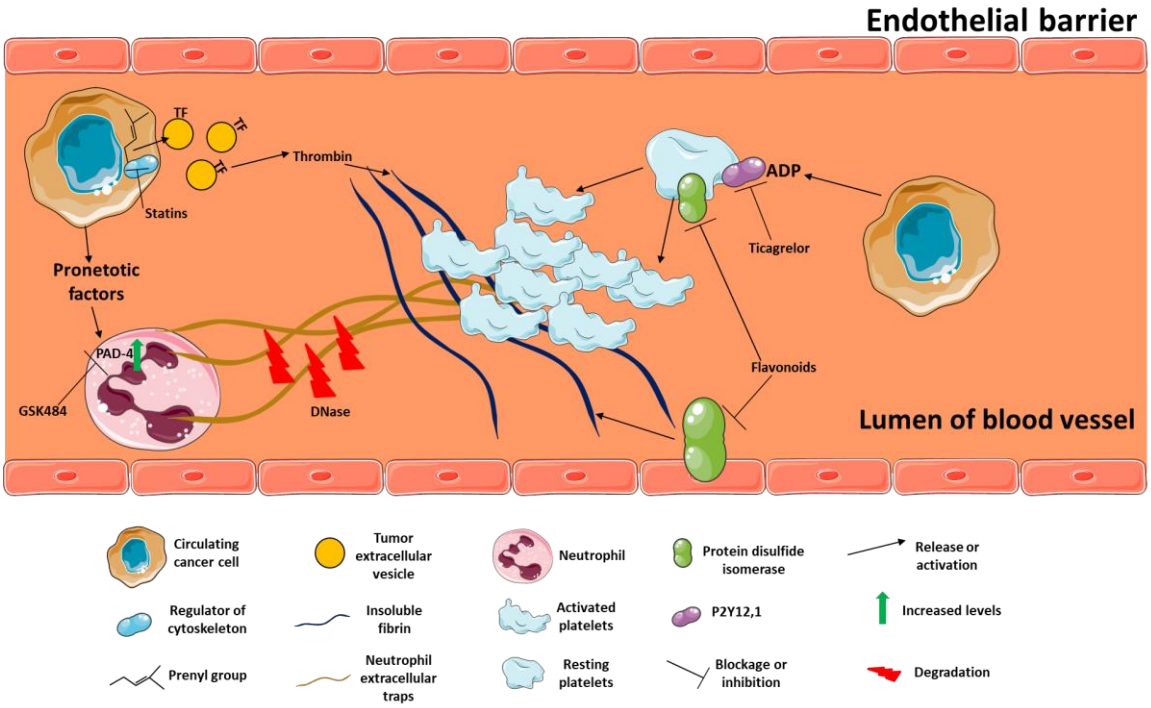

Figure 5. Potential molecular targeted therapies for prevention of cancer-associated thrombosis. This figure highlights various agents that can block pathways through which cancer cells promote thrombosis. Statins prevent the prenylation of proteins that regulate the actin cytoskeleton thereby reducing the release of tissue factor-bearing extracellular vesicles from the tumor cells membrane. GSK484 is an inhibitor of peptidyl-arginine deiminase-4, a promoter of NETosis. DNases degrade neutrophil extracellular traps, which would otherwise induce thrombus formation. Ticagrelor blocks adenosine diphosphate (ADP)-induced activation of platelet receptors, preventing platelet aggregation. Flavonoids inhibit protein disulfide isomerase activity, a key enzyme for fibrin deposition and platelet aggregation. *ADP*: adenosine diphosphate; *PAD-4*: peptidyl-arginine deiminase-4; *P2Y12,1*: purinergic receptor P2Y, G-protein coupled, 12 or 1; *TF*: tissue factor. Figure created with *Power Point* and *Medical Server ART*.

2. CANCER-ASSOCIATED THROMBOSIS IN GASTROINTESTINAL TUMORS

2.1. AN OVERVIEW

Gastrointestinal (GI) tumors refer to neoplasms originated in the digestive system, including gastric, pancreatic, liver and colorectal cancers. Many of these cancers are highly prevalent in the general population, and are associated with high rates of mortality (71), partly due to the high prevalence of thromboembolic events in this group of cancers. In a study of 220 consecutive GI cancer patients, 60 subjects (27.3%) experienced a total of 83 thromboembolic events, of which 38.60% were DVT and 20.50% were PE (72). Another study involving 87,069 Danish patients with GI cancer found that the 1-year cumulative incidence of VTE from the time of cancer diagnosis was 4.40% (73). However, VTE risk varies by GI tumor type. In the Danish study, the 1-year cumulative

incidences rated were 7.80, 4.80, 3.60 and 3.60% for pancreatic, gastric, liver and CRC, respectively. Figure 6 shows the distribution of VTE incidence accross different tumor types. Since 2010, among the 5 most thrombogenic tumors, 4 of them belong to digestive system cancers, with pancreatic cancer at the top of the ranking. This tumor type is followed by gastric and liver cancer in terms of thrombosis risk, while colorectal tumors are comparatively less thrombogenic (Figure 6) (19).

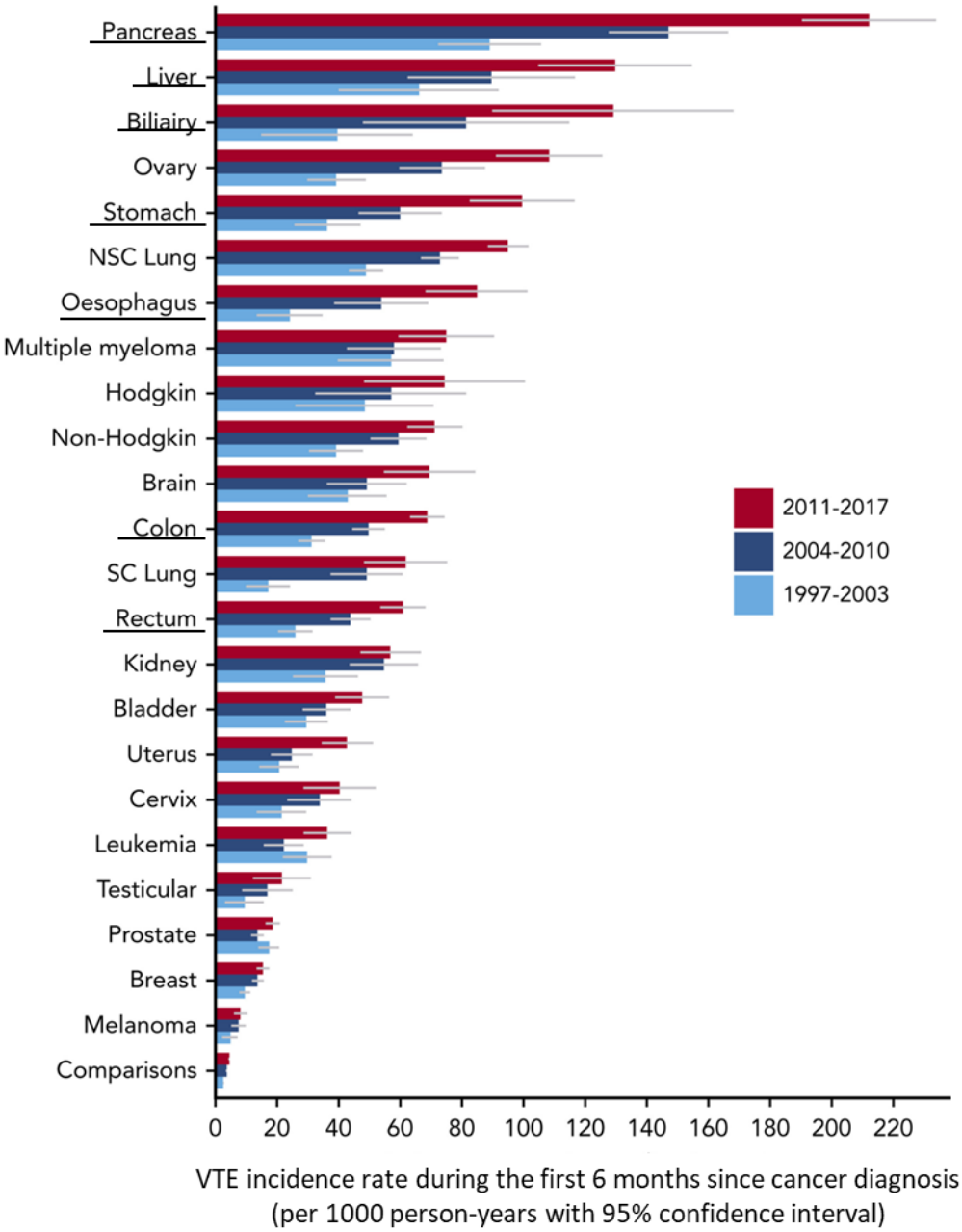

Figure 6. Venous thromboembolism incidence according to cancer primary site in different time intervals. Gastrointestinal tumors are underlined. *NSC*: non-small cell; *SC*: small cell; *VTE*: venous thromboembolism. Figure adapted from reference *19. Mulder FI, Horváth-Puhó E, van Es N, van Laarhoven HWM, Pedersen L, Moik F, Ay C, Büller HR, Sørensen HT. 2021. Venous thromboembolism in cancer patients: a population-based cohort study. Blood. 137(14):1959-1969.*

The differential risk of CAT in GI tumors likely arises from specific thrombogenic mechanisms. For instance, pancreatic cancer is associated with a particularly high overexpression of TF. Among all cancer types, the highest levels of TF-bearing EV are observed in patients with pancreatic neoplasms, making pancreatic cancer the only tumor type where an association between these microvesicles and VTE has been identified. A potential explanation for these findings is the pancreas's endocrine function, which provides TF-EV an accessible route to the bloodstream (74). CAT in other GI tumors involves distinct thrombogenic mechanisms. In the case of portal vein thrombosis, one of the most common VT in liver cancer patients, associated link has been established with hepatitis-B virus infection. According to the literature, the viral X protein upregulates the production of metastatic tumor antigen 1 and forkhead box M1, which are proteins involved in vascular invasion of tumor cells, thus enhancing interaction between cancer cells and the hemostatic system (75). Additionally, hypoxia is considered to exert a prothrombotic effect in hepatocellular neoplasms. Hypoxia, often caused by liver cirrhosis and rapid tumor growth, restricts blood flow and oxygen supply to the tissue. This condition upregulates proteins, such as tryptophan 5-monooxygenase activation protein, that inhibit degradation of hypoxia-inducible factor alpha (HIF-α). The resulting stability of HIF-α drives overexpression of genes linked to epithelial-mesenchymal transition, which increase the risk of metastasis and vascular invasion (75).

In GC, numerous studies have noted its association with NET (76-78). One study, which reported both patient clinical data and in vitro experiments, showed that NET were more prevalent in the blood and tumor tissues of GC patients than in comparable tissues of healthy individuals. In the same study, gastric tumor cells increased the formation of NET in vitro more than healthy cells from the mucosa. Additionally, the authors showed that these NET promoted platelet activation (79).

CRC has been associated with leukocytosis. Some studies have shown that cancer patients with elevated leukocyte counts also present high levels of myeloid growth factors (G-CSF and GM-CSF). Leukocytosis has been associated to VTE, based on capacity of cells such as neutrophils or monocytes to release NET or overexpress TF, respectively (74). These GI cancer-specific thrombogenic mechanisms are summarized in Figure 7.

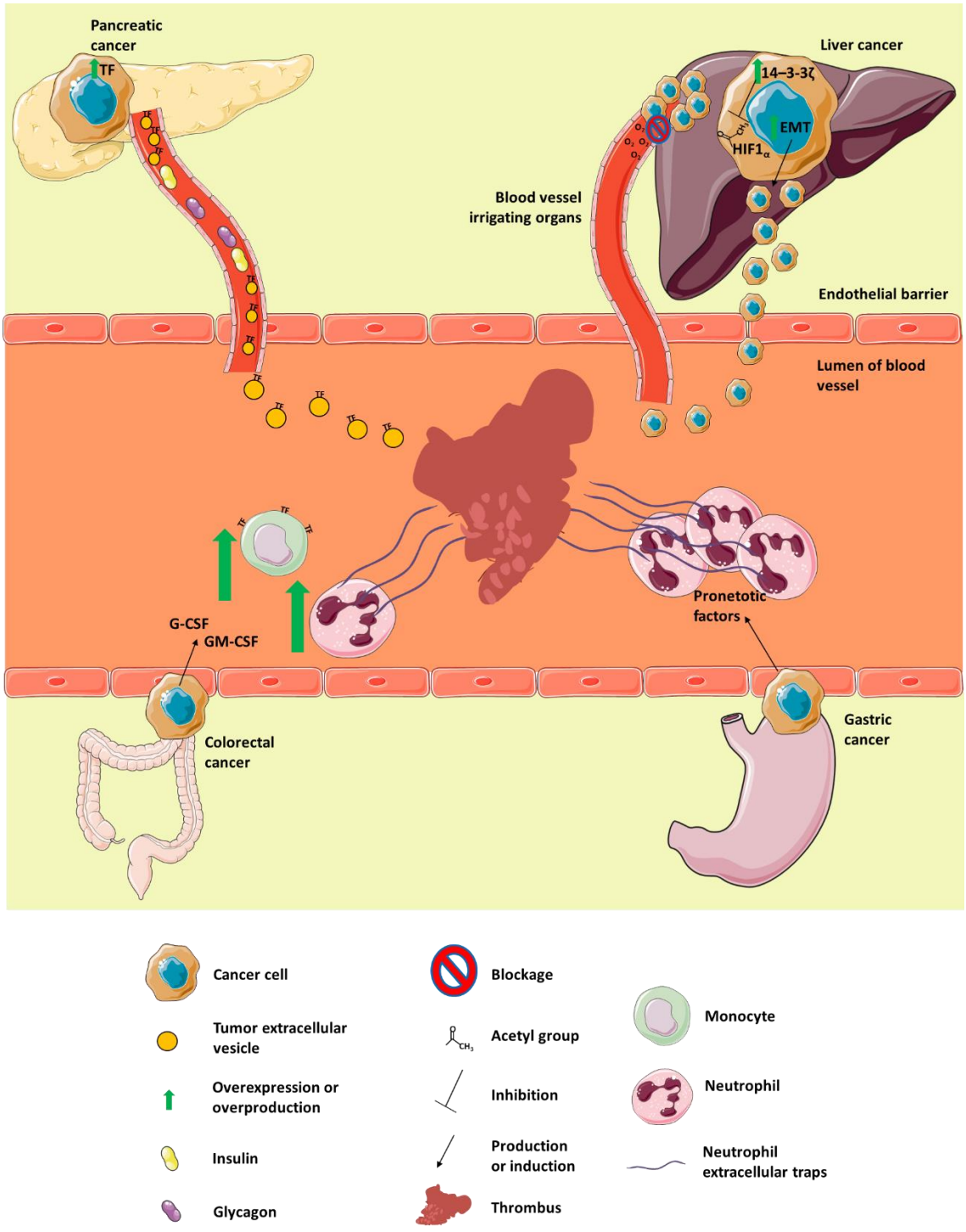

Figure 7. Gastrointestinal cancer-specific thrombogenic mechanisms. Given the high vascularization of pancreatic cancer, related to its endocrine role, tumor cells can readily export their tissue factor-bearing microparticles into the bloodstream. In liver cancer, tumor cells-induced hypoxia promotes upregulation of proteins that prevent acetylation and degradation of hypoxia inducible factor alpha (HIF1α). This stabilization of HIF1α induces overexpression of epithelial-mesenchymal transition genes, increasing vascular invasion of cancer cells. In colorectal cancer, tumor cells release myeloid growth factors that elevate blood levels of monocytes or neutrophils, potentially promoting thrombosis through tissue factor expression or neutrophil extracellular traps, respectively. Gastric

cancer cells are associated with the promotion of neutrophil extracellular traps. *EMT*: epithelial-mesenchymal transition genes; *G-CSF*: granulocyte colony stimulating factor; *GM-CSF*: granulocyte-monocyte colony stimulating factor; *HIF1$_\alpha$*: hypoxia-inducible factor alpha; O_2: oxygen; *TF*: tissue factor; *14–3-3ζ*: tryptophan 5-monooxygenase activation protein. Figure created with *Power Point* and *Servier Medical ART*.

Regarding VTE prevention in GI cancer patients, recent studies are focused on the use of LMWH and DOAC. In a cohort of 1,172 patients with digestive tumors, use of LMWH significantly reduced the incidence of VTE (relative risk = 0.52; p-value = 0.04), compared to untreated patients (80). In another study of 130 patients with GI cancer, apixaban significantly reduced the 6-month cumulative incidence of VTE, with a hazard ratio of 0.27 (p-value < 0.01) (81). However, PTP in GI cancer carries the same limitations as in general cancer population, with a heightened risk of bleeding becoming particularly significant in patients with digestive neoplasms.

As previously mentioned, DOAC are overshadowing LMWH treatment due to their easier administration and generally more effective prevention of VTE. Nevertheless, in GI cancer patients, it has been shown that DOAC significantly increase the risk of bleeding compared to heparins-based treatments. In the Hokusai VTE Cancer Trial, 13.20% of patients receiving edoxaban suffered a major bleeding event during follow-up, compared to 2.40% in those treated with dalteparin (p-value = 0.02). Similarly, in the "Anticoagulation therapy in selected cancer patients at risk of recurrence of venous thromboembolism study", which included esophageal cancer patients, major bleeding occurred in 36% of those treated with rivaroxaban compared to 5% in the dalteparin-treated group (82).

GI cancers are thus marked by a high simultaneous risk of bleeding and VTE. While PTP can reduce the risk of VTE, it may also increase the risk of hemorrhages. For this reason, it is crucial to identify risk factors to select GI cancer patients with the highest risk of thrombosis, for whom anticoagulation would be warranted despite the risk of bleeding. This need varies by primary tumor site. For instance, in pancreatic cancer, there is little controversy over anticoagulation due to its high risk of VTE, making both DOAC and LMWH highly effective, with a low number needed to treat for benefit from thromboprophylaxis (67). However, for other GI cancers with lower risk of VTE, the benefit is less clear. In three different studies involving 1,932 patients with GC or CRC, neither DOAC nor LMWH significantly reduced VTE risk, and the number of patients needed to treat for benefit from anticoagulation was 78 (83).

For this reason, this thesis focuses on two different GI cancers-CRC and GC-that carry a VTE risk high enough to worsen prognosis, but not sufficient to justify routine thromboprophylaxis. Patients with these tumors would benefit from the identification of new thrombotic biomarkers to help select individuals at the highest VTE risk, to whom PTP should be administered despite the associated risk of bleeding.

2.2. CANCER-ASSOCIATED THROMBOSIS IN COLORECTAL CANCER

In the ranking of most frequent neoplasms, CRC is the second most commonly diagnosed cancer in the world, with 10% of detected tumors originating in the colon and rectum. It ranks as the second most frequent cancer in women and the third in men, although it is approximately 25% more prevalent in men in absolute numbers. The highest incidence of CRC is seen in the most developed countries, although its diagnosis is also increasing in developing regions (84). This GI tumor is responsible for approximately 900,000 deaths annually, making it the fourth most deadly tumor worldwide. In addition, the prognosis for patients varies considerably depending on the tumor stage, with a 5-year overall survival rate exceeding 90% for stage I and slightly over 10% for stage IV (84, 85). Although CRC is associated with a lower thrombotic risk compared to other GI tumors such as liver, gastric or pancreatic cancers, its incidence of thromboembolic events remains high, and it worsens prognosis of patients. In a study conducted in the Netherlands, involving 68,238 CRC patients and 136,476 matched controls, the 1-year cumulative incidence of VTE was 1.93% for tumor patients versus 0.24% for controls, while the 1-year cumulative incidence of ATEE was 2.74% for CRC patients compared to 1.88% for controls. Both types of thrombotic events significantly increased mortality (hazard ratios of 3.68 and 3.05 for VTE and ATEE, respectively, compared to controls) (86). In a Chinese study published in 2023, the incidence of short-term VTE after CRC diagnosis in 1,836 patients was 11.20% (87). Another study involving 68,142 CRC patients from California reported a 24-month cumulative incidence of VTE of 3.10%, with a higher incidence of 5% observed at 6 months after cancer diagnosis. This study also concluded that VTE significantly reduced 1-year overall survival (88). In a study conducted in South Korea, involving 12,093 CRC patients undergoing chemotherapy, the 6-month cumulative incidence of VTE and ATEE was 3.28% and 0.32%, respectively (89). A different Asian study involving CRC patients in 2006 found that VTE increased 2-year mortality, with a significant hazard ratio of 4.20% (90). In summary, CRC is associated with a high incidence of thrombotic events that increase the mortality rate among patients.

Apart from its well-known implication in overall survival (85), tumor stage is also a key risk factor for thrombosis in CRC. In the previously mentioned Asian study (90), the 2-year cumulative incidence of VTE was 0.30%, 0.90%, 1.40% and 6.40% for tumor stages I, II, III and IV, respectively. Similarly, the mentioned study in the Netherlands (86) observed this trend. Using stage I as the reference level, hazard ratios for 1-year VTE for stages II, III and IV were 1.75, 2.70 and 6.31 (all with p-value < 0.05), respectively. For ATEE, these hazard ratios were 1.15, 1.19 and 1.30 (all with p-value < 0.05), respectively. In another study involving 516 CRC patients diagnosed with stages II and III, researchers found that metastatic relapse after primary tumor surgery significantly increased the risk of VTE (hazard ratio = 13.03, 95% CI, 4.39 - 38.74) (91). Thus, CRC

invasion and progression increases thrombotic risk in patients, and all these complications worsen their prognosis.

Regarding strategies focused on preventing thrombosis in CRC, PTP is one of the most commonly used; however, its low efficacy and increased risk of bleeding make this therapy controversial. In a review of 7 studies including 5,302 CRC patients, VTE incidence in patients treated and untreated with LMWH was 1.10% and 1.90%, respectively, with no significant differences. The review also indicated that using PTP resulted in an overall incidence of bleeding complications of 7.80%, compared with 0% in patients not receiving LMWH (92). Another study of 121 patients with CRC reported a 1-month cumulative incidence of VTE of 12.30% and 11.90% for patients treated and untreated (respectively) with enoxaparin. In addition, 1.80% of patients on LMWH suffered a bleeding event, compared to 0% among those not on PTP (93). In a study involving 950 CRC patients treated with either enoxaparin or nadroparin following tumor surgery, VTE rates remained high (12.60 – 15.90%), alongside a substantial risk of major bleeding (7.30 – 11.50%) (94). DOAC are generally more effective than LMWH for preventing VTE in CRC. For example, in "Prophylaxis of VTE after laparoscopic surgery for CRC study II", involving 582 patients, incidence of VTE in patients treated and untreated with rivaroxaban was 1.00 and 3.90%, respectively (p-value = 0.03) (95). However, DOAC also carry a considerable risk of bleeding, even exceeding that of LMWH. In a study of 1,019 CRC patients undergoing endoscopic submucosal dissection, DOAC such as dabigatran, apixaban, edoxaban and rivaroxaban reduced the rate of thromboembolic events to 0.29%. Yet, bleeding rates ranged from 7.21% to 18.26%, depending on the DOAC used, similar to the bleeding rate observed with warfarin (11.76%) (96). In a study involving 498 CRC patients, the use of low doses of rivaroxaban in 363 patients reduced the 2-month incidence of VTE to 0.60%. However, the risk of bleeding events for these patients was 7.20%, double that of patients not on anticoagulation (97). As exposed in this paragraph, use of PTP in CRC is often inefficient, and the significant increase of bleeding risk creates considerable controversy among clinicians regarding the use of anticoagulants in this high-risk population. Consequently, various thrombotic risk markers have been explored over time to identify CRC patients for whom PTP may be beneficial.

Regarding thrombosis risk scores, one of the main limitations in predicting incidence of the disease within the same cancer type, is the omission of tumor site variable, which is commonly used in scores such as Khorana, Vienna and PROTECHT (Table 4). Thus, these models lose effectiveness when predicting VTE incidence in CRC. For example, in a study carried out in 1,380 CRC patients, only 0.20% were classified as high-risk for VTE according to the Khorana score. In addition, the incidence of VTE events was similar between the intermediate-risk (rate of VTE = 6.40%) and low-risk (rate of VTE = 4.80%) groups (98). This limitation may be addressed by using models with a larger number of variables. The Caprini risk score, for instance, includes more than 25 variables (e.g., history of inflammatory bowel disease, age over 75, planned major surgery, serious trauma…), and can stratify patients into four different VTE risk groups (99). In a study of

148 patients with CRC, the Caprini score effectively stratified groups with significantly different risk of DVT, achieving a p-value of less than 0.01 and an area under the receiver operating characteristic curve (AUC) of 0.70 (100). Another useful model for predicting VTE in CRC is derived from the study "Prospective comparison of methods for thromboembolic risk assessment with clinical perceptions and awareness in real life patients–cancer associated thrombosis". This score includes factors such as anthracycline- or anti-hormonal-based therapies, time since tumor diagnosis, catheters in the central venous system, cancer stage, previous thrombosis risk factors, recent hospital admission due to an acute medical condition, history of VTE, and platelet count. According to these variables, CRC patients classified as low-intermediate-risk and high-risk exhibited 6-month VTE rates of 1.70 and 13.30%, respectively. In addition, the AUC for VTE prediction with this score was 0.85 (101). Aiming to improve the thrombosis risk scores based on clinical variables in CRC, research has focused on combining them with biomarkers. For example, D-dimer, a marker of fibrin formation and degradation, has shown to enhance the Caprini risk score. In a study of 171 CRC patients, the AUC for DVT prediction was 0.79 for Caprini score, 0.74 for D-dimer alone, and 0.87 when combined (102). In another study involving 80 CRC patients, the use of thrombodynamic test (measuring parameters such as clot formation velocity, size of the thrombus and clot density) further improved the Caprini score's predictive power for VTE risk. In this study, the AUC for the Caprini model alone and combined with thrombodynamic test was 0.84 and 0.92, respectively (103). Over time, the number of studies focused on thrombosis biomarkers in CRC has increased. A study involving 166 CRC patients found that *KRAS* mutations significantly elevated VTE risk (odds ratio = 2.76, 95% CI, 1.55 – 4.90) throughout follow-up since cancer diagnosis (104). Similar findings were reported by Ades and colleagues in 2015 (105). In their study of 172 metastatic CRC patients, VTE incidence at 6 months post-diagnosis, or any time thereafter, was significantly higher among patients with mutated *KRAS* compared to wild-type patients (odds ratio = 2.21, 95% CI, 1.08 - 4.53). Other VTE biomarkers include proteins measured in the primary tumor. High expression of both alpha-1-antitrypsin and regenerating islet-derived protein 4 was significantly associated with VTE risk in a cohort of 418 CRC patients (106). CRC-associated thrombosis can also be predicted through circulating plasma proteins, such as prothrombin fragment 1+2 and thrombin-antithrombin complexes, which are significantly elevated in CRC patients compared to individuals with benign colorectal diseases. These proteins predict DVT risk throughout follow-up since CRC diagnosis (107). A summary of the different tools used to predict thrombosis in CRC is shown in Figure 8.

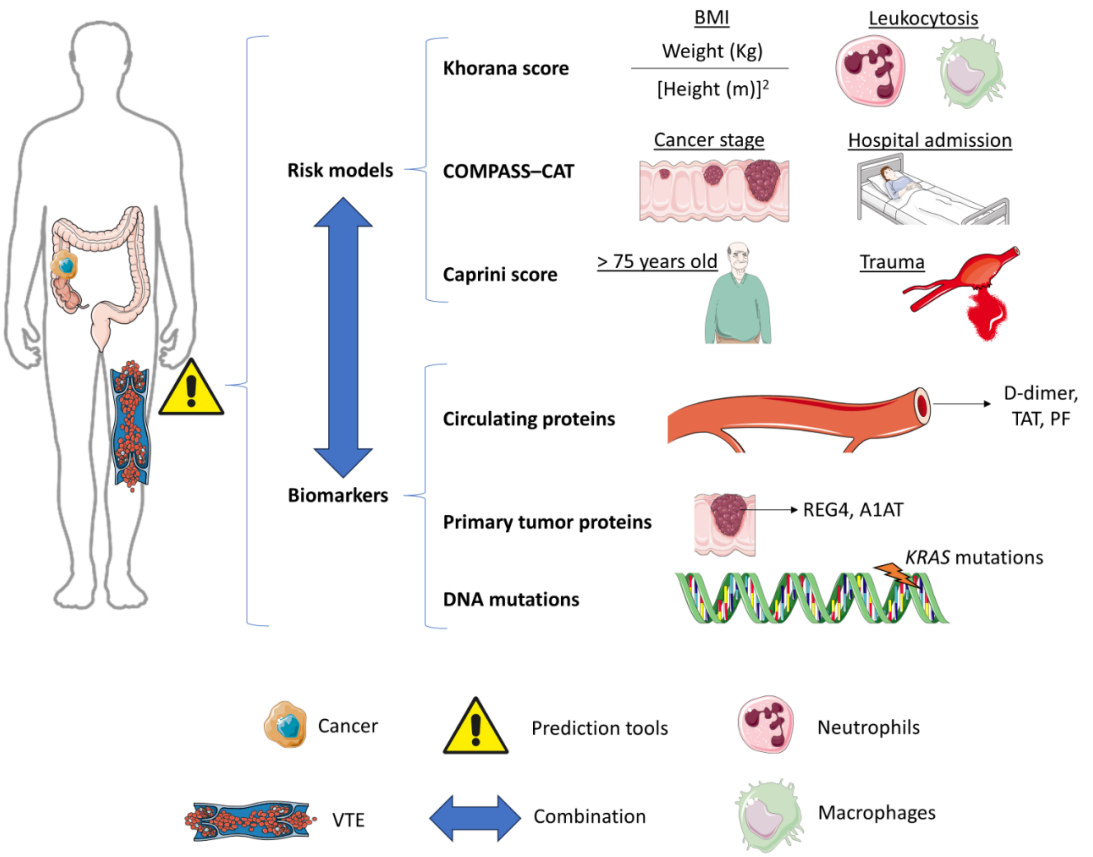

Figure 8. Predictors of venous thromboembolism in colorectal cancer patients. This figure shows the different clinical risk models and biomarkers that have been used over time to predict a high risk of venous thromboembolism in colorectal cancer patients. Risk models rely on clinical variables such as age, leukocyte count or tumor stage, while biomarkers typically involve specific protein levels or DNA variants. To enhance risk scores based on clinical variables, biomarkers are usually incorporated into these models, thus improving their predictive accuracy. *A1AT*: alpha-1-antitrypsin; *BMI*: body mass index; *COMPASS-CAT*: prospective comparison of methods for thromboembolic risk assessment with clinical perceptions and awareness in real life patients–cancer associated thrombosis; *DNA*: deoxyribonucleic acid; *KRAS*: kirsten rat sarcoma viral oncogene homolog; *PF*: prothrombin fragment 1+2; *REG4*: regenerating islet-derived protein 4; *TAT*: thrombin-antithrombin complexes; *VTE*: venous thromboembolism. Figure created with *Power Point* and *Servier Medical ART*.

The identification of new thrombotic biomarkers in CRC is a valuable strategy for better selecting who should receive PTP treatment. Furthermore, understanding the mechanisms by which these biomarkers induce a hypercoagulable state could aid in identifying new therapeutic targets and drugs to complement existing anticoagulants, which often remain ineffective at preventing thrombosis in CRC. Supporting the previously noted relationship between tumor stage and thrombosis in CRC (86, 90, 91), various targets are known to promote both prothrombotic effect and a more aggressive tumor phenotype. Thus, targeting these factors could provide a dual benefit by mitigating both cancer progression and thrombosis in CRC. For example, high platelet count is well-known to correlate with

VTE in different cancers, including CRC (47-51). Furthermore, thrombocytosis has been linked to tumor growth, poor prognosis and increased mortality in CRC patients (108, 109). In vitro studies have shown that EV released by platelets from CRC patients, increase the prometastatic and prothrombotic phenotype of CRC cells (110). Thus, targeting platelets in this cancer type could potentially prevent both VTE and metastasis. A 2016 study found that in vitro co-culture of physiological platelets with a CRC cell line led to a more invasive phenotype in tumor cells, while platelets were more prone to aggregate. However, these effects were suppressed by different anti-platelet agents, such as aspirin and ticagrelor (111). Another example of target is TF, a procoagulant protein known to be associated with VTE across different cancer types (25). In CRC patients, TF is overexpressed and correlates with advanced tumor stage and metastasis to distant organs, such as the liver. Furthermore, high TF levels are also linked to poor prognosis. TF's protumor effects are believed to stem from its ability to activate protease-activated receptor-2 (PAR-2), which triggers signaling pathways that promote migration, proliferation and angiogenesis in cancer cells. Given the protumor and prothrombotic effects of TF, different potential inhibitors are under study, as they could have a positive effect on the prognosis of tumors overexpressing TF, such as CRC (112).

In the context of thrombosis risk stratification and prevention of this comorbidity in CRC, it would be worthwhile to expand the arsenal of biomarkers to better identify high–risk patients, as well as developing treatments targeting factors that drive prothrombotic and prometastatic effects. In this context, our group focused on a type-II transmembrane serine protease called hepsin (HPN) (113). This protein was selected for its potential as both a biomarker and a therapeutic target for thrombosis and metastasis. According to literature, HPN can activate coagulation factor VII, which together with TF, is the responsible for the initiation of extrinsic coagulation pathway (114, 115). This is consistent with HPN's function as a serine protease, as many proteins belonging to this group play roles in the coagulation cascade that leads to fibrin formation (116). HPN overexpression has been observed in several cancer types, and it is associated with tumor progression, invasion and poor prognosis (117-119). In CRC, data on these effects are sparse, although one study found that HPN serum levels were significantly higher among metastatic CRC patients compared to those with localized disease (120). Based on this background, HPN could serve as a useful biomarker for both thrombosis risk and cancer progression in CRC. Beyond its potential use as a predictive tool, targeting HPN could also help prevent thromboembolic events and metastasis in CRC patients, addressing two of the main causes of mortality in cancer patients.

2.3. CANCER-ASSOCIATED THROMBOSIS IN GASTRIC CANCER

According to International agency for research on cancer "Global cancer observatory project", in 2018, GC was diagnosed in 1,033,701 patients worldwide, and it provoked 782,685 deaths. Nowadays, this GI tumor ranks among the top five most common and

deadly cancers. Globally, GC is rare among individuals under 50 years old, with incidence rising from this age and reaching a plateau between ages 55 and 80. GC is more common in men than in women, ranking as the fourth most frequent in men and the seventh in women (121). The incidence and mortality associated to GC vary significantly across different regions of the world. East Asia has a particularly high prevalence of GC. For example, in China, GC is the third most frequent and deadly tumor among all cancer types, accounting for 44.00% of global new diagnosis of GC and 48.60% of worldwide GC deaths (122). In terms of prognosis, GC is associated with a low survival rate. Globally, the 5-year overall survival is around 20% (123). As with many other tumors, this parameter depends on cancer stage: up to 70% of patients with early stage GC are still alive 5 years post-diagnosis, while the 5-year survival for those with advanced stages drops to about 4% (124). Regarding thrombosis risk, GC is among the five most thrombogenic cancers worldwide (19). This comorbidity worsens patient prognosis, and its incidence depends on cancer stage. In a study involving 2,085 GC patients, the cumulative 24-month VTE incidence was 3.80%, though this varied significantly by tumor stage: 0.50, 3.30, 3.60 and 24.40% for stages I, II, III and metastatic stage, respectively. In addition, VTE events occurring within the first year post-diagnosis significantly increased early mortality (hazard ratio = 1.90, 95% CI, 1.10 - 3.20), and this effect remained significant for stage IV patients when analyzed separately (125). In another study with 3,095 advanced gastric cancer (AGC) patients, the 1-year cumulative incidence of VTE was 3.50%, and this comorbidity significantly reduced overall survival, with median survival of 4.50 months for patients with a VTE event, compared to 10.70 months for those without a VTE (126). A 2013 study involving 375 GC patients reported a 1-month VTE incidence of 2.40%, with rates of 1.40, 2.40 and 9.70% for stages I, II-III and IV, respectively (127). Another study with 964 AGC patients found a VTE cumulative incidence of 10.10% at 1-year post-diagnosis, and these events significantly worsened overall survival (128). Overall, these studies indicate that among GC patients, the highest VTE rates are observed in those with the most advanced stages.

Taking into account the high risk of VTE in AGC and its negative impact on prognosis, prevention of this comorbidity would likely benefit patients. However, anticoagulation treatment is controversial as development of AGC is associated with a high rate of bleeding events, which usually reduce patients´ quality of life (129). This tumor's inherent risk of hemorrhages is further increased by anticoagulants. For instance, in a study of 188 AGC patients, 18% suffered a VTE during chemotherapy. Of these patients, 71% were treated with DOAC, but 54% of them had to discontinue treatment because of a bleeding event (130). Another study involving 192 AGC patients found that the use of antiplatelet or other anticoagulant agents significantly increased the risk of bleeding (odds ratio = 3.22, p-value = <0.01). This comorbidity significantly reduced median overall survival, being 6.50 months for patients with bleeding compared to 18.50 months for those without bleeding (131). More examples of anticoagulation-induced bleeding are displayed in the literature. In one study recruiting 340 GC patients, those pretreated with aspirin and/or unfractionated heparin had a significantly higher rate of bleeding (8.10%) throughout follow-up compared to patients without PTP (0.70%). In addition, incidence

of thromboembolic events was similar between the two groups (132). In another article published in 2021, the authors recruited 176 patients with GC, of whom 89 were treated with rivaroxaban and 87 with placebo. During follow-up, 3.90% of the rivaroxaban group and 6.90% of the placebo group suffered a VTE. However, this positive effect of rivaroxaban was overshadowed by its adverse effect on bleeding, with hemorrhage rate being 3 times higher in the rivaroxaban group compared to the placebo group (133). In this context, risk of bleeding leads many clinicians to avoid the use of PTP in AGC patients, thus leaving them exposed to a high risk of thrombosis. In another study involving 671 patients, 150 (22%) suffered a VTE event, and of these, more than 80% had not received PTP (134).

Taking into account this bidirectional effect of AGC on thrombosis and bleeding, many studies are focused on developing VTE predictive models, using different clinical markers and biomarkers, to identify patients at the highest VTE risk, who should receive anticoagulation despite the risk of bleeding. As with CRC, clinical scores that stratify thrombosis risk by primary tumor site are not useful for determining VTE risk in a population with the same cancer type. Thus, even when Khorana, Vienna or PROTECHT scores classify GC as one of the most thrombogenic neoplasms (Table 4), they fail to distinguish between different groups of VTE risk within the AGC population. For this reason, new clinical and biological parameters have been studied as alternatives ways to predict VTE. In a study involving 2,129 AGC patients, the cumulative incidence of VTE at 3- and 6-month post-diagnosis was 5.70% and 8.20%, respectively. Among baseline clinical variables, high tumor burden and cisplatin-based treatment were significantly associated with VTE occurrence in the first 2 to 3 months of follow-up, but this effect diminished in the following months. In addition, the presence of signet-ring cells consistently predicted VTE risk, with an associated cumulative sub-hazard ratio of 1.47 (95% CI, 1.06-2.05) (135). In another study with 671 GC patients, 150 of whom suffered a VTE during follow-up, metastatic patients who received multiple lines of chemotherapy had a significantly higher incidence of VTE (48.20%) compared to those with a single line of chemotherapy (19.40%) (134). In one article published in 2018, involving 188 AGC patients, low levels of serum albumin were significantly correlated with VTE incidence (p-value = 0.01) (130). To address limitations in existing VTE prediction models, recent studies based on machine learning have combined single clinical variables and biomarkers to establish new thrombosis risk models for GC. In 2023, Xu and colleagues used machine learning algorithms to build different prediction models based on 11 variables collected from 3,092 GC patients. This approach yielded a VTE prediction model using tumor stage, prior blood transfusion, age, D-dimer levels and other fibrinogen degradation derivatives. The AUC of this model was 0.83 (136). This work exemplifies VTE prediction based on a combination of clinical variables and biomarkers. Over time, research has continued to explore biomarkers to complement the limitations of clinical parameters. For instance, in a study of 241 AGC patients, the 1-year cumulative incidence of VTE was 12.40%. In this work, baseline D-dimer levels were associated with VTE risk (hazard ratio = 1.32, 95% CI, 1.00 - 1.75, p-value = 0.05) (137). In another study of 126 GC patients, the authors aimed to identify biomarkers of VTE

during the post-surgery period. Four percent of patients suffered a VTE during this interval, with significantly higher D-dimer and soluble fibrin levels on the first day post-surgery compared to the rest of patients. The AUC for D-dimer and fibrin was 0.97 and 0.87, respectively (138). Additionally, many studies support the observation of increased NET formation in GC patients compared to healthy controls, highlighting its role in promoting a hypercoagulable state (44, 79, 139). A summary of different predictive tools of VTE in GC are displayed in Figure 9.

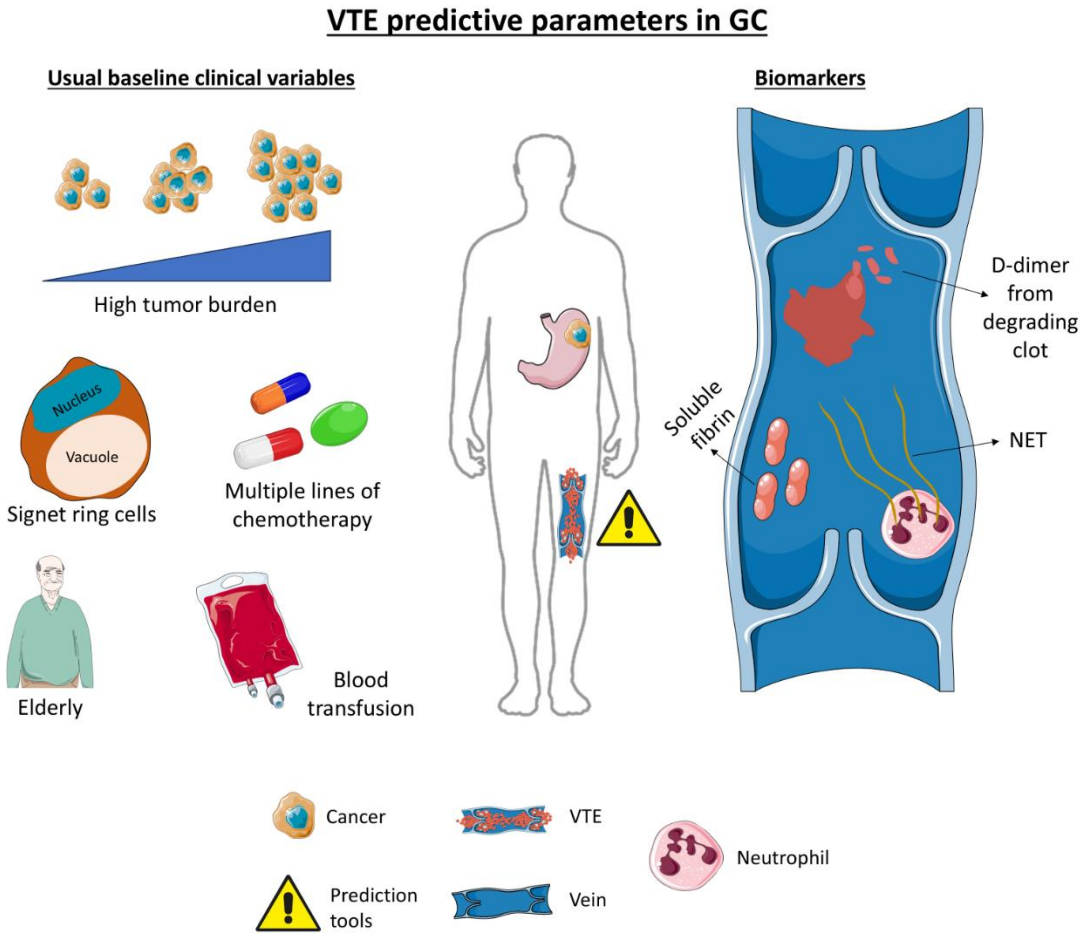

Figure 9. Predictors of venous thromboembolism in gastric cancer patients. This figure shows the different prediction tools developed over time to predict a high risk of venous thromboembolism in gastric cancer patients. Clinical parameters, typically measured at cancer diagnosis, can be distinguished from biomarkers that are collected from bloodstream or other parts of the body. *GC*: gastric cancer; *NET*: neutrophil extracellular traps; *VTE*: venous thromboembolism. Figure created with *Power Point* and *Servier Medical ART*.

Despite the different tools developed to predict VTE in GC patients, AGC remains a highly thrombogenic tumor where the use of PTP is unclear, as those tools do not predict a thrombosis risk high enough to justify anticoagulation. For this reason, further research is needed to identify new biomarkers that address the limitations of existing markers.

3. HYPOTHESIS

In the context of CRC, HPN could serve as a biomarker for both thrombosis and cancer progression, because of its ability to activate coagulation factor VII and to promote invasion across various cancer types, respectively. In addition, understanding the underlying mechanisms by which HPN promotes thrombus formation and cancer invasion could advance research on HPN inhibitors, potentially preventing both thrombosis and cancer progression in CRC patients, thereby improving their prognosis. Taking into account these hypotheses, studies on HPN could simultaneously address two severe and interrelated complications, as tumor stage is associated with thrombosis occurrence in CRC.

Regarding AGC, current clinical models do not predict a high enough VTE risk to justify PTP. Over the years, the discovery of new thrombotic biomarkers has enhanced the predictive accuracy of these models. Thus, identifying novel VTE-related molecules could aid in stratifying patients at significantly high risk and, in the future, further enhance predictive clinical models.

OBJECTIVES

OBJECTIVES

Due to the simultaneous high risks of thrombosis and bleeding in CRC and AGC, the administration of PTP in patients with these GI tumors is highly controversial. **The main objective of this thesis** was to identify new thrombotic biomarkers to help stratify patients at the highest VTE risk, for whom anticoagulation should be administered despite the risk of hemorrhage. Regarding CRC, this thesis focused on HPN as a potential biomarker for both thrombosis and cancer progression. In the case of AGC, the aim was to identify new genes associated with the hypercoagulable state in these patients. Thus, the specific objectives of this thesis are:

1. To study HPN as a predictor of thrombosis and cancer progression (Article 1).

2. To investigate the cellular mechanisms by which HPN promotes thrombosis and progression (Article 2).

3. To identify new HPN inhibitors that suppress its effects (Articles 2 & 3).

4. To identify new genes whose expression is related to VTE occurrence in AGC patients (Article 4).

5. To validate these genes as predictors of VTE risk in a new cohort of AGC patients, and establish a potential gene expression signature (Article 5).

ARTICLES OF THE THESIS

1. IMPLICATION OF HEPSIN FROM PRIMARY TUMOR IN THE PROGNOSIS OF COLORECTAL CANCER PATIENTS

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JOURNAL NAME

Cancers

ABSTRACT

Hepsin is a type II transmembrane serine protease whose deregulation promotes tumor invasion by proteolysis of the pericellular components. In colorectal cancer, the implication of hepsin is unknown. Consequently, we aimed to study the correlations between hepsin expression and different clinical-histopathological variables in 169 patients with localized colorectal cancer and 118 with metastases. Tissue microarrays were produced from samples at diagnosis of primary tumors and stained with an anti-hepsin antibody. Hepsin expression was correlated with clinical-histopathological variables by using the chi-square and Kruskal–Wallis tests, Kaplan–Meier and Aalen–Johansen estimators, and Cox and Fine and Gray multivariate models. In localized cancer patients, high-intensity hepsin staining was associated with reduced 5-year disease-free survival (p-value = 0.16). Medium and high intensity of hepsin expression versus low expression was associated with an increased risk of metastatic relapse (hazard ratio 2.83, p-value = 0.035 and hazard ratio 3.30, p-value = 0.012, respectively), being a better prognostic factor than classic histological variables. Additionally, in patients with

localized tumor, 5-year thrombosis cumulative incidence increased with the increment of hepsin expression (p-value = 0.038). Medium and high intensities of hepsin with respect to low intensity were associated with an increase in thrombotic risk (hazard ratio 7.71, p-value = 0.043 and hazard ratio 9.02, p-value = 0.028, respectively). This relationship was independent of previous tumor relapse (p-value = 0.036). Among metastatic patients, low hepsin expression was associated with a low degree of tumor differentiation (p-value < 0.001) and with major metastatic dissemination (p-value = 0.023). Hepsin is a potential thrombotic and metastatic biomarker in patients with localized colorectal cancer. In metastatic patients, hepsin behaves in a paradoxical way with respect to differentiation and invasion processes.

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2. VENETOCLAX IS A POTENT HEPSIN INHIBITOR THAT REDUCES THE METASTATIC AND PROTHROMBOTIC PHENOTYPES OF HEPSIN-EXPRESSING COLORECTAL CANCER CELLS

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JOURNAL NAME

Frontiers in Molecular Biosciences

ABSTRACT

Introduction: Hepsin is a type II transmembrane serine protease and its expression has been linked to greater tumorigenicity and worse prognosis in different tumors. Recently, our group demonstrated that high hepsin levels from primary tumor were associated with a higher risk of metastasis and thrombosis in localized colorectal cancer patients. This study aims to explore the molecular role of hepsin in colorectal cancer. Methods: Hepsin levels in plasma from resected and metastatic colorectal cancer patients were analyzed by ELISA. The effect of hepsin levels on cell migration, invasion, and proliferation, as well as on the activation of crucial cancer signaling pathways, was performed *in vitro* using colorectal cancer cells. A thrombin generation assay determined the procoagulant function of hepsin from these cells. A virtual screening of a database containing more than 2000 FDA-approved compounds was performed to screen hepsin inhibitors, and selected compounds were tested *in vitro* for their ability to suppress hepsin effects in colorectal cancer cells. Xenotransplantation assays were done in zebrafish larvae to study the impact of venetoclax on invasion promoted by hepsin. Results: Our results showed higher plasma hepsin levels in metastatic patients, among which, hepsin was higher in those suffering thrombosis. Hepsin overexpression increased colorectal cancer cell invasion, Erk1/2 and STAT3 phosphorylation, and thrombin generation in plasma. In addition, we identified venetoclax as a potent hepsin inhibitor that reduced the metastatic and prothrombotic phenotypes of hepsin-expressing colorectal cancer cells. Interestingly, pretreatment with Venetoclax of cells overexpressing hepsin reduced their invasiveness *in vivo*. Discussion: Our results demonstrate that hepsin overexpression correlates with a more aggressive and prothrombotic tumor phenotype. Likewise, they demonstrate the antitumor role of venetoclax as a hepsin inhibitor, laying the groundwork for molecular-targeted therapy for colorectal cancer.

URL ADDRESS

https://www.frontiersin.org/journals/molecular-biosciences/articles/10.3389/fmolb.2023.1182925/full

3. SURAMIN, A DRUG FOR THE TREATMENT OF TRYPANOSOMIASIS, REDUCES THE PROTHROMBOTIC AND METASTATIC PHENOTYPES OF COLORECTAL CANCER CELLS BY INHIBITING HEPSIN

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JOURNAL NAME

Biomedicine & Pharmacotherapy

ABSTRACT

Recently, our group identified serine-protease hepsin from primary tumor as a biomarker of metastasis and thrombosis in patients with localized colorectal cancer. We described hepsin promotes invasion and thrombin generation of colorectal cancer cells in vitro and in vivo and identified venetoclax as a hepsin inhibitor that suppresses these effects. Now, we aspire to identify additional hepsin inhibitors, aiming to broaden the therapeutic choices for targeted intervention in colorectal cancer. Methods: We developed a virtual screening based on molecular docking between the hepsin active site and 2000 compounds from DrugBank. The most promising drug was validated in a hepsin activity

assay. Subsequently, we measured the hepsin inhibitor effect on colorectal cancer cells with basal or overexpression of hepsin via wound-healing, gelatin matrix invasion, and plasma thrombin generation assays. Finally, a zebrafish model determined whether hepsin inhibition reduced the invasion of colorectal cancer cells overexpressing hepsin. Results: Suramin was the most potent hepsin inhibitor (docking score: -11.9691 Kcal/mol), with an IC50 of 0.66 µM. In Caco-2 cells with basal or overexpression of hepsin, suramin decreased migration and significantly reduced invasion and thrombin generation. Suramin did not reduce the thrombotic phenotype in the hepsin-negative colorectal cancer cells HCT-116 and DLD-1. Finally, suramin significantly reduced the in vivo invasion of Caco-2 cells overexpressing hepsin. Conclusion: Suramin is a novel hepsin inhibitor that reduces its protumorigenic and prothrombotic effects in colorectal cancer cells. This suggests the possibility of repurposing suramin and its derivatives to augment the repertoire of molecular targeted therapies against colorectal cancer.

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https://www.clinicalkey.es/#!/content/playContent/1-s2.0-S0753332223016128?returnurl=https:%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS0753332223016128%3Fshowall%3Dtrue&referrer=https:%2F%2Fpubmed.ncbi.nlm.nih.gov%2F

4. IDENTIFICATION OF THROMBOSIS-RELATED GENES IN PATIENTS WITH ADVANCED GASTRIC CANCER: DATA FROM AGAMENON-SEOM REGISTRY

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JOURNAL NAME

Biomedicines

ABSTRACT

Advanced gastric cancer is one of the most thrombogenic neoplasms. However, genetic mechanisms underlying this complication remain obscure, and the molecular and histological heterogeneity of this neoplasm hinder the identification of thrombotic biomarkers. Therefore, our main objective was to identify genes related to thrombosis regardless of Lauren subtypes. Furthermore, in a secondary exploratory study, we seek to discover thrombosis-associated genes that were specific to each TCGA molecular subtype. We designed a nested case-control study using the cohort of the AGAMENON national advanced gastric cancer registry. Ninety-seven patients were selected—48 with and 49 without venous thromboembolism (using propensity score matching to adjust for confounding factors)—and a differential gene expression array stratified by Lauren histopathological subtypes was carried out in primary tumor samples. For the secondary objective, the aforementioned differential expression analysis was conducted for each TCGA group. Fifteen genes were determined to be associated with thrombosis with the same expression trend in both the intestinal and diffuse subtypes. In thrombotic subjects, *CRELD1*, *KCNH8*, *CRYGN*, *MAGEB16*, *SAA1*, *ARL11*, *CCDC169*, *TRMT61A*, *RIPPLY3* and *PLA2G6* were underexpressed (adjusted-p<0.05), while *PRKD3*, *MIR5683*, *SDCBP*, *EPS8* and *CDC45* were overexpressed (adjusted-p<0.05), and correlated, by logistic regression, with lower or higher thrombotic risk, respectively, in the overall cohort. In each TCGA molecular subtype, we identified a series of genes differentially expressed in thrombosis that appear to be subtype-specific. We have identified several genes associated with venous thromboembolism in advanced gastric cancer that are common to Lauren intestinal and diffuse subtypes. Should these genetic factors be validated in the future, they could be complemented with existing clinical models to bolster the ability to predict thrombotic risk in individuals with advanced gastric adenocarcinoma.

URL ADDRESS

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5. NOVEL PREDICTORS OF VENOUS THROMBOEMBOLISM RISK IN ADVANCED GASTRIC CANCER: DATA FROM AGAMENON-SEOM REGISTRY

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JOURNAL NAME

Gastric Cancer

ABSTRACT

Background: Venous thromboembolism (VTE) is common in advanced gastric cancer (AGC), but bleeding risk discards thromboprophylaxis. We identified 15 genes whose expression was associated with VTE in AGC. These genes may select patients at enough VTE risk to warrant anticoagulation. Thus, we aimed to validate them as predictors of VTE risk in an independent AGC cohort (44 VTE vs 39 non-VTE patients).

Methods: We measured absolute expression of the 15 genes in the primary tumor using digital PCR. We analyzed expression at cancer diagnosis and its association with VTE occurrence, assessing whether it stratified patients with significantly different VTE risk (Fine&Gray regression). We combined validated genes to improve the prediction model of the best single marker.

Results: EPS8, SDCBP and PRKD3 were significantly overexpressed (Mann-Whitney p-value < 0.050) in patients who developed VTE within the first 6 months after cancer diagnosis. SAA1 was significantly underexpressed in patients with VTE within the first 3 months. Patients with <26.28 copies/µL of SAA1, >347.3 copies/µL of PRKD3 or >385.6 copies/µL of EPS8 at diagnosis had higher risk of VTE (p-value of Fine&Gray regression < 0.050) from the first months (3-6 months) to the first 2 years after diagnosis (SAA1 and PRKD3) or to the end of follow-up (EPS8, 65 months). SAA1 expression improved the prediction model based on EPS8 (AUC of ROC curves = 0.700-0.750 at 6 and 24 months post-diagnosis).

Conclusions: PRKD3 and the combination of EPS8 and SAA1 expression are novel predictors of VTE risk in AGC.

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Under review for publication.

CONCLUSIONS

CONCLUSIONS

1. Hepsin expression in the primary tumor at the time of cancer diagnosis is a potential biomarker for thrombosis risk and metastatic relapse in patients with localized colorectal cancer. In metastatic patients, however, hepsin showed no prognostic value and was, paradoxically, significantly reduced in the most aggressive tumors.

2. In colorectal cancer cells, hepsin increases levels of phosphorylated oncogenic proteins, promotes invasion both *in vitro* and *in vivo*, and contributes to thrombin generation in plasma incubated with tumor cells. These findings may explain prior associations of hepsin in patients with localized colorectal cancer.

3. As a potential therapeutic target, hepsin can be inhibited by Venetoclax (a BCL-2 inhibitor) and Suramin (commonly used for the treatment of trypanosomiasis), both approved by the Food & Drug Administration. These inhibitors suppress the protumor and prothrombotic effects of hepsin in colorectal cancer cells.

4. At the time of cancer diagnosis, the differential expression of 15 genes in the primary tumor is associated with venous thromboembolism occurrence in a cohort of advanced gastric cancer, independently of Lauren histopathological subtype.

5. In a validation cohort of advanced gastric cancer, the expression of *EPS8*, *PRKD3* and *SAA1* (previously associated with venous thromboembolism in the original cohort) in the primary tumor at cancer diagnosis stratify patients at a significantly high risk for venous thromboembolism. As a potential predictive gene signature, the combination of *EPS8* and *SAA1* represents the most accurate predictive model for venous thromboembolism in the validation cohort.

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APPENDIX

1. SCIENTIC PRODUCTION RELATED TO MY THESIS

1.1. PUBLISHED ARTICLES

- **Zaragoza-Huesca D**, Garrido-Rodríguez P, Jimenez-Fonseca P, Martínez de Castro E, Sánchez-Cánovas M, Visa L, Custodio A, Fernández-Montes A, Peñas-Martínez J, Morales Del Burgo P, Gallego J, Luengo-Gil G, Vicente V, Martínez-Martínez I, Carmona-Bayonas A. 2022. Identification of thrombosis-related genes in patients with advanced gastric cancer: data from AGAMENON-SEOM registry. *Biomedicines*. 10(1):148.
- **Zaragoza-Huesca D**, Nieto-Olivares A, García-Molina F, Ricote G, Montenegro S, Sánchez-Cánovas M, Garrido-Rodríguez P, Peñas-Martínez J, Vicente V, Martínez F, Lozano ML, Carmona-Bayonas A, Martínez-Martínez I. 2022. Implication of hepsin from primary tumor in the prognosis of colorectal cancer patients. *Cancers (Basel)*. 14(13):3106.
- Rodenas MC, Peñas-Martínez J, Pardo-Sánchez I, **Zaragoza-Huesca D**, Ortega-Sabater C, Peña-García J, Espín S, Ricote G, Montenegro S, Ayala-De La Peña F, Luengo-Gil G, Nieto A, García-Molina F, Vicente V, Bernardi F, Lozano ML, Mulero V, Pérez-Sánchez H, Carmona-Bayonas A, Martínez-Martínez I. 2023. Venetoclax is a potent hepsin inhibitor that reduces the metastatic and prothrombotic phenotypes of hepsin-expressing colorectal cancer cells. *Front Mol Biosci*. 10:1182925.
- **Zaragoza-Huesca D**, Rodenas MC, Peñas-Martínez J, Pardo-Sánchez I, Peña-García J, Espín S, Ricote G, Nieto A, García-Molina F, Vicente V, Lozano ML, Carmona-Bayonas A, Mulero V, Pérez-Sánchez H, Martínez-Martínez I. 2023. Suramin, a drug for the treatment of trypanosomiasis, reduces the prothrombotic and metastatic phenotypes of colorectal cancer cells by inhibiting hepsin. *Biomed Pharmacother*. 168:115814.
- Under review: **Zaragoza-Huesca D**, Teruel-Montoya R, Carmona-Bayonas A, Peñas-Martínez J, Ricote G, Jimenez-Fonseca P, Visa L, Hernández R, Martínez de Castro E, Fernández-Montes A, Lozano ML, Martínez-Martínez I. 2024. Novel predictors of venous thromboembolism risk in advanced gastric cancer: Data from AGAMENON-SEOM registry. *Gastric Cancer*.

1.2. ORAL COMMUNICATIONS IN CONGRESSES

- Ródenas-Bleda MC, Baroni M, Peñas-Martínez J, Martinelli N, Ortega-Sabater C, Castagna A, Espín-García S, Luengo-Gil G, **Zaragoza-Huesca D**, Corral de la Calle J, Vicente-García V, Carmona-Bayonas A, Bernardi F, Martínez-Martínez I. 2020. Hepsin is involved in the tumorigenicity and in the hipercoagulability of

colon and gastric cancer cells. XXVIII Congress of the International Society on Thrombosis and Haemostasis. Virtual congress.

- Ródenas-Bleda MC, Peñas-Martínez J, Espín-García S, **Zaragoza-Huesca D**, de la Morena-Barrio ME, Corral J, Vicente-García V, Carmona-Bayonas A, Martínez-Martínez I. 2020. Thrombin generation increment by serine-protease hepsin on the gastric and colorectal cancer cells. XXXVI Congress of Sociedad Española de Trombosis y Hemostasia. Virtual congress.
- **Zaragoza-Huesca D**, Garrido-Rodríguez P, Jiménez-Fonseca P, Sánchez-Cánovas M, Visa L, Custodio A, Fernández-Montes A, Peñas-Martínez J, Vicente V, Martínez-Martínez I, Carmona-Bayonas A. 2021. Transcriptomics and identification of potential thrombotic biomarkers in advanced gastric cancer. VI Jornadas Doctorales EIDUM. Virtual congress.
- **Zaragoza-Huesca D**, Ródenas-Bleda MC, Peñas-Martínez J, Pardo-Sánchez I, Ortega-Sabater C, Peña-García J, Espín-García S, Ricote G, Montenegro-Luis S, Ayala de la Peña F, Luengo-Gil G, Nieto A, García-Molina F, Vicente-García V, Bernardi F, Mulero-Méndez V, Pérez-Sánchez H, Carmona-Bayonas A, Martínez-Martínez I. 2021. Pro-tumor and prothrombotic activities of hepsin in colorectal cancer cells and suppression by venetoclax. VI Jornadas IMIB. Murcia, Spain.
- **Zaragoza-Huesca D**, Nieto-Olivares A, García-Molina F, Peñas-Martínez J, Ródenas MC, Pardo-Sánchez I, Ortega-Sabater C, Ricote G, Montenegro S, Sánchez-Cánovas M, Peña-García J, Garrido-Rodríguez P, Vicente V, Martínez F, Lozano ML, Mulero V, Pérez-Sánchez H, Carmona-Bayonas A, Martínez-Martínez I. 2022. Functional and clinical implication of hepsin in the thrombogenesis and tumorigenicity of colorectal cancer. VII Jornadas Doctorales EIDUM. Virtual congress.
- **Zaragoza-Huesca D**, Nieto-Olivares A, García-Molina F, Peñas-Martínez J, Ródenas-Bleda MC, Pardo-Sánchez I, Ortega-Sabater C, Ricote G, Montenegro S, Sánchez-Cánovas M, Peña-García J, Garrido-Rodríguez P, Vicente V, Martínez F, Lozano ML, Mulero V, Pérez-Sánchez H, Carmona-Bayonas A, Martínez-Martínez I. 2022. Plenary session: Clinical and functional implication of hepsin in the tumorigenesis and thrombogenesis of colorectal cancer. XXXVIII Congress of Sociedad Española de Trombosis y Hemostasia. Barcelona, Spain.
- **Zaragoza-Huesca D**, Peñas-Martínez J, Garrido-Rodríguez P, Jiménez-Fonseca P, Martínez de Castro E, Sánchez-Cánovas M, Visa L, Custodio A, Fernández-Montes A, Lozano ML, Martínez C, González-Conejero R, Carmona-Bayonas A, Martínez-Martínez I. 2023. New thrombotic biomarkers and their underlying mechanisms in advanced gastric cancer. XXXIX Congress of Sociedad Española de Trombosis y Hemostasia. Sevilla, Spain.
- **Zaragoza-Huesca D**, Peñas-Martínez J, Teruel R, Ricote G, Carmona-Bayonas A, Fernández-Montes A, Jiménez-Fonseca P, Macia-Rivas L, Morales del Burgo P, Visa L, Hernández R, Martínez de Castro E, Pereira Elorrieta A, de la Morena-Barrio ME, Garrido-Rodríguez P, Lozano ML, Martínez C, González-Conejero R, Martínez-Martínez I. 2024. MIR5683 predicts venous thromboembolism in advanced gastric cancer through regulation of thrombin generation, fibrinolysis and

endothelial TFPI expression. XXXII Congress of the International Society on Thrombosis and Haemostasis. Bangkok, Thailand.

- **Zaragoza-Huesca D**, Peñas-Martínez J, Teruel-Montoya R, Garrido-Rodríguez P, Ricote G, Carmona-Bayonas A, Fernández-Montes A, Jiménez-Fonseca P, Macia Rivas L, Morales del Burgo P, Visa L, Hernández R, Martínez de Castro E, Pereira Elorrieta A, Lozano ML, Martínez-Martínez I. 2024. Identification of new biomarkers of high risk of venous thromboembolism in advanced gastric cancer. XL Congress of Sociedad Española de Trombosis y Hemostasia. Mallorca, Spain.

1.3. PATENTS

- Pérez-Sánchez H, Martínez-Martínez I, Peña-García J, Carmona-Bayonas A, Ródenas-Bleda MC, Peñas-Martínez J, **Zaragoza-Huesca D**, Mulero-Méndez V, Pardo-Sánchez I, Espín-García S. 2023. New treatment for colorectal cancer. Holders of rights: Fundación para la formación e investigación sanitarias de la Región de Murcia; Fundación universitaria San Antonio. Nº application: P202130871. Spain.
- Conesa-Zamora P, Pérez-Sánchez H, Martínez-Martínez I, Peña-García J, Carmona-Bayonas A, Ródenas-Bleda MC, Peñas-Martínez J, **Zaragoza-Huesca D**. 2023. New treatment for colorectal cancer. Holders of rights: Fundación para la formación e investigación sanitarias de la Región de Murcia; Fundación universitaria San Antonio. Nº application: P202130064. Spain.

2. SCIENTIFIC PRODUCTION NON-RELATED TO MY THESIS

2.1. PUBLISHED ARTICLES

- Peñas-Martínez J, Luengo-Gil G, Espín S, Bohdan N, Ortega-Sabater C, Ródenas MC, **Zaragoza-Huesca D**, López-Andreo MJ, Plasencia C, Vicente V, Carmona-Bayonas A, Martínez-Martínez I. 2021. Anti-tumor functions of prelatent antithrombin on glioblastoma multiforme cells. *Biomedicines*. 9(5):523.
- **Zaragoza-Huesca D**, Martínez-Cortés C, Banegas-Luna AJ, Pérez-Garrido A, Vegara-Meseguer JM, Peñas-Martínez J, Rodenas MC, Espín S, Pérez-Sánchez H, Martínez-Martínez I. 2022. Identification of kukoamine A, zeaxanthin, and clexane as new furin inhibitors. *Int J Mol Sci*. 23(5):2796.
- Agüera-Sánchez A, Martínez-Diaz F, Pagán-Muñoz I, Martínez-Pérez M, Cano-Mármol PLR, Martínez-Martínez I, **Zaragoza-Huesca D**, García-Molina F. 2024. Duodenal pseudolipomatosis. Literature review and case report of a very unusual finding. *Rev Esp Enferm Dig*. 116(10):562-563.

2.2. ORAL COMMUNICATIONS IN CONGRESSES

- Peñas-Martínez J, Ródenas-Bleda MC, **Zaragoza-Huesca D**, Espín-García S, Vicente-García V, Martínez-Martínez I. 2020. Prelatent antithrombin regulates VEGF-A pathway in glioblastome-astrocytoma cells. XXXVI Congress of Sociedad Española de Trombosis y Hemostasia. Virtual congress.
- Peñas-Martínez J, **Zaragoza-Huesca D**, Espín S, Vicente V, Carmona-Bayonas A, Martínez-Martínez I. 2021. Analysis of antitumor effect of prelatent antithrombin on glioblastome multiforme cells by means of transcriptomics. XXXVII Congress of Sociedad Española de Trombosis y Hemostasia. Navarra, Spain.
- Peñas-Martínez J, Mariappan A, **Zaragoza-Huesca D**, Garrido-Rodríguez P, Vicente V, Lozano ML, Gopalakrishnan J, Martínez-Martínez I. 2022. Antitumor effect of antithrombin on a 3-D model of organoids of human brain and patient-derived glioma stem cells. XXXVIII Congress of Sociedad Española de Trombosis y Hemostasia. Barcelona, Spain.
- Peñas-Martínez J, **Zaragoza-Huesca D**, Espín S, Zuazu I, Cano H, Lozano ML, Martínez-Martínez I. 2023. New antidote of low molecular weight heparins based on a recombinant antithrombin. XXXIX Congress of Sociedad Española de Trombosis y Hemostasia. Sevilla, Spain.
- Peñas-Martínez J, **Zaragoza-Huesca D**, Garrido-Rodríguez P, Cuenca-Zamora E, Navarro E, Lozano ML, González-Conejero R, Martínez C, Martínez-Martínez I. 2023. Plenary session: Anti-proliferative, anti-angiogenic and anti-invasive effect of antithrombin in glioblastoma multiforme. XXXIX Congress of Sociedad Española de Trombosis y Hemostasia. Sevilla, Spain.
- Peñas-Martínez J, **Zaragoza-Huesca D**, Garrido-Rodríguez P, Cuenca-Zamora E, Navarro E, Lozano ML, González-Conejero R, Martínez C, Vicente V, Martínez-Martínez I. 2023. Anti-proliferative, anti-angiogenic and anti-invasive effect of antithrombin in glioblastoma multiforme. VII Jornadas IMIB. Murcia, Spain.
- Peñas-Martínez J, **Zaragoza-Huesca D**, Espín S, Martínez C, González-Conejero R, Lozano ML, Vicente V, Martínez-Martínez I. 2024. Anti-proliferative, anti-angiogenic and anti-invasive effect of antithrombin in glioblastoma multiforme. XXXII Congress of the International Society on Thrombosis and Haemostasis. Thailand, Bangkok.

2.3. PATENTS

- Pérez-Sánchez H, Martínez-Martínez I, **Zaragoza-Huesca D**, Martínez-Cortés C, Banegas-Luna AJ, Pérez-Garrido A, Vegara-Meseguer J, Peñas-Martínez J, Ródenas-Bleda MC, Espín-García S. 2022. Kukoamine-A for the prevention and treatment of viral infection, preferably by coronavirus. Holders of rights: Fundación para la formación e investigación sanitarias de la Región de Murcia; Fundación universitaria San Antonio. Nº application: P202130872. Spain.
- Pérez-Sánchez H, Martínez-Martínez I, **Zaragoza-Huesca D**, Martínez-Cortés C, Banegas-Luna AJ, Pérez-Garrido A, Vegara-Meseguer J, Peñas-Martínez J,

APPENDIX

Ródenas-Bleda MC, Espín-García S. 2022. Zeaxanthin for the prevention and treatment of viral infection, preferably by coronavirus. Holders of rights: Fundación para la formación e investigación sanitarias de la Región de Murcia; Fundación universitaria San Antonio. Nº application: P202130873. Spain.