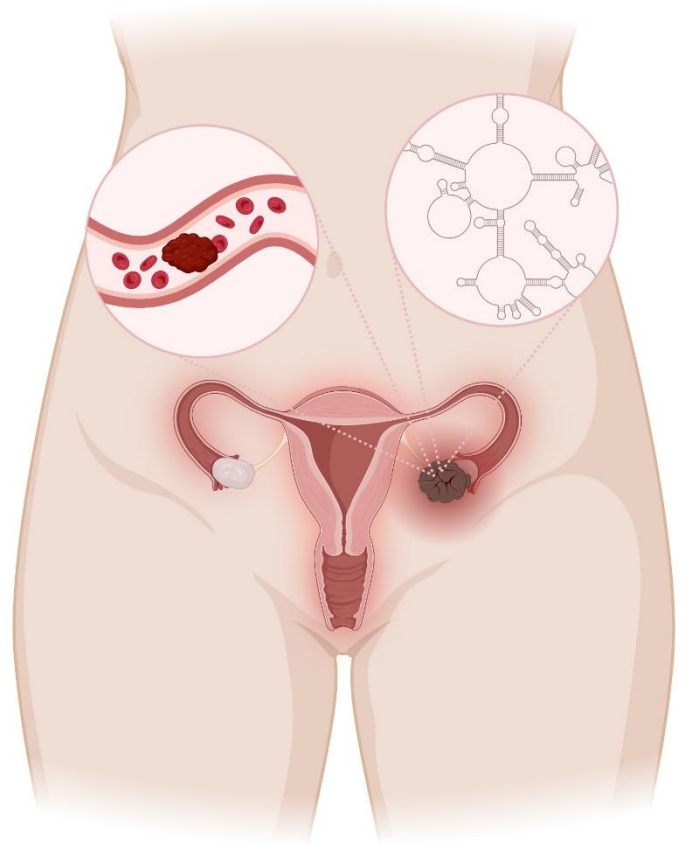
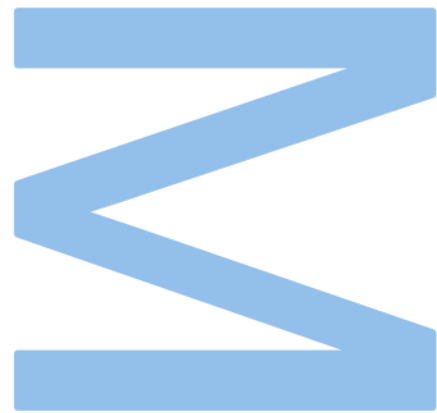




Long non-coding RNAs associated with venous thromboembolism: impact on the prognosis of ovarian cancer patients



Inês Soares Marques

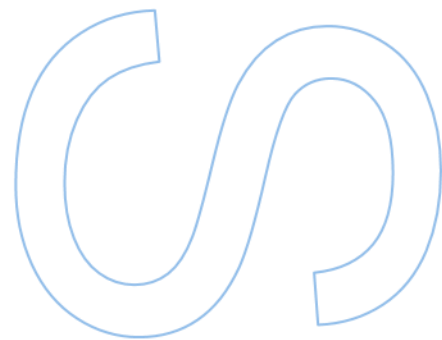
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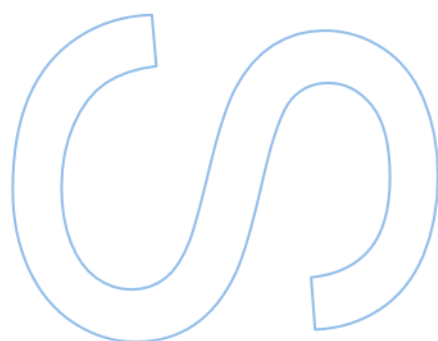
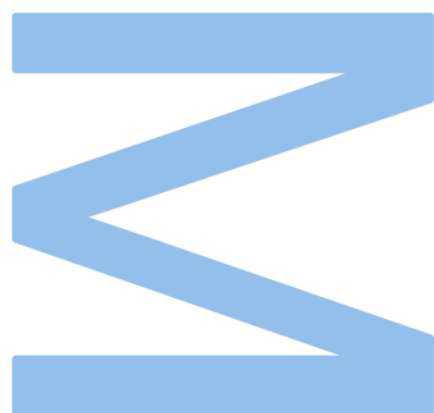
Professor Doctor Rui Manuel de Medeiros Melo Silva, Pharm.D., PhD

Head of the research group of Molecular Oncology and Viral Pathology at the research centre of IPOP (CI-IPOP)

Doctor Deolinda Pereira, MD, PhD

Director of the Medical Oncology Department of IPOP





*To my beloved nephews,
May you never settle and always keep exploring.*

Publications

The author declares that information derived from the publications mentioned below has been incorporated into the development of this dissertation. Additionally, the author highlights their significant contribution to the planning and execution of the involved activities, as well as to the analysis and discussion of the results, and the drafting of the manuscripts.

Publications from which data was used for the composition of this thesis:

1. Neto, B. V. †, Tavares, V. †, da Silva, J. B., Liz-Pimenta, J., **Marques, I. S.**, Carvalho, L., Salgado, L., Pereira, D., & Medeiros, R. (2023). Thrombogenesis-associated genetic determinants as predictors of thromboembolism and prognosis in cervical cancer. *Sci Rep*, 13(1), 9519. <https://doi.org/10.1038/s41598-023-36161-w> (**Attachment 1**).
2. **Marques, I. S.**, Tavares, V., Neto, B. V., Mota, I. N. R., Pereira, D., & Medeiros, R. (2023). Long Non-Coding RNAs in Venous Thromboembolism: Where Do We Stand? *International Journal of Molecular Sciences*, 24(15), 12103. <https://www.mdpi.com/1422-0067/24/15/12103> (**Attachment 2**).
3. Tavares, V. †, Neto, B. V. †, **Marques, I. S.** †, Assis, J., Pereira, D., & Medeiros, R. (2023) Cancer-associated thrombosis: What about microRNAs targeting the tissue factor coagulation pathway? *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 2024. 1879(1): p. 189053. <https://doi.org/10.1016/j.bbcan.2023.189053> (**Attachment 3**).

† Jointed first authors.

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Resumo

O cancro de ovário (CO) é a segunda neoplasia ginecológica mais mortal, sendo o seu prognóstico desfavorável atribuído ao diagnóstico tardio e à aquisição de quimiorresistência. Para além disso, as doentes com CO são frequentemente diagnosticadas com tromboembolismo venoso (TEV), um distúrbio cardiovascular que, para além de deteriorar a sua qualidade de vida, é potencialmente fatal. Estas duas patologias têm uma relação próxima, na qual as células tumorais recrutam o sistema hemostático para favorecer o seu crescimento e disseminação. Os RNAs longos não codificantes (do inglês *long non-coding RNAs* (lncRNAs)) parecem estar implicados na patogénese de TEV na população geral. No entanto, os seus papéis em TEV associado a cancro permanecem por explorar. Compreender como os lncRNAs impactam a trombogénese venosa e a tumorigénese em CO pode levar à identificação de novos biomarcadores para a prevenção e/ou tratamento de TEV assim como o tratamento de CO. Neste presente estudo, o papel de cinco lncRNAs, nomeadamente MALAT1, TUG1, NEAT1, XIST e MEG8, foi avaliado numa coorte de 40 doentes com CO. Um total de sete doentes desenvolveram TEV associado a CO. Considerando aqueles que sofreram TEV após diagnóstico de CO (n=5), a ocorrência de trombose foi associada a uma pior sobrevivência global (teste de log-rank, $p=0,028$). Níveis elevados de MEG8 pré-quimioterapia em células do sangue periférico foram um preditor de TEV após o diagnóstico de CO (teste de Man Whitney, $p=0,037$; teste X^2 , $p=0,033$). Contrariamente, baixos níveis de MEG8 foram associados a um maior risco de progressão de CO (razão de risco ajustada (do inglês, *adjusted hazard ratio* (aHR)) = 3,00; intervalo de confiança de 95% (IC), 1,06-8,51; $p=0,039$). Adicionalmente, baixos níveis de NEAT1 pré-quimioterapia em células do sangue periférico foram associados a um maior risco de morte de CO (aHR=6,25; 95%CI, 1,60-24,43; $p=0,008$). Relativamente aos restantes lncRNAs, não se verificou nenhuma associação significativa com a incidência de TEV, progressão tumoral e sobrevivência das doentes. Futuramente, estudos de validação deverão ser realizados a fim de explorar os papéis destes lncRNAs em doentes com CO.

Palavras-chave: neoplasias do ovário, trombose venosa, RNAs longos não codificantes, terapia, trombopprofilaxia, prognóstico.

Abstract

Ovarian cancer (OC) is the second deadliest gynaecological tumour. Its poor prognosis is attributed to the common late diagnosis of the disease and the acquisition of chemoresistance. Patients with OC are commonly diagnosed with venous thromboembolism (VTE), a cardiovascular disorder that deteriorates their quality of life, being also possibly life-threatening. These two diseases have a close relationship, in which tumour cells capture the haemostatic system to boost their growth and dissemination. Long non-coding RNAs (lncRNAs) seem to be implicated in VTE pathogenesis in the general population. Yet, their roles in cancer-associated VTE are unexplored. Comprehending how lncRNAs impact venous thrombogenesis and ovarian tumorigenesis may help identify valuable biomarkers for both VTE and OC management, which are currently needed. In this study, the roles of five lncRNAs, namely MALAT1, TUG1, NEAT1, XIST, and MEG8, were evaluated in a cohort of 40 OC patients. Seven patients developed OC-related VTE. For those with VTE after OC diagnosis ($n=5$), venous thrombogenesis was associated with worse overall survival (log-rank test, $p=0.028$). Elevated prechemotherapy MEG8 levels in peripheral blood nucleated cells (PBNCs) were a predictor of VTE after OC diagnosis (Mann Whitney test, $p=0.037$; χ^2 test, $p=0.033$). In opposition, its low levels were linked to a higher risk of OC progression (adjusted hazard ratio (aHR) = 3.00; 95% confidence interval (CI), 1.06-8.51; $p=0.039$). Furthermore, low prechemotherapy NEAT1 levels in PBNCs were associated with a higher risk of death (aHR=6.25; 95%CI, 1.60-24.43; $p=0.008$). As for the remaining lncRNAs, no significant association with VTE incidence, OC progression or related mortality was observed. Future investigation with external validation is needed to explore the roles of the evaluated lncRNAs in OC patients.

Keywords: ovarian neoplasms, venous thrombosis, long non-coding RNAs, thromboprophylaxis, drug therapy, prognosis.

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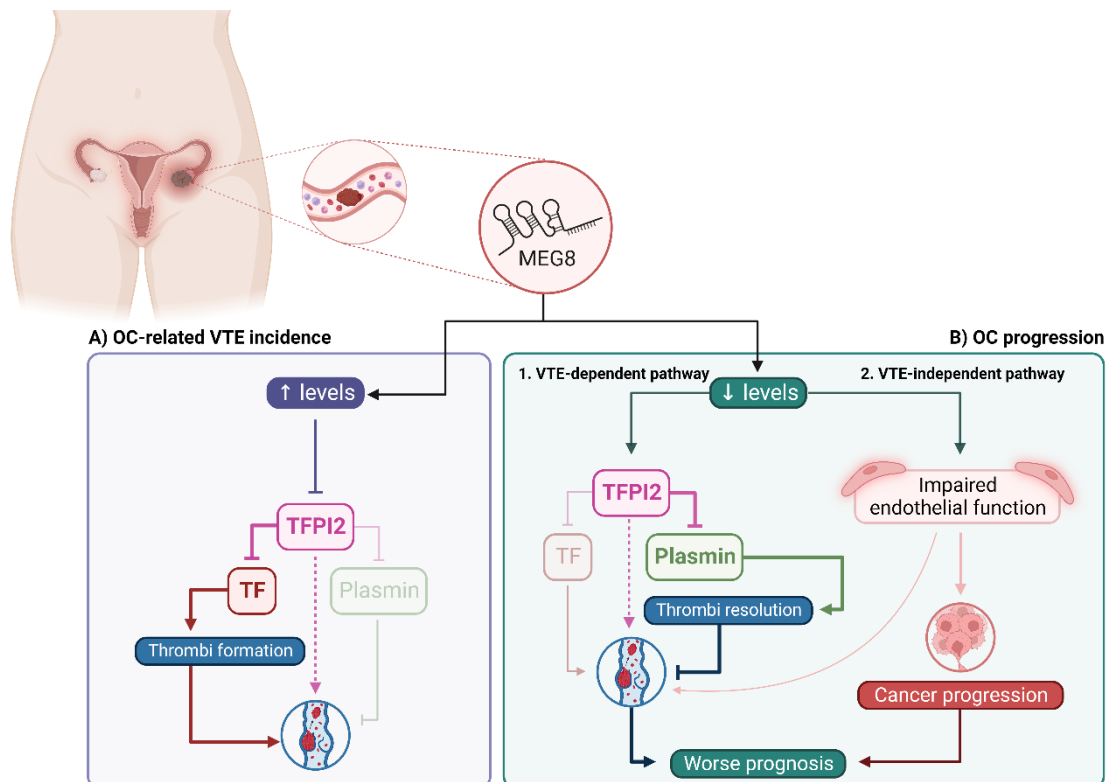


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Low levels of MEG8 are known to impair endothelial function and elevate TFPI2 expression [275]. TFPI2, in turn, has inhibitory activity towards TF and plasmin [64]. While the former initiates the coagulation cascade, the latter primarily contributes to blood clot removal [63, 64]. **a)** In this study, expression levels of MEG8 were elevated in OC patients who developed a VTE event after cancer diagnosis. The underlying hypothetical mechanism relies on the fact that TFPI2 inhibition towards TF might be more significant at thrombi formation. Specifically, as MEG8 inhibits TFPI2, the latter, when unavailable, cannot inhibit TF, which consequently is able to initiate the coagulation cascade leading to VTE. **b)** Low expression levels of MEG8 were linked to an increase in the risk of disease progression, which can be explained by VTE-dependent and independent pathways. **b1)** A possible VTE-dependent hypothesis could be that TFPI2 inhibits plasmin, preventing it to execute its profibrinolytic functions, hampering VTE resolution and promoting OC progression given the plasmin roles in the remodelling of extracellular matrix [64, 286]. **b2)** Other possible mechanism consists in a VTE-independent pathway where impaired endothelial function promotes cancer progression, aggravating OC patient's prognosis [285]. Parallely, impaired endothelial function contributes also to VTE [83]. Dashed arrows represent the final result through which TFPI2 impacts VTE development. MEG8 expression: ↓ downregulated and ↑ upregulated. Transparency indicates the possible less relevant TFPI2-inhibitory activity in the respective pathway. Abbreviations: MEG8, maternally expressed 8; OC, ovarian cancer; VTE, venous thromboembolism; TFPI2, tissue factor pathway inhibitor. The figure was created with BioRender.com..... 56

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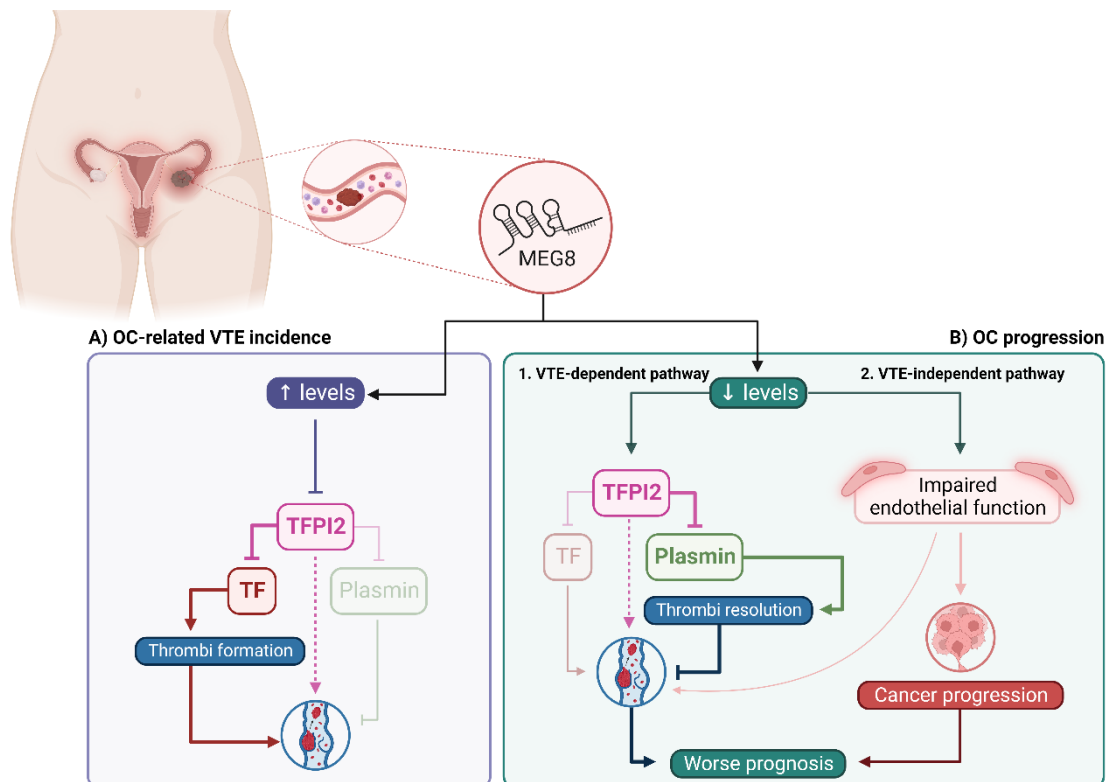


Figure 15. Hypothesis of MEG8 roles in a) OC-related VTE incidence and b) OC progression..... 56

Low levels of MEG8 are known to impair endothelial function and elevate TFPI2 expression [275]. TFPI2, in turn, has inhibitory activity towards TF and plasmin [64]. While the former initiates the coagulation cascade, the latter primarily contributes to blood clot removal [63, 64]. **a)** In this study, expression levels of MEG8 were elevated in OC patients who developed a VTE event after cancer diagnosis. The underlying hypothetical mechanism relies on the fact that TFPI2 inhibition towards TF might be more significant at thrombi formation. Specifically, as MEG8 inhibits TFPI2, the latter, when unavailable, cannot inhibit TF, which consequently is able to initiate the coagulation cascade leading to VTE. **b)** Low expression levels of MEG8 were linked to an increase in the risk of disease progression, which can be explained by VTE-dependent and independent pathways. **b1)** A possible VTE-dependent hypothesis could be that TFPI2 inhibits plasmin, preventing it to execute its profibrinolytic functions, hampering VTE resolution and promoting OC progression given the plasmin roles in the remodelling of extracellular matrix [64, 286]. **b2)** Other possible mechanism consists in a VTE-independent pathway where impaired endothelial function promotes cancer progression, aggravating OC patient's prognosis [285]. Parallely, impaired endothelial function contributes also to VTE [83]. Dashed arrows represent the final result through which TFPI2 impacts VTE development. MEG8 expression: ↓ downregulated and ↑ upregulated. Transparency indicates the possible less relevant TFPI2-inhibitory activity in the respective pathway. Abbreviations: MEG8, maternally expressed 8; OC, ovarian cancer; VTE, venous thromboembolism; TFPI2, tissue factor pathway inhibitor. The figure was created with BioRender.com..... 56

List of Abbreviations

A

ACK	AMMONIUM-CHLORIDE-POTASSIUM
ADAMTS13	A DISINTEGRIN AND METALLOPROTEASE WITH THROMBOSPONDIN TYPE 1 MOTIF
ADP	ADENOSINE DIPHOSPHATE
AGAP2-AS1	AGAP2 ANTISENSE RNA 1
AHR	ADJUSTED HAZARD RATIO
AKT	PROTEIN KINASE B
ANRIL	ANTISENSE NON-CODING RNA IN THE INK4 LOCUS
APL	ACUTE PROMYELOCYTIC LEUKAEMIA
APTT	ACTIVATED PARTIAL THROMBOPLASTIN TIME
ATRA	ALL-TRANS RETINOIC ACID

B

BCL2L11	BCL2-LIKE 11
BMI	BODY MASS INDEX
BRCA	BREAST CANCER GENE

C

CA-125	CANCER ANTIGEN 125
CAT	CANCER-ASSOCIATED THROMBOSIS
CCC	CLEAR CELL CARCINOMA
CDKN2A	CYCLIN DEPENDENT KINASE INHIBITOR 2A
CDKN2B	CYCLIN DEPENDENT KINASE INHIBITOR 2B
CERNAS	COMPETITIVE ENDOGENOUS RNAS
CI	CONFIDENCE INTERVAL
CI-IPOP	RESEARCH CENTRE OF PORTUGUESE INSTITUTE OF ONCOLOGY OF PORTO
CP	CANCER PROCOAGULANT
CRNDE	COLORECTAL NEOPLASIA DIFFERENTIALLY EXPRESSED
CT	CYCLE THRESHOLD
CXCL12	CHEMOKINE 12

D

DD	D-DIMER
DOAC	DIRECT ORAL ANTICOAGULANT
DPCR	DIGITAL POLYMERASE CHAIN REACTION

DVT	DEEP VEIN THROMBOSIS
E	
ECOG	EASTERN COOPERATIVE ONCOLOGY GROUP
EDTA	ETHYLENEDIAMINE TETRAACETIC ACID
EMT	EPITHELIAL-MESENCHYMAL TRANSITION
EOC	EPITHELIAL OVARIAN CANCER
EPCR	ENDOTHELIAL PROTEIN C RECEPTOR
EPCS	ENDOTHELIAL PROGENITOR CELLS
F	
FGFS	FIBROBLAST GROWTH FACTORS
FI	COAGULATION FACTOR I
FIA	ACTIVATED COAGULATION FACTOR I
FIGO	INTERNATIONAL FEDERATION OF GYNECOLOGY AND OBSTETRICS
FII	COAGULATION FACTOR II
FIIA	ACTIVATED COAGULATION FACTOR II
FIII	COAGULATION FACTOR III
FIV	CALCIUM IONS OR COAGULATION FACTOR IV
FIX	COAGULATION FACTOR IX
FIXA	ACTIVATED COAGULATION FACTOR IX
FVA	ACTIVATED COAGULATION FACTOR V
FVII	COAGULATION FACTOR VII
FVIIA	ACTIVATED COAGULATION FACTOR VII
FVIII	COAGULATION FACTOR VIII
FVIII A	ACTIVATED COAGULATION FACTOR VIII
FX	COAGULATION FACTOR X
FXA	ACTIVATED COAGULATION FACTOR X
FXI	COAGULATION FACTOR XI
FXIA	ACTIVATED COAGULATION FACTOR XI
FXII	COAGULATION FACTOR XII
FXIIA	ACTIVATED COAGULATION FACTOR XII
FXIII	COAGULATION FACTOR XIII
FXIIIA	ACTIVATED COAGULATION FACTOR XIII

G

GOMPV	MOLECULAR ONCOLOGY AND VIRAL PATHOLOGY
GSK-3B	GLYCOGEN SYNTHASE KINASE-3 BETA
GUSBP5-AS	GUSB PSEUDOGENE 5 ANTISENSE
GWAS	GENOME-WIDE ASSOCIATION STUDIES

H

HGSC	HIGH-GRADE SEROUS CARCINOMA
HIPEC	HEATED INTRAPERITONEAL CHEMOTHERAPY
HMGB1	HIGH-MOBILITY GROUP BOX 1
HMGCR	3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE
HNF1B	HEPATOCYTE NUCLEAR FACTOR 1-BETA
HRR	HOMOLOGOUS RECOMBINATION REPAIR
HUVECS	HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

I

IL	INTERLEUKIN
IL-1B	INTERLEUKIN-1B
IL-6	INTERLEUKIN-6
IL-8	INTERLEUKIN-8
INR	INTERNATIONAL NORMALIZED RATIO
IPOP	PORTUGUESE INSTITUTE OF ONCOLOGY OF PORTO
IQR	INTERQUARTILE RANGE

K

KS	KHORANA SCORE
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L

LGSC	LOW-GRADE SEROUS CARCINOMA
LINC00473	LONG INTERGENIC NON-PROTEIN CODING RNA 473
LINC00659	LONG INTERGENIC NON-CODING RNA 00659
LINC01123	LONG INTERGENIC NON-PROTEIN CODING RNA 1123
LINC RNAS	INTERGENIC LONG NON-CODING RNAS
LMWH	LOW-MOLECULAR-WEIGHT HEPARIN
LNC RNAS	LONG NON-CODING RNAS
LRG1	LEUCINE-RICH ALPHA-2-GLYCOPROTEIN 1

M

MALAT1	METASTASIS-ASSOCIATED LUNG ADENOCARCINOMA TRANSCRIPT 1
MEG8	MATERNALLY EXPRESSED 8
MEG9	MATERNALLY EXPRESSED GENE 9
MIR OR MIRNA	MICRORNA
MRI	MAGNETIC RESONANCE IMAGING
MRNAS	MESSANGER RNAS

N

NCCN	NATIONAL COMPREHENSIVE CANCER NETWORK
NCRNAS	NON-CODING RNAS
NEAT1	NUCLEAR ENRICHED ABUNDANT TRANSCRIPT 1 OR NUCLEAR PARASPECKLE ASSEMBLY TRANSCRIPT 1
NETS	NEUTROPHIL EXTRACELLULAR TRAPS
NF-KB	NUCLEAR FACTOR KB

O

OC	OVARIAN CANCER
OS	OVERALL SURVIVAL

P

PAI-1	PLASMINOGEN ACTIVATOR INHIBITOR-1
PARP	POLY (ADP-RIBOSE) POLYMERASE
PBNCS	PERIPHERAL BLOOD NUCLEATED CELLS
PBS	PHOSPHATE-BUFFERED SALINE
PDGFS	PLATELET-DERIVED GROWTH FACTORS
PD-L1	PROGRAMMED DEATH-LIGAND 1
PDPN	PODOPLANIN
PE	PULMONARY EMBOLISM
PET	POSITRON EMISSION TOMOGRAPHY
PFS	PROGRESSION-FREE SURVIVAL
PI3K	PHOSPHOINOSITIDE 3-KINASE
PML	PROMYELOCYTIC LEUKAEMIA
PS	PHOSPHATIDYL SERINE
PT	PROTHROMBIN TIME

ZINC FINGER E-BOX BINDING HOMEBOX 1

Introduction

I. Introduction

1. Ovarian cancer

Worldwide, ovarian cancer (OC) ranks eighth in both incidence and fatality among malignancies in women, with approximately 313, 959 new cases and 207, 252 deaths reported in 2020 (**Figure 1a**). In Portugal, OC is the thirteenth most diagnosed cancer and the eighth deadliest in women, with 561 new cases and 408 deaths reported in the same year (**Figure 1b**) [1]. Though OC rates have fallen over the years, mainly due to the increased use of oral contraceptive pills, this malignancy remains the second deadliest gynaecological cancer after cervical cancer [2, 3]. In fact, despite being relatively rare, OC stands out as the most lethal gynaecological tumour. This is partially attributed to the often late diagnosis of the disease owing to its nonspecific symptoms and lack of screening methods with adequate sensitivity and specificity [2-4].

Characterized as a heterogenous disease, OC encompasses three main types: epithelial (the most frequent), germ cell and sex-cord-stromal tumours, with the latter two accounting for only about 5% of all cases. Epithelial OC (EOC) or ovarian carcinoma has four primary histologic subtypes: serous (70-80%), clear cell (CCC; 10%), endometrioid (10%) and mucinous tumours (3%). As well, depending on the molecular profile and clinical behaviour, EOC can be divided into two groups: type I and type II carcinomas [4]. While type I is linked to continued ovulation cycles, inflammation, and endometriosis and usually originates in the ovary, type II is associated with a high degree of genetic instability (i.e. mutations in *breast cancer gene 1/2* (*BRCA1/2*), *tumour protein 53* (*TP53*) and other tumour-suppressing genes), has his origin typically in the fallopian tube and correlates with more fatal outcomes [4-7]. Serous tumours, the most common EOC subtype, can be classified as either high-grade (HGSC) or low-grade serous carcinomas (LGSC). While LGSCs make up less than 5% of all EOC subtypes, HGSCs account for 70% to 80% in the same group and are accordantly associated with a worse prognosis [4]. Each OC subtype has a distinct aetiology, differentiation, pathogenesis and molecular profiles, which is reflected in differences in the response to therapeutic approaches and the overall clinical outcome of the patients [8].

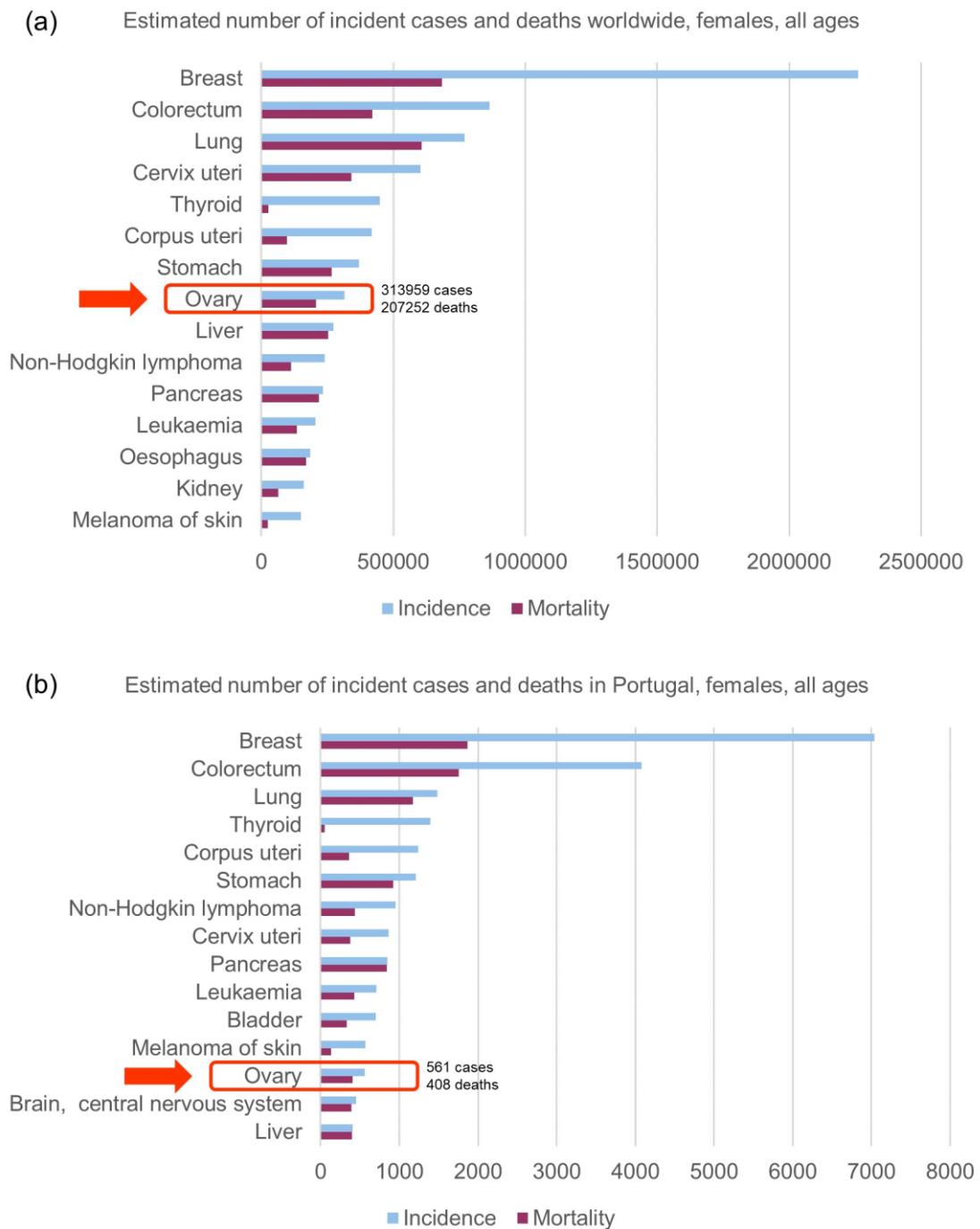


Figure 1. Estimated number of incident cancer cases and deaths **a)** worldwide and in **b)** Portugal among women of all ages in 2020.

Worldwide, ovarian cancer ranks eighth in both frequency and fatality among malignancies in women, with approximately 313, 959 new cases and 207, 252 deaths reported in 2020. In Portugal, ovarian cancer is the thirteenth most diagnosed malignancy and the eighth deadliest with 561 new cases diagnosed and 408 deaths reported in 2020. Data retrieved from GLOBOCAN 2020 [1].

Regarding the disease aetiology, several modifiable and nonmodifiable risk factors are thought to play a role in ovarian tumorigenesis (**Figure 2**). Although with inconsistent

data, the modifiable determinants appear to include hormonal replacement therapy, smoking, alcohol consumption, physical inactivity, unhealthy diet, obesity and nulliparity [4, 9]. Nonmodifiable risk factors, in turn, encompass a family history of tumours, mainly due to the hereditary breast and ovarian cancer syndrome (includes mostly germline mutations in *BRCA1* and *BRCA2*) and Lynch syndrome (germline mutations in genes related to DNA mismatch repair), with advanced age, early menarche, late menopause, endometriosis and ethnicity/race constituting also important risk determinants [4, 10]. For instance, OC is more incident in non-Hispanic white women (12.0 per 100,000), followed by Hispanic (10.3 per 100,000), non-Hispanic black (9.4 per 100,000) and Asian/Pacific Islander women (9.2 per 100,000) [11]. However, African populations are reported to have the highest related mortality rate, most likely due to the imbalanced healthcare access and non-standard treatment protocols provided in these communities. Additionally, genetic susceptibility, the presence of co-existing health conditions or the higher prevalence of modifiable risk factors also contribute to this ethnicity/race discrepancy [12]. In opposition, other factors can be pointed out as protective or associated with a lower risk of OC development. The list includes oral contraceptive use, pregnancy, breastfeeding, tubal ligation or hysterectomy and prophylactic oophorectomy [9]. Concordantly, studies have shown that the fewer ovulatory cycles a woman completes, the lower the predisposition for OC, possibly due to the proinflammatory response that occurs in the fallopian tubes during ovulation, which increases the malignant tendencies in the ovaries [4, 7, 13]. A better comprehension of OC aetiology and the identification of risk determinants for OC is important to better tailor prevention measures.

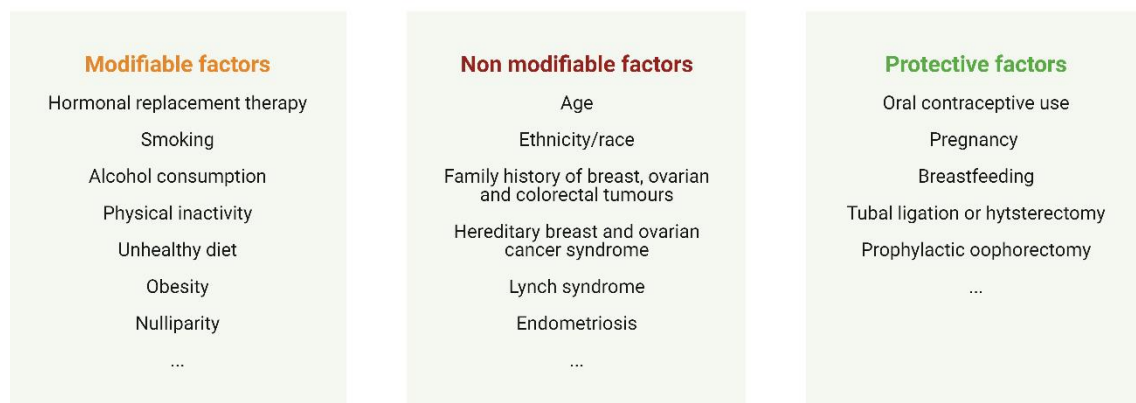


Figure 2. Risk determinants associated with ovarian tumorigenesis.

The aetiology of ovarian cancer is diverse and not entirely understood. Several risk and protective determinants have been reported, the most established being advanced age and genetic predisposition. The figure was created with BioRender.com.

Currently, there is no surefire way to prevent OC. Still, the Society of Gynecologic Oncology lists the use of oral contraceptives, tubal sterilization and salpingo-oophorectomy for women at high risk as possible prophylactic options, particularly for those with hereditary syndromes related to the disease. As such, genetic counselling and testing should be offered to women with a family history of the disease [13].

Another challenge related to OC is its nonspecific symptoms and lack of screening methods with appropriate performance [4]. Pelvic examination, transvaginal ultrasound and the cancer antigen 125 (CA-125) blood test, which were proposed as possible candidates for the disease screening, have been consistently showing poor sensitivity and specificity in trials considering women in the general and the high-risk population [14-16]. Additionally, screening for asymptomatic and average-risk women is not recommended due to the low prevalence of the disease and the high probability of obtaining false-positive results, which might lead to unnecessary interventions [17, 18]. As a result, OC patients are frequently (~70%) diagnosed at advanced stages (III and IV), which, as previously mentioned, significantly impacts their prognosis [4]. Of note, current diagnostic strategies encompass the combination of medical history evaluation, gynaecological exam, imaging tests (transvaginal ultrasonography, computed tomography, magnetic resonance imaging (MRI), and/or positron emission tomography (PET)) and serum CA-125 measurement, requiring also a histopathological examination for a definitive diagnosis and staging [19-21].

At diagnosis, the International Federation of Gynecology and Obstetrics (FIGO) staging system is one of the most important tools used to predict the clinical outcome of OC patients and evaluate their therapeutical options [22]. This system was first published in 1973 and was last revised in 2018 [18, 22]. It comprises four stages, namely I, II, III, and IV, each one with several subdivisions. Stage I integrates tumours confined to either the ovary (one or both ovaries) or the fallopian tubes, while, at stage II, the tumour has already spread beyond the ovaries or fallopian tubes, with pelvic extension. In stage III, cancer cells spread to the peritoneum outside the pelvis, and there might be metastasis to the retroperitoneal lymph nodes. Lastly, stage IV, the most advanced one, is characterized by the dissemination of the tumour to other body parts beyond the pelvis and abdomen [18].

Regarding the disease treatment, it relies on several factors, including the disease stage, histologic subtype, the patient's general well-being and medical background and genetic mutations. The standard treatment encompasses debulking surgery, with the removal of the affected tissue, followed by chemotherapy with a combination of platinum-containing drugs (cisplatin and carboplatin, the latter with lower toxicity without compromising

survival) with or without taxane family agents (paclitaxel and docetaxel) [4, 23, 24]. Furthermore, at advanced stages, for those not suitable for primary debulking, it may be necessary to perform neoadjuvant chemotherapy to lower the tumour burden prior to surgery. In opposition, for patients in the early stages, in some cases, surgery followed by observation is the recommended approach [18]. Another possible therapeutic approach is radiotherapy. However, except for its use in palliative care, it is no longer used as a standard practice due to its high toxicity rates and less effectiveness when compared to platinum-based chemotherapy. However, with modern and less toxic radiotherapy techniques, its use is being reassessed [25-27]. Importantly, following cytoreductive surgery and chemotherapy, most OC patients attain a state of complete clinical remission. Nonetheless, approximately 50-70% of these individuals encounter recurrence around 18 months, often linked to resistance to chemotherapy [4, 28, 29]. Intratumor heterogeneity is the primary driver behind treatment ineffectiveness and the development of drug resistance in OC, with both of them posing hurdles in the disease management [30]. For women resistant to platinum and/or with recurrent disease, hormonal therapy can be used as a treatment option, as stated by National Comprehensive Cancer Network (NCCN) OC guidelines (2021) and the European Society of Medical Oncology/European Society of Gynecological Oncology guidelines [15, 18]. Other alternative approaches include gemcitabine and doxorubicin-based chemotherapy [24]. In addition to the first line of treatment, maintenance therapy for OC patients is also important, particularly to minimize the relapse rate [18]. Over the last decade, new treatments have been emerging and with them, the use of poly (ADP-ribose) polymerase (PARP) inhibitors. As maintenance therapy, PARP inhibitors seem to have a positive effect in patients with platinum-sensitive recurrent OC. They also prove beneficial as monotherapy for those with recurrent disease [18, 31]. Examples of drugs in this category include Olaparib and Rucaparib, which are used in combination after first-line chemotherapy [4]. In terms of function, PARP proteins are known to help cells self-regenerate after they suffer damage, being crucial for DNA damage repair. In the context of malignancy, tumour cells can use these proteins to repair the damage caused by chemotherapy [4, 31]. For OC patients with homologous-recombination deficiency, meaning their homologous recombination repair (HRR) pathway is not functioning effectively (mainly due to mutations in *BRCA1* and *BRCA2*), PARP inhibitors leverage a vulnerability in tumour cells rendering these cells unable to efficiently repair specific types of DNA damage, thus promoting their apoptosis [31, 32]. Consequently, these inhibitors arrest the regeneration process of tumour cells after chemotherapy, effectively inhibiting their growth [4]. Another established maintenance approach for patients at advanced stages involves the combination of the antiangiogenic drug, Bevacizumab, with a regimen of carboplatin and paclitaxel, followed by subsequent administration of

Bevacizumab alone [33]. Additionally, also for individuals with advanced disease, combining Bevacizumab and Olaparib as maintenance therapy is proven to be beneficial, regardless of the presence of a BRCA mutation [33]. This angiogenesis inhibitor acts by attaching to circulating vascular endothelial growth factor (VEGF), blocking its ability to bind to receptors present on the surface of vascular endothelial cells (vECs). Consequently, this hinders the formation of new blood vessels and the pressure of interstitial fluid within tumours is reduced, creating a more favourable environment for chemotherapeutic drugs to effectively reach their designated targets [34].

As for the prognosis assessment, serum CA-125, FIGO stage, histologic subtype, tumour differentiation, degree of tumour reduction surgery and course of chemotherapy are independent prognostic factors of OC patients widely used to help clinical decision making to improve patient's clinical outcome [35-37]. Among these biomarkers, serum CA-125 is the one used not only to assess prognosis but also to monitor the disease recurrence. The levels of this OC marker are associated with clinical staging and survival. Specifically, patients with lower levels of preoperative CA-125 tend to have a higher survival than their counterparts [38]. However, non-tumour cells, such as mesothelial cells, can also secrete CA-125 in a proinflammatory environment, having this biomarker limited specificity [35, 36, 39]. Regarding the tumour stage, those at stage I have a 90% 5-year survival rate, while for those with regional (when cancer spreads to nearby tissues) and metastatic disease, the survival rates fall to 80% and 25%, respectively [4, 40]. Concerning the histological subtype, the survival rate also varies, with type I EOC, germ cell and sex-cord-stromal tumours being generally associated with higher survival rates [4, 41]. The quality of surgery, which encompasses the extent of tumour removal (R0, R1 and R2), the dissection of the lymph nodes and even the prevention of tumour rupture, is known to significantly affect the prognosis of OC patients [40, 42]. For instance, achieving an R0 cytoreductive surgery, which means complete resection of the tumour tissue, is regarded as the optimal goal given its significant enhancement in survival rates compared to suboptimal cytoreduction (R1 and R2) for OC patients [43-45]. Regarding chemotherapy, the optimal approach relies essentially on cancer stage, grade and histology. Different adjuvant chemotherapy approaches have markedly improved the survival rates in both early and advanced-stages. Examples of these strategies include the combination of paclitaxel with platinum-based chemotherapy, administration of intraperitoneal cisplatin for optimally cytoreduced cases (heated intraperitoneal chemotherapy (HIPEC)), transitioning to a dose-dense weekly paclitaxel regimen instead of the conventional three-week schedule [45, 46].

In summary, OC patients have a poor prognosis that can be explained by the lack of effective screening tools and the common acquisition of chemoresistance followed by

tumour recurrence. There is currently a need for more and better therapeutic options and adequate prognostic biomarkers to improve the clinical outcome of these patients, which requires a better knowledge of ovarian tumorigenesis.

2. Venous thromboembolism

The blood circulatory system (or the cardiovascular system) is responsible for transporting and delivering various compounds, including nutrients and gases, being also engaged in removing waste products [47]. As such, ensuring the integrity of the blood vessels in this complex network is vital, a function carried out by the haemostatic system, which allows the continuous blood circulation [48].

Haemostasis is a complex and dynamic process that rapidly responds to vascular injury. Several biochemical and cellular processes are triggered following vascular damage to form a blood clot, a process known as blood coagulation (or blood clotting). This mechanism stops blood loss, restores haemostasis and initiates vascular repair [49]. Haemostasis is, however, under tight regulation to limit blood coagulation to sites of vascular damage, preventing haemorrhage and the formation of unnecessary blood clots known as thrombi. The generation of a thrombus (i.e., thrombosis) can be life-threatening as it might restrict or even block the blood circulation inside the vessels, preventing the transportation of compounds and exchange of gases [50, 51]. Thus, proclotting and anticlotting mechanisms that regulate haemostasis must be balanced to avoid excessive bleeding and thrombogenesis. To maintain the haemostatic balance, four key players - vECs, platelets, the coagulation cascade, and the fibrinolytic system - interact in an intricate and tightly controlled manner (**Figure 3**). In short, the damage to blood vessels activates platelets, which adhere to the exposed subendothelial matrix. Simultaneously, the coagulation cascade is triggered to stabilize the platelet plug with fibrin and form a blood clot at the injury site to stop the bleeding [49, 52].

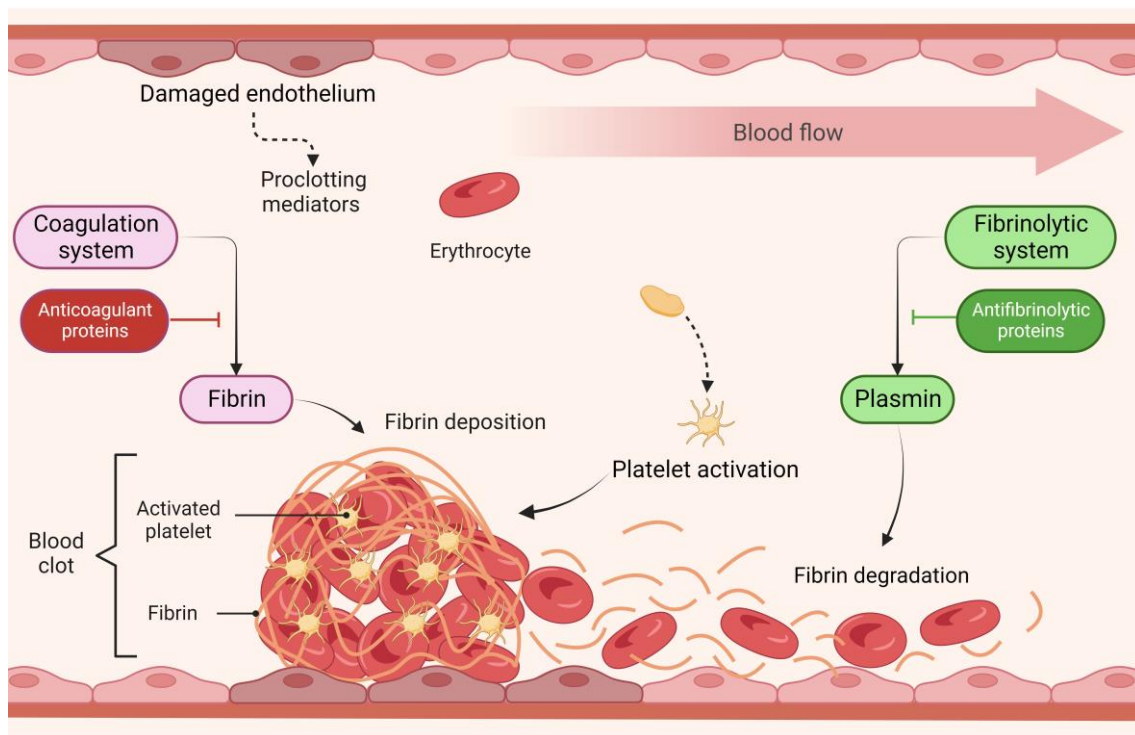


Figure 3. Interplay between the components of the haemostatic system.

To maintain haemostasis and prevent excessive bleeding or thrombosis, a delicate equilibrium between proclotting and anticoagulant mechanisms is mandatory. This balance is regulated by four essential components: vascular endothelial cells, platelets, the coagulation cascade and the fibrinolytic system. When vascular damage occurs, platelets are activated and adhere to the exposed subendothelial matrix. Parallely, the coagulation cascade is triggered to fortify the platelet plug with fibrin, creating a blood clot at the injury site to stop the bleeding. Figure retrieved from Marques *et. al* [53].

The coagulation cascade encompasses a series of sequential proteolytic activation reactions, with a complex interplay between several proteins named coagulation factors that culminate in fibrin formation. There are three major coagulation pathways according to the waterfall model: extrinsic (also called the tissue factor (TF) coagulation pathway) and intrinsic (contact coagulation pathway), with both converging on a common pathway (**Figure 4**) [54]. Coagulation factor III (FIII), thromboplastin or TF is a transmembrane protein located constitutively on the surface of all cells, except for endothelial and blood cells. When damage to blood vessels occurs, TF is released into the bloodstream initiating the extrinsic coagulation pathway. By combining with the coagulation factor VII (FVII), the complex TF-FVII (extrinsic tenase complex) activates coagulation factor X (FX) [55]. Activated FX (FXa) then participates in the common coagulation pathway. On a phospholipid surface, FXa forms a complex with activated coagulation factor V (FVa), known as prothrombinase complex, that converts prothrombin (coagulation factor II (FII)) into thrombin. Next, thrombin transforms fibrinogen (coagulation factor I (FI)) into fibrin that deposits at the vascular injury site, forming a network together with platelets to create a blood clot [56]. Parallely, the intrinsic coagulation pathway is initiated by

collagen and other negatively charged surfaces exposed at the injury site. Once activated, the coagulation factor XII (FXII) activates coagulation factor XI (FXI), which then activates coagulation factor IX (FIX). In the presence of calcium ions (coagulation factor IV (FIV)), activated FIX (FIXa) forms a complex with coagulation factor VIII (FVIII) that generates FXa [57]. Both extrinsic and intrinsic pathways thus produce FXa, which then participates in the common coagulation pathway leading to thrombin generation and ultimately fibrin deposition [56]. To be noted, thrombin also activates thrombin activable fibrinolysis inhibitor (TAFI), FV, FVIII, FXI and coagulation factor XIII (FXIII) creating a positive feedback loop that enhances clot formation. In addition, thrombin also promotes platelet adhesion by inactivating a disintegrin and metalloprotease with thrombospondin type 1 motif (ADAMTS13). On the other hand, thrombin has also an anticoagulant activity to avoid thrombogenesis by activating the protein C anticoagulation pathway, which then inhibits FVa and FVIIIa [58]. Antithrombin, in turn, can inhibit all coagulation factors but primarily targets thrombin FXa and FIXa [59]. Other noteworthy proteins in the coagulation cascade are tissue factor pathway inhibitor 1 (TFPI1) and 2 (TFPI2), two homologous proteins encoded by different genes, which are recognized as innate inhibitors of the TF coagulation pathway [60-62]. In opposition to TFP1, TFPI2 is, however, reported to have a weak inhibition activity towards the TF-mediated pathway, being instead a strong inhibitor of plasmin, a protein responsible for fibrin degradation [60, 63, 64]. Hence, TFPI2 may present a dual role in haemostasis that might vary according to the context [64].

Subsequently to the action of the coagulation cascade, the fibrinolytic system is responsible for clot lysis (i.e., fibrinolysis), which prevents thrombosis and restores blood flow. Fibrinolysis is primarily regulated by proteases and their inhibitors, with plasmin acting as the central orchestrator in this system [65]. Specifically, plasmin is responsible for inactivating coagulation factors (FV, FVIII, FIX, and FX), breaking down the deposited fibrin into soluble fibrin degradation products and degrade base membranes or extracellular matrices to promote tissue remodelling and facilitate cell migration [63, 65]. As for the vECs, these cells play several roles in haemostasis maintenance and thrombus development. In fact, these cells can express and/or release a set of proteins that modulate the platelet activity, the coagulation cascade and fibrinolysis [52].

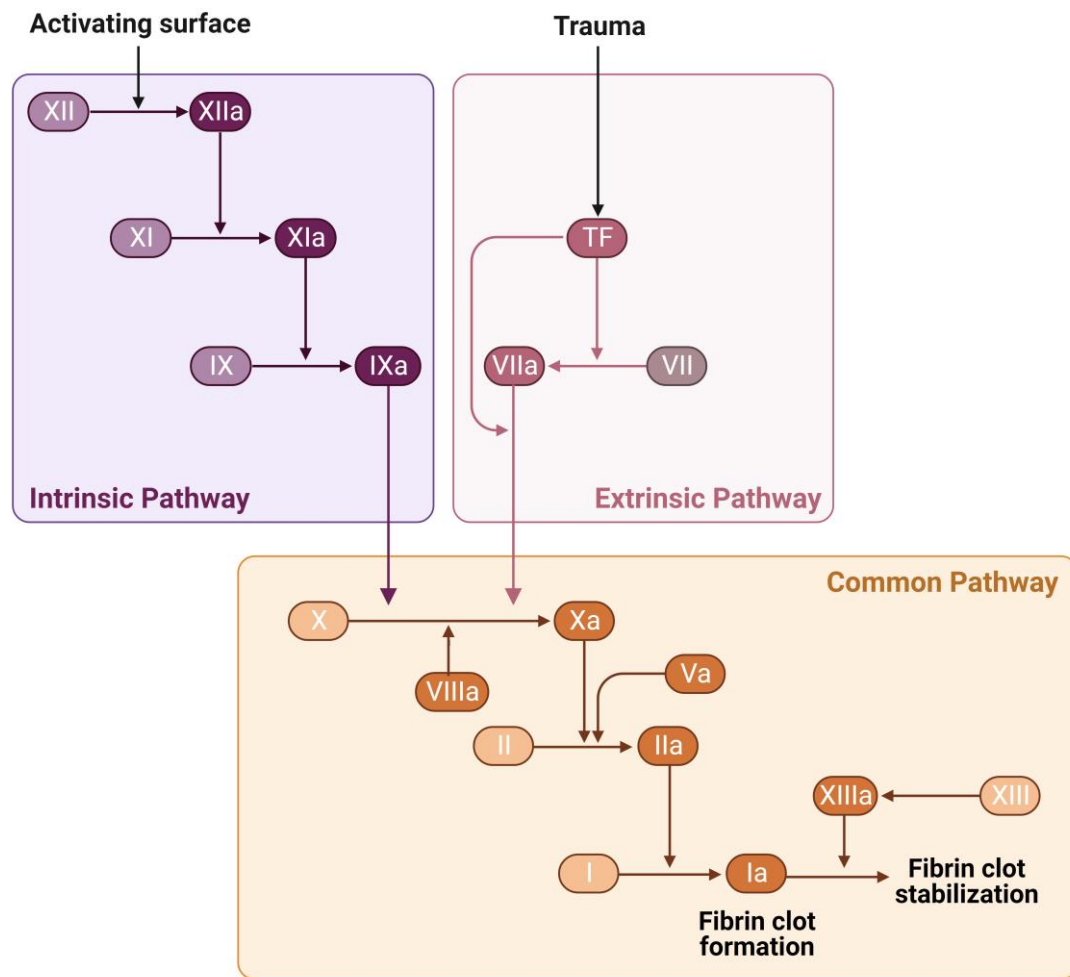


Figure 4. Blood coagulation pathways based on the waterfall model.

The coagulation cascade encompasses a series of sequential proteolytic activation reactions that culminate in fibrin formation and can be divided into three major coagulation pathways: extrinsic (also called the tissue factor (TF) coagulation pathway) and intrinsic (contact coagulation pathway), which converge on a common coagulation pathway. Abbreviations: XII: coagulation factor XII; XIIa: activated coagulation factor XII; XI: coagulation factor XI; XIa: activated coagulation factor XI; TF: Tissue Factor; VII: coagulation factor VII; VIIa: activated coagulation factor VII; IX: coagulation factor IX; IXa: activated coagulation factor IX; X: coagulation factor X; Xa: activated coagulation factor X; VIIIa: activated coagulation factor VIII; Va: activated coagulation factor V; II: coagulation factor II or prothrombin; IIa: activated coagulation factor II or thrombin; I: coagulation factor I or fibrinogen; Ia: activated coagulation factor I or fibrin; XIII: coagulation factor XIII; XIIIa: activated coagulation factor XIII. The figure was created with BioRender.com.

When one of the four main components of the haemostatic system is malfunctioning, the haemostatic balance can be disturbed leading to bleeding or thrombotic disorders [51]. Deep vein thrombosis (DVT) and pulmonary embolism (PE) are two thrombotic events that collectively are known as venous thromboembolism (VTE). The former occurs when a thrombus forms within a deep vein (usually leg veins) leading to abnormal swelling and ulceration [66-69]. As for PE, this is a frequent complication of DVT that occurs when the thrombus migrates to the lungs, representing the most fatal VTE manifestation. Worldwide, every year VTE affects nearly ten million people [10]. If not treated properly,

this cardiovascular disorder can be lethal, resulting in approximately 15% of related mortality within the first three months following diagnosis [67-69]. However, even with successful treatment, the disease is also associated with a spectrum of potential complications that lead to substantial morbidity, namely VTE recurrent events, post-thrombotic syndrome, pulmonary hypertension, major bleeding under anticoagulation therapy and long-term disability [70, 71]. Currently, anticoagulation therapy is the standard therapeutic approach for VTE, which, however, has proven to not always be efficient in the removal of the existing thrombus. Thus, it is crucial to dissect the molecular pathways involved in VTE pathogenesis to identify novel biomarkers, improve risk stratification, disease prevention and treatment [66, 71, 72].

Like other common diseases in the general population, VTE has a complex pathophysiology with both acquired and genetic risk determinants modulating the disease susceptibility. Advanced age, comorbidities, physical trauma, obesity, prolonged immobilization, previous VTE events and active cancer constitute the most common acquired risk factors [70, 73-75]. As for genetic factors, Factor V Leiden gene polymorphism (*F5* rs6025), the prothrombin gene polymorphism 20210 (*F2* rs1799963), mutations causing inherited deficiencies of antithrombin, protein C and protein S, as well as the antiphospholipid antibody syndrome, account for about 7% to 22% of the elderly population attributable risk [73, 74]. These factors can trigger what is known as Virchow's triad, which encompasses three conditions that prompt VTE development, namely blood hypercoagulability, stasis of blood flow within the vessels (i.e., venous stasis) and vascular endothelial damage [76].

As Virchow's triad postulates, endothelial injury is a major risk factor for thrombogenesis [77]. As previously mentioned, vECs cells assume important roles in the maintenance of haemostasis and thrombus development [78]. In fact, several studies have confirmed the close relationship between vECs and the occurrence of DVT [79]. Under physiological conditions, these cells act as a barrier, preventing coagulation factors and platelets from activating and forming blood clots. Additionally, they secrete anticoagulant factors such as TFPI1, nitric oxide and prostacyclin. They also exert an anticoagulant activity by expressing molecules such as heparin-like molecules, as well as thrombomodulin and endothelial protein C receptor (EPCR), the latter two implicated in the protein C anticoagulant pathway. Besides the anticoagulant activity, vECs also favour fibrinolysis via tissue-type plasminogen activator (tPA) production, which by converting plasminogen to plasmin, dissolves fibrin clots [80]. Contrarily, under pathological conditions (for instance inflammation), vECs can express adhesion molecules such as P-selectin, which facilitates blood clot formation. Inclusively, under these conditions, these cells can become dysfunctional, which compromises their antithrombotic properties, increasing

VTE risk [81-84]. In addition to mature vECs, two cell populations are of relevance when it comes to dissecting the endothelium biology, namely the endothelial progenitor cells (EPCs) and human umbilical vein endothelial cells (HUVECs) (**Figure 5**). The former are multipotent cells derived from bone marrow that circulate in the peripheral blood and can differentiate into mature vECs when needed [72, 79]. Inclusively, studies have already shown that EPCs have the potential to prevent thrombus propagation and recurrence and to promote endothelial regeneration, revascularization, and vein wall remodelling [79, 85]. When there is a vascular injury, a series of mechanisms are triggered to repair the damage. One of these mechanisms is angiogenesis, during which new vessels are formed from pre-existing ones, allowing the restoration of normal vascular function and thrombus resolution [79, 86]. During this process, several angiogenic factors are released such as VEGFs, fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs), which together promote angiogenesis and stimulate the proliferation and migration of vECs to the injury site to help in the repair process [84]. Additionally, vECs can bind to platelets and inhibit their role in thrombus formation [87, 88]. As for HUVECs, they are primary cells present in the vein of the umbilical cord, which are used as a model system for studying the biology of the endothelium and its response to environmental cues, angiogenesis, and the development of cardiovascular diseases [89, 90].

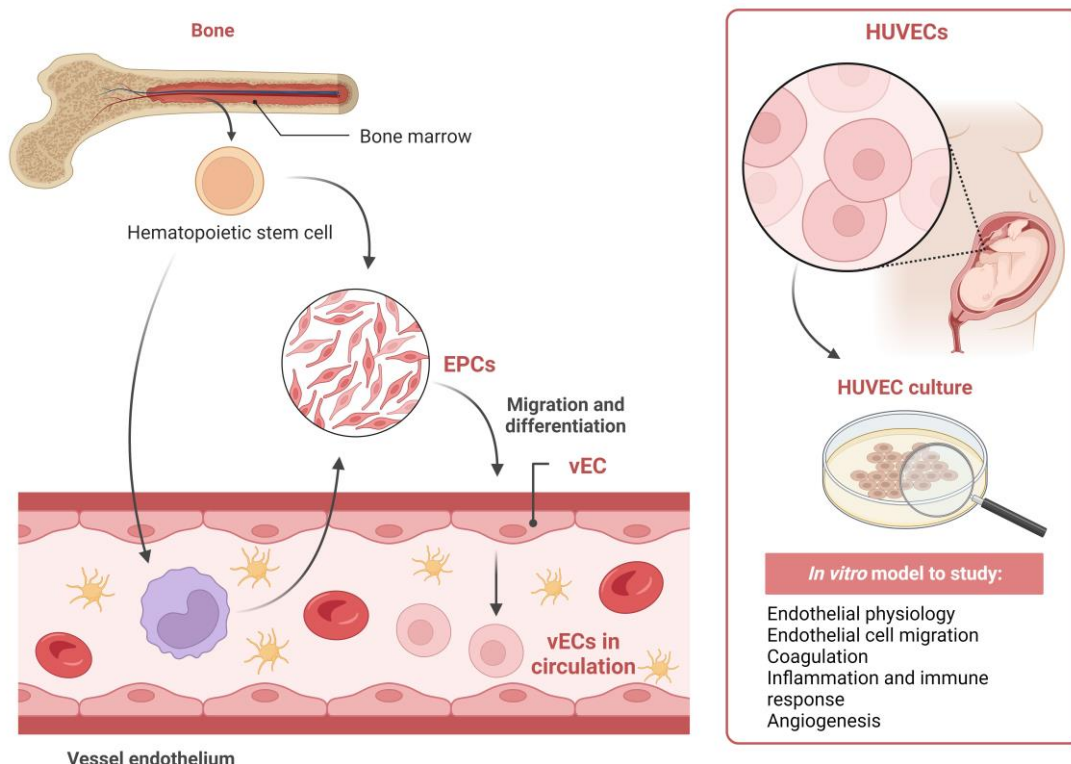


Figure 5. Biology of vascular endothelial cells.

Vascular endothelial cells (VECs) encompass three populations of relevance: mature VECs, endothelial progenitor cells (EPCs) and human umbilical vein endothelial cells (HUVECs). While EPCs are multipotent cells derived from bone marrow that circulate in the peripheral blood and can differentiate into mature VECs when needed, HUVECs are primary cells present in the vein of the umbilical cord and are commonly used as an *in vitro* model system for studying the biology of the endothelium, in addition to other processes such as angiogenesis, coagulation and cardiovascular diseases. Abbreviations: EPC, endothelial progenitor cell; HUVEC, human umbilical vein endothelial cell. Figure retrieved from Marques *et. al* [53].

The most clinically used biomarker for VTE prediction and diagnosis is D-dimer (DD), which is a product resultant from fibrinolysis [91]. In addition to first VTE events, DD is also associated with the rate of recurrent events and mortality [92]. This thrombotic biomarker was first identified in the 1980s and has been used since in clinical practice, particularly given its long half-life [91, 93]. However, DD levels are significantly elevated in several other conditions, such as inflammation, stroke, long hospitalization periods and individuals' advanced age, being unspecific of VTE presence [91, 93, 94]. To improve the specificity in detection, DD testing is combined with other diagnostic methods including duplex ultrasonography for DVT and/or computed tomography for PE diagnosis [95, 96]. Other suggested VTE biomarkers incorporate markers for thrombophilia, for instance, soluble P-selectin and C-reactive protein, which are still under evaluation and can also lack specificity and/or sensitivity [97, 98]. Thus, novel and trustworthy biomarkers are required to reduce the time of diagnosis, and consequently improve the morbidity and mortality rates associated with VTE.

3. Cancer associated-thrombosis

Cancer-associated thrombosis (CAT) can refer to both arterial and venous thromboembolic events among cancer patients. However, there's still scarce evidence concerning arterial events contrary to the greatly heightened VTE risk in this group [99]. Therefore, cancer-associated VTE is the focus of the present work.

Cancer-related VTE was first described by Trousseau in 1865 in his chapter titled *Phlegmasia alba dolens* [100]. Unfortunately, he himself ended up suffering from gastric cancer and was conscious about the lethal outcome of his illness. Now, 156 years after his death, cancer-related VTE, also known as Trousseau syndrome, is a more well-known phenomenon, although not yet completely understood [101]. Cancer patients are four to seven times more prone to develop VTE compared to those without malignancy. In fact, cancer is an independent predictor of venous thrombogenesis, with approximately 15% of the patients presenting at least one VTE episode during their therapeutic intervention [102, 103]. Moreover, cancer-related VTE (including mainly VTE

events occurring six months before to two years after cancer diagnosis) accounts for 20% of the overall VTE incidence [104, 105]. Inversely, cancer risk is elevated at least 2 years after a first idiopathic episode of VTE [103, 105, 106]. Beyond being common, CAT is the second most common cause of death among these patients, the first being the cancer itself [107]. Given the implications for the prognosis of oncological patients, this condition has been widely studied over the years.

Interestingly, even in the absence of thrombosis, most cancer patients display haemostatic changes that can be detected with laboratory coagulation tests [108]. Namely, prothrombin time (PT) and activated partial thromboplastin time (aPTT) are coagulation tests used to evaluate the functions of most of the coagulation factors involved in the coagulation cascade [109, 110]. In turn, the international normalized ratio (INR) is a mathematical derivation done to the PT result when monitoring anticoagulant therapy, ensuring that the results are standardized when considering thromboplastin and the instrument used [111]. While PT and INR quantify the activity of the extrinsic route, aPTT, on the other hand, evaluates the intrinsic coagulation pathway [112]. These tests thus reflect the coagulation and haemostatic status and might suggest the presence of abnormalities in these processes [112, 113]. However, their interpretation is highly context-dependent. Oncological patients often exhibit abnormal results in these tests, indicating the presence of a subclinical hypercoagulability status, which reinforces that coagulation is closely intertwined with cancer [108].

The pathogenesis of CAT can be explained by factors related to the patient (mentioned in section 2. venous thromboembolism), the treatments and the cancer itself, with the latter two carrying a more preponderant role (**Figure 6**) [75, 114]. Concerning the treatment, antineoplastic therapies are known to significantly increase the risk of VTE among cancer patients, even though the underlying mechanisms are not yet completely understood [115]. However, induction of TF procoagulant activity, reduction of endogenous anticoagulants, vascular endothelial damage and activation of platelets are some of the reported mechanisms [116]. In the context of OC treatment, the currently used protocols, namely the cytotoxic platinum-based chemotherapy, pelvic surgery and the use of antiangiogenic-agents such as Bevacizumab, elevate the incidence of VTE. Among OC patients, the incidence of VTE after surgery or during postoperative chemotherapy revolves around 13 – 27% and about 75% of thrombotic events occur within the first week of the postoperative period. Moreover, the conditions often present after surgery, such as long periods of rest, postoperative complications or prolonged stay in the hospital, also aid in the risk of VTE in these patients [117]. Plus, asymptomatic VTE events are frequently detected before treatment, and so, they might progress and manifest after surgery [118].

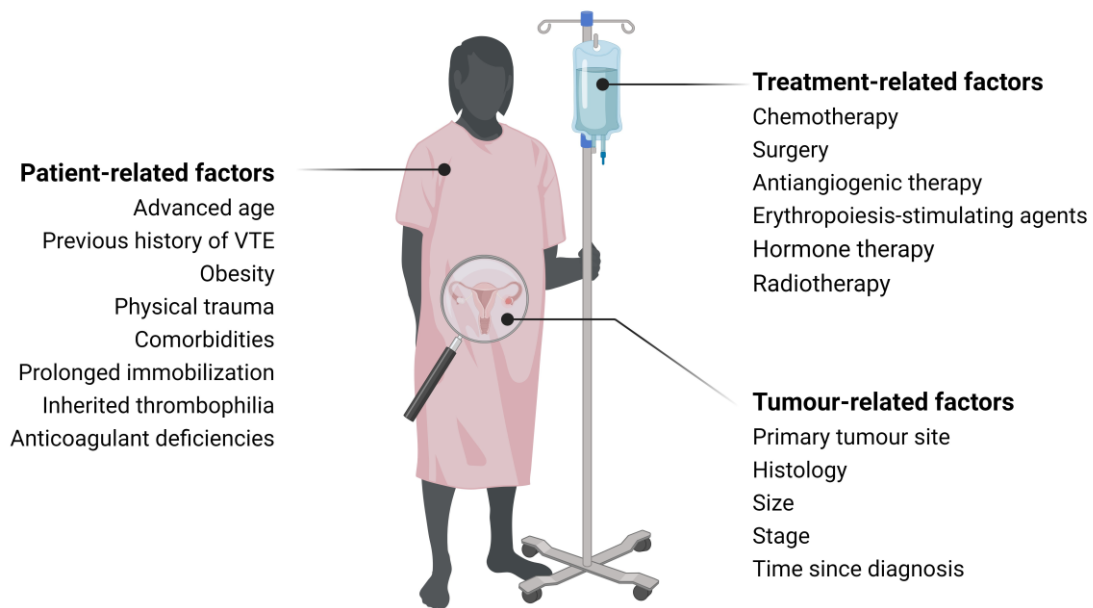


Figure 6. Risk determinants associated with cancer-related venous thromboembolism.

The risk factors related to the pathogenesis of cancer-related venous thromboembolism can be grouped into patient- (as advanced age), treatment- (as chemotherapy) and cancer-related factors (as primary tumour site), with the latter two carrying a more preponderant role in this population subgroup. The figure was created with BioRender.com.

Regarding the cancer-related factors, the primary tumour site, histology, size and stage, as well as the time since cancer diagnosis constitute important clinical factors in the setting of VTE among oncological patients [75]. The impact of these factors relies on the bidirectional well-known phenomenon between the tumour and the host coagulation system, whereas the tumour activates the haemostatic system, which in turn supports cancer progression (**Figure 7**) [119]. Specifically, tumour cells are recognized for promoting thrombogenesis via direct or indirect mechanisms, with the latter involving the activation of platelets, vECs and immune cells [75]. Focusing on the direct mechanisms, TF plays a crucial role in VTE occurrence. Several tumours are known to overexpress TF, which as mentioned before, triggers the extrinsic coagulation pathway, generating FXa. Parallely, these cells can also produce cancer procoagulant (CP) that also activates FX, leading to the amplification of the coagulation cascade. Plasminogen activator inhibitor-1 (PAI-1) is also secreted by tumour cells during this process and is responsible for inhibiting the fibrinolytic process contributing to thrombogenesis [105, 120]. Regarding the indirect mechanisms, tumour cells can release promoters of platelet activation (e.g., podoplanin (PDPN), adenosine diphosphate (ADP) and thrombin), proangiogenic factors (e.g., VEGF and PDGF), adhesive or aggregating factors (e.g., phosphatidyl serine (PS)) and proinflammatory cytokines (e.g. tumour necrosis factor

alpha (TNF- α), interleukin (IL)-1 β (IL-1 β), IL-6 and IL-8) [75, 121, 122]. Like platelets and vECs, immune cells under the influence of tumour cells are also key players in thrombogenesis, specifically in a process called immunothrombosis [123]. Proinflammatory cytokines and adhesion molecules released by the cancer are responsible for the recruitment and activation of leucocytes, such as monocytes and neutrophils [108]. When activated, monocytes are conditioned to express TF at their surface and release TF-positive microparticles, which contribute to the generation of a prothrombotic cascade. Likewise, neutrophil extracellular traps (NETs) can serve as a structure able to capture and activate platelets, leading also to TFPI1 inactivation, fibrin buildup and capturing of red blood cells, all inducing and worsening the formation of blood clots [121, 122, 124]. In addition to the above-mentioned risk factors, some cancers can even increase the number of platelets and leucocytes, which also increases the risk of VTE [114]. Thrombocytosis, defined as a high platelet count, occurs frequently among oncological patients, namely with ovarian, breast, lung and gastrointestinal tumours. The condition alongside elevated levels of IL-6 is considered an independent predictive factor of VTE among those with ovarian CCC [115]. In mice with ovarian tumours, tumour-derived IL-6 was shown to induce the expression of hepatic thrombopoietin, which stimulates the production of megakaryocytes and thrombocytosis [125]. Additionally, approximately one-third of the patients newly diagnosed with OC present thrombocytosis, which is associated with reduced survival [125]. Conversely, the components responsible for normal blood clotting that are exploited by tumour cells can also drive the tumour's advancement, for example by favouring tumour growth, neoangiogenesis, immune evasion and metastasis [75]. As a consequence of these complex interactions, the tumour behaviour becomes more aggressive and individuals with active cancer become more prone to develop thrombotic events, worsening their overall prognosis [126, 127].

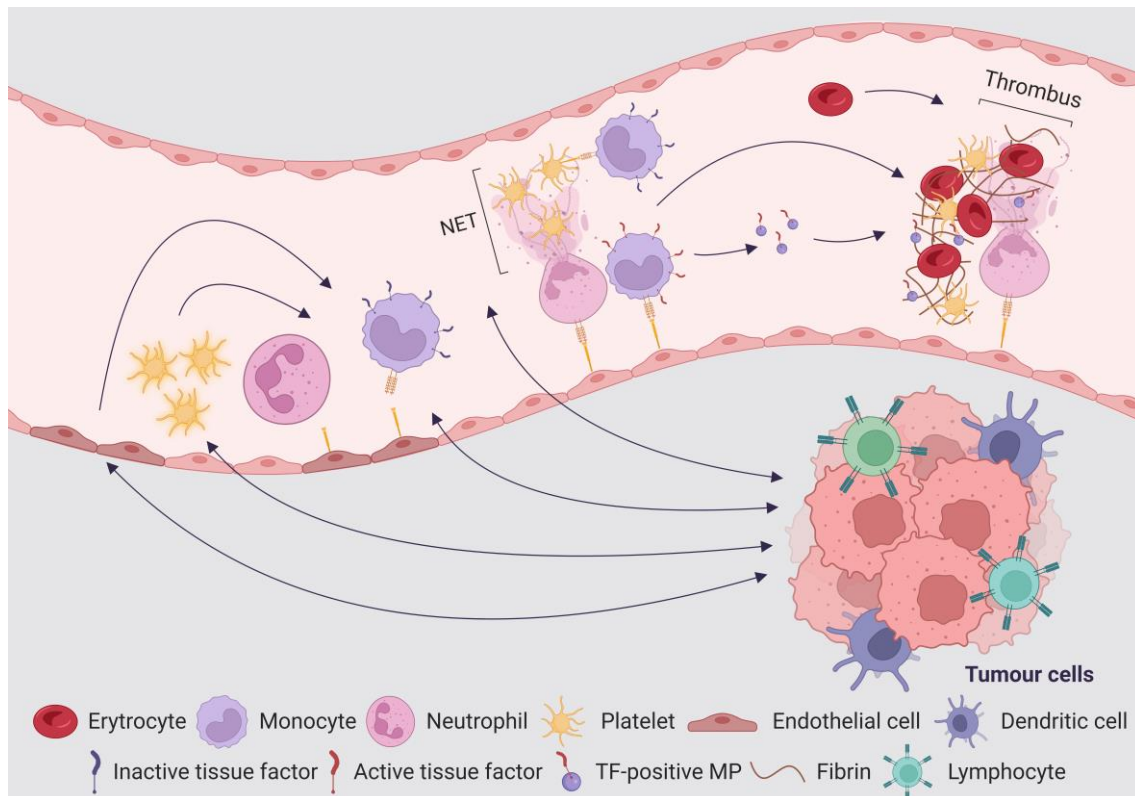


Figure 7. Crosstalk between tumour cells and platelets, leukocytes, and endothelial cells.

There is a well-known bidirectional phenomenon between the tumour and the host coagulation system, where the tumour affects the haemostatic system, while the coagulation system promotes tumour growth. Tumour cells are recognized for initiating a cascade that promotes blood clot formation. In this process, the activation of platelets, vascular endothelial cells and immune cells (such as monocytes and neutrophils) occurs. Conversely, these components are also responsible for secreting several factors (such as inflammatory cytokines and chemokines), known to enhance tumour cells. Consequently, the tumour behaviour becomes more aggressive and individuals with active cancer become more prone to develop thrombotic events, worsening their overall prognosis. Abbreviations: NET: neutrophil extracellular trap; TF-positive MP: tissue factor positive microparticle. The figure was created with BioRender.com.

Pancreas, lung and stomach cancers are commonly associated with an elevated risk of VTE, while prostate, breast and skin cancers are generally associated with a relatively low risk [115, 121]. Among gynaecological malignancies, which are linked to high rates of VTE, endometrial cancer presents a VTE risk of approximately 6%, while cervical cancer is associated with a risk ranging from 0 to 34% [128, 129]. Inclusively, in a previous study conducted by our research group, a VTE incidence of 8.5% was reported among 400 cervical cancer patients [130]. As for OC, despite the inconsistent data, the disease is considered to have an intermediate-to-high associated risk, with an incidence ranging from 5% to over 20% depending on the time of follow-up, cancer subtype and stage and treatments [131-138]. Interestingly, OC patients present mostly silent or asymptomatic thromboembolic events, meaning without clinical manifestation [118]. The subtype of EOC, as well as the stage and histological grade, influences VTE-risk heavily [117]. Among the different subtypes, CCC is the one associated with the highest risk of VTE development [139]. The same can be said for advanced stages and high histological

grades of ovarian malignancies. In advanced stages, massive ascites might occur, and their presence is considered an independent risk factor for VTE due to the accumulation of fluids that, by compressing pelvic veins, can impair blood flow [118, 140, 141].

Identifying cancer patients who are at a higher risk of developing VTE is crucial to better tailor prevention (thromboprophylaxis) strategies. Although other risk assessment models (RAMs) have been proposed throughout the years, the Khorana score (KS) remains the most studied one to assess cancer-related VTE susceptibility. This score was first introduced in 2008 and it is currently used in the clinical practice to select cancer patients suitable for thromboprophylaxis. This assessment comprises five predictive factors: the site of the primary tumour and the prechemotherapy haemoglobin level, platelet and leucocyte counts and body mass index (BMI) (**Figure 8**) [116, 142]. Based on the original cut-off score, VTE risk can be divided into 3 categories: low (score 0), intermediate (score 1-2) and high (score ≥ 3). In a median follow-up period of 2.5 months, the rates of symptomatic VTE in the categories of low, intermediate, and high risk are 0.3%, 2%, and 6.7% respectively [142]. More recently, the optimal cut-off score was defined as ≥ 2 (0 as low, 1 as intermediate and 2 or higher as high risk) [115]. This RAM is extremely useful since not every patient presents the same risk for VTE and, as such, not all of them should receive anticoagulation treatment due to the high risk of major bleeding [105]. However, KS as well as other proposed RAMs have demonstrated limited sensitivity [75, 143-145]. Consequently, novel risk stratification methods are imperative to avoid the deterioration in prognosis for these individuals.

PATIENT CHARACTERISTICS	RISK SCORE
Site of cancer	
Very high risk (stomach, pancreas)	2
High risk (lung, lymphoma, gynaecologic, bladder, testicular)	1
Prechemotherapy platelet count $\geq 350 \times 10^9/L$	1
Haemoglobin level $< 100g/L$ or use of red cell growth factors	1
Prechemotherapy leukocyte count $> 11 \times 10^9/L$	1
Body mass index $\geq 35 \text{ kg/m}^2$	1

Figure 8. Khorana risk score.

Khorana risk stratification score is used in the clinical practice to select cancer patients suitable for thromboprophylaxis (i.e., preventive measures taken to reduce the risk of blood clot formation) and comprises five predictive factors. In initial studies, the original cut-off score was low risk, 0; intermediate risk, 1-2; high risk, ≥ 3 . In recent studies, the score of ≥ 2 has been used as the preferred cut-off score, where: low risk, 0; intermediate risk, 1; high risk, ≥ 2 [115, 142]. The figure was created with BioRender.com.

Thromboprophylaxis should be considered in outpatient, hospitalized, and postoperative scenarios. Also, even though primary prophylaxis in ambulatory CAT is controversial, in surgical cancer patients, mainly those submitted to major abdominal procedures, prophylactic anticoagulation is recommended for at least 30 days [105]. As cancer-related VTE treatment, the safety and efficacy of anticoagulation differs compared to the disease management among non-cancer patients. The primary issues include major bleeding and VTE recurrence, which correlates to cancer extension [105]. The currently recommended anticoagulants for CAT treatment are low-molecular-weight heparins (LMWH) and direct oral anticoagulants (DOAC). The former binds to antithrombin III, while DOACs act by inhibiting either thrombin or FXa, depending on the drug used [146]. Several variables must be taken into account when deciding on which one of them should be given to the patient, namely the primary tumour site, the risk for haemorrhaging, the performance status, eventual drug-drug interactions, the presence of comorbidities and the patient's choice [75].

Summarizing, cancer-related VTE is an important health burden, which drastically affects the patient's prognosis. Identifying predictive biomarkers is crucial to design prevention strategies. In addition, these biomarkers could be valuable tools to assess prognosis among cancer patients. Conversely, over the past few years, emerging evidence has been suggesting a role of long non-coding RNAs (lncRNAs) in thrombosis [53].

4. Long non-coding RNAs

lncRNAs are non-coding RNAs (ncRNAs) longer than 200 nucleotides with the particularity of forming secondary structures, which enables them to bind to other biomolecules such as DNA, RNA and even proteins, thus affecting gene expression at different levels [147-150]. Another feature of these ncRNAs is the fact that they can function in the nucleus, where they are transcribed, but also at the cytoplasm, with most of them operating in both. In fact, lncRNAs can move between the two compartments in response to signals, even though the underlying mechanisms are unclear [151]. Similarly to messenger RNAs (mRNAs), lncRNAs are also transcribed by RNA Polymerase II and are often capped and polyadenylated, presenting also evolutionarily conserved patterns and a high level of tissue specificity [150].

According to the NONCODE database (version 6.0) (<http://www.noncode.org>), there are 96,411 human lncRNA genes [152]. Based on their genomic position, orientation, and relative location to nearby protein-coding genes, these ncRNAs can be classified as intergenic, antisense, sense, intronic, and bi-directional (**Figure 9**) [151]. Specifically,

intergenic lncRNAs (lincRNAs), as the name indicates, don't overlap with any other genes [153]. As for those that do overlap, they can be sense or antisense depending on whether they possess sequence elements that will pair with other RNAs in the same or the opposite direction, respectively [154, 155]. Intronic lncRNAs are located within the introns of other genes, while bi-directional lncRNAs correspond to those that are transcribed from the same region in the genome as another gene but in the opposite direction [151]. In addition to linear lncRNAs, another class is circular lncRNAs, which are formed by back-splicing of coding and non-coding transcripts [156].

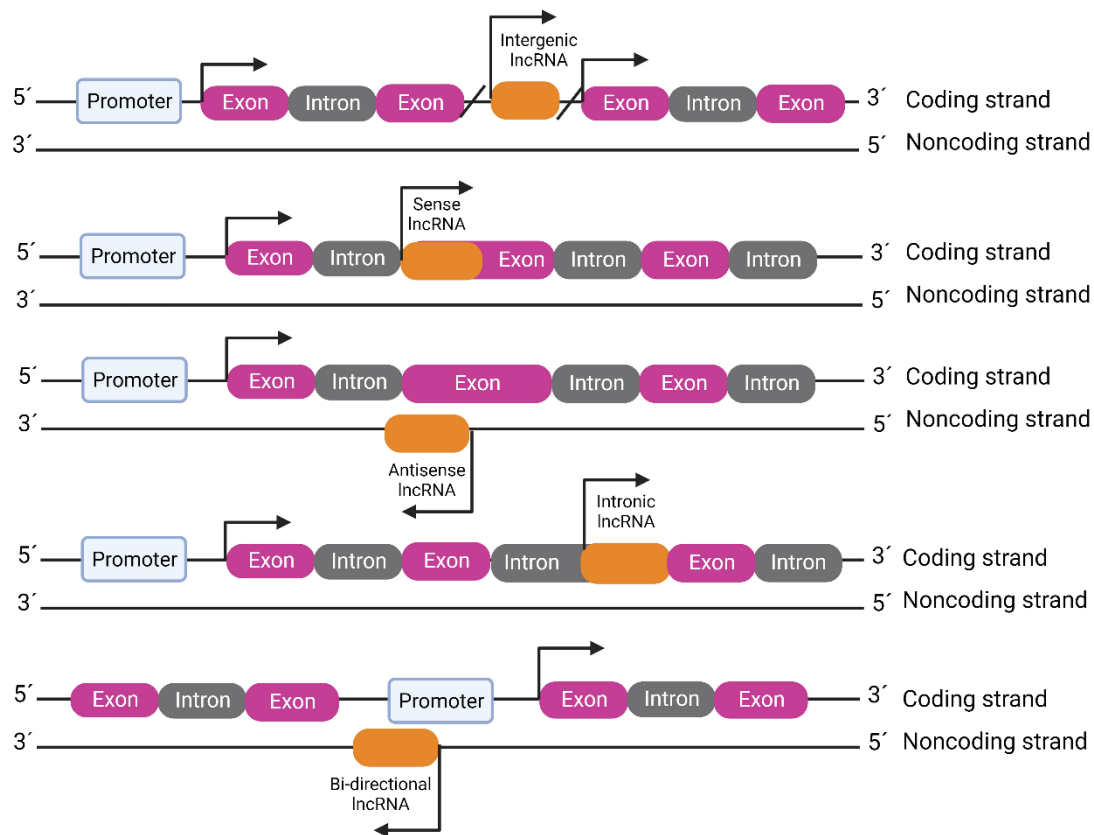


Figure 9. Genomic localization of long non-coding RNAs (lncRNAs).

lncRNAs can be categorized as intergenic, sense, antisense, intronic and bi-directional, based on their genomic position, orientation and proximity to neighbouring protein-coding genes. Figure retrieved from Marques *et. al* [53].

Despite the inconsistency in classification, the roles of lncRNAs in gene expression regulation can be divided into four main non-exclusive archetypes: signalling, decoy, guide, and scaffold functions (**Figure 10**) [148]. As signalling molecules, lncRNAs can operate as spatiotemporal markers, reflecting the biological effects of transcription factors or signalling pathways. As decoys, lncRNAs can sequester transcription factors and other proteins into nuclear subdomains or away from chromatin. Decoy lncRNAs can also operate as competitive endogenous RNAs (ceRNAs), functioning as microRNA (miR or miRNA) sponges, consequently influencing the expression of miRNAs' targets

(i.e., mRNAs). On the other hand, guide lncRNAs can recruit RNA-binding proteins to target genes, while scaffolds may bring together several proteins to build complexes with specific biological roles, influencing transcription. Of note, lncRNAs localized in the nucleus can modify chromatin, and activate or repress transcription of target genes by interacting with DNA sequences or transcription factors, while cytoplasmic lncRNAs are mostly associated with mRNA stability and translation, promoting or repressing protein synthesis [150, 157, 158]. The versatility in these functions (chromatin regulation, transcriptional activation and repression, and RNA and protein modifications) makes lncRNAs important players in normal physiology and in the development of a variety of diseases, such as cancer, cardiovascular and neurodegenerative diseases, in which their dysregulated levels have been consistently observed [67, 72, 150, 157, 159-166]. Despite their lower abundance, lncRNAs possess specific traits that make them promising biomarkers for these conditions with clinical applicability, namely convenient accessibility, potential detection in bodily fluids (such as whole blood, serum, plasma and urine), frequent encapsulation (e.g., within extracellular vesicles, proteins and apoptotic bodies, which protect them from RNase cleavage), and cell and/or tissue and disease-specificity [167, 168]. However, the study of these ncRNAs is still in its early stages [169].

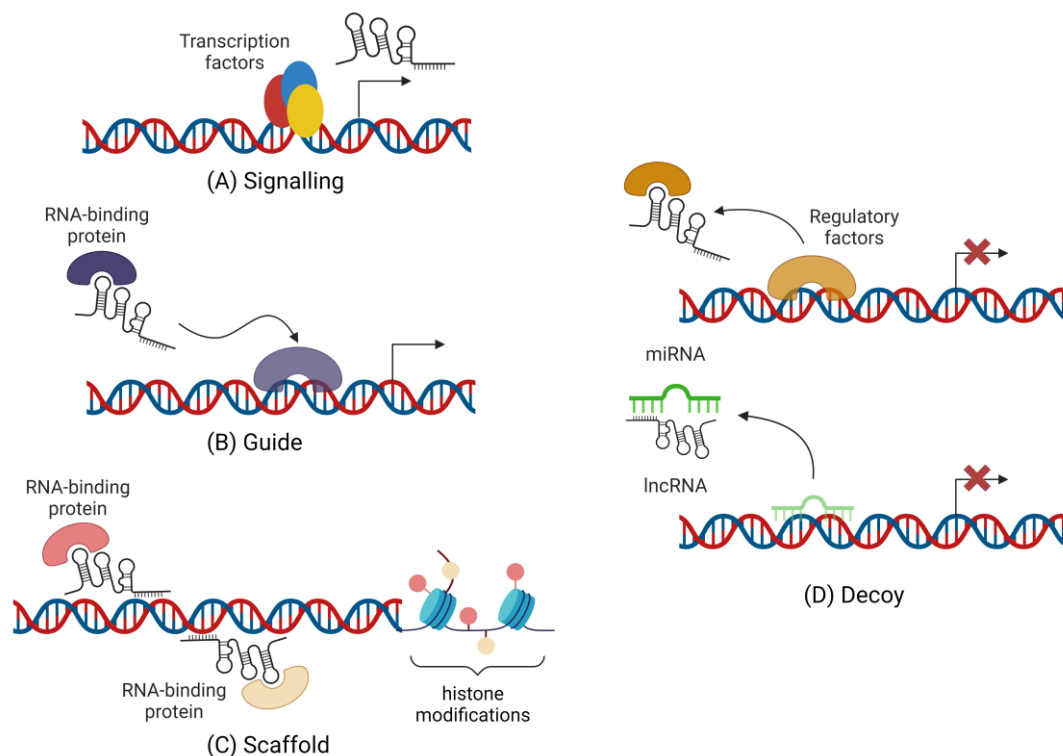


Figure 10. Four archetypes of long non-coding RNA (lncRNA) functions.

Overall, the functions of lncRNAs in regulating gene expression can be broadly categorized into four main non-exclusive archetypes: signalling, decoy, guide and scaffold functions. Figure retrieved from Marques *et al* [53].

The first report of a putative implication of lncRNAs in cardiovascular diseases came from genome-wide association studies (GWAS). These studies independently identified single-nucleotide polymorphisms (SNPs) located in a *locus* close to the protein-coding genes *cyclin dependent kinase inhibitor 2A* (*CDKN2A*) and *cyclin dependent kinase inhibitor 2B* (*CDKN2B*) as related to susceptibility to coronary artery disease [170, 171]. This *locus* (9p21) is adjacent to the lncRNA named antisense non-coding RNA in the *INK4 locus* (*ANRIL*), indicating a potential role of this lncRNA in coronary artery disease, which was later corroborated by subsequent studies [172]. In the context of VTE, reported lncRNAs include antisense non-coding RNA in the *INK4 locus* (*ANRIL*), *GUSB* pseudogene 5 antisense (*GUSBP5-AS*), maternally expressed gene 9 (*MEG9*), metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), sirtuin1 antisense lncRNA (*Sirt1-AS*), long intergenic non-protein coding RNA 1123 (*LINC01123*), long intergenic non-coding RNA 00659 (*LINC00659*), colorectal neoplasia differentially expressed (*CRNDE*), taurine upregulated gene 1 (*TUG1*), maternally expressed 8 (*MEG8*), nuclear enriched abundant transcript 1 (*NEAT1*; more recently designed as nuclear paraspeckle assembly transcript 1) and X inactive-specific transcript (*XIST*). These RNAs impact VTE through several mechanisms, namely the direct promotion of clot formation, regulation of platelet and vECs activity and modulation of thrombus resolution (**Figure 11**) [53, 173].

Additionally, given the central role of the coagulation system, lncRNAs targeting this system may have a role in VTE pathogenesis. Conversely, in recent years, several *TFPI2*-targeting lncRNAs have been pinpointed in the context of other disorders, including glioblastoma, preeclampsia, non-small-cell lung cancer and ischemic heart disease [53]. The list of lncRNAs encompasses maternally expressed 8 (*MEG8*), *TFPI2* antisense RNA 1 (*TFPI2AS1*), AC003092.1, *AGAP2* antisense RNA 1 (*AGAP2-AS1*) and long intergenic non-protein coding RNA 473 (*LINC00473*). In the context of VTE, they may achieve this interaction either by upregulating *TFPI2* expression (as seen in *TFPI2AS1* and AC003092.1) or by repressing it (as observed in *MEG8*, *AGAP2-AS1* and *LINC00473*).

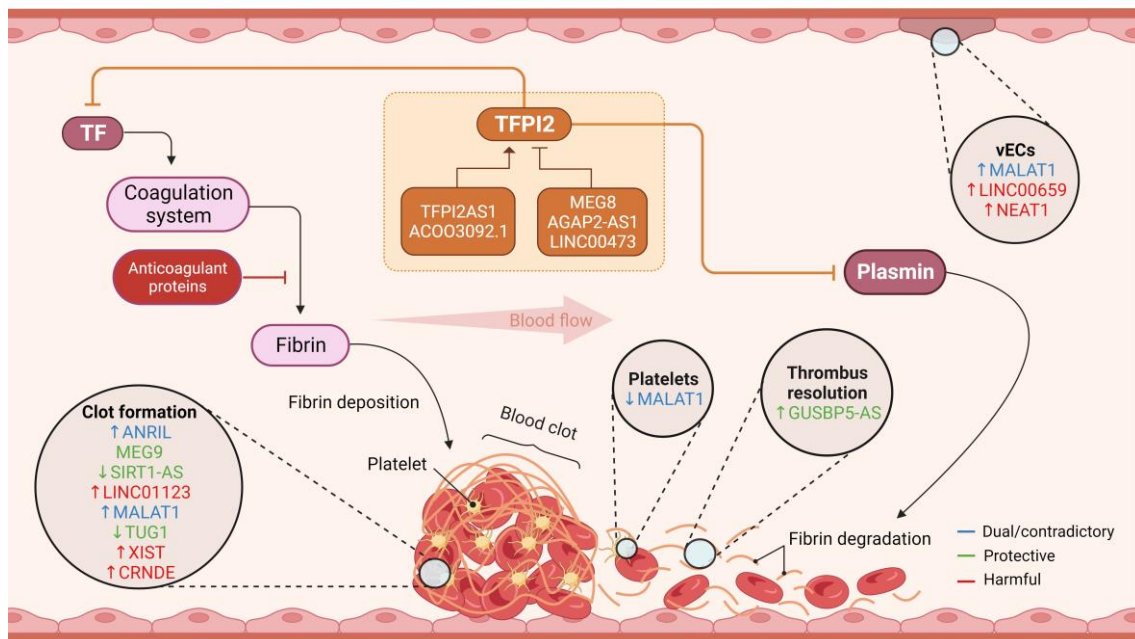


Figure 11. Established and potential roles of long non-coding RNAs (lncRNAs) in venous thromboembolism (VTE).

Several lncRNAs target the coagulation system, and in most cases, their expression is deregulated in VTE. In orange are the lncRNAs whose relationship with VTE has not yet been established; however, these can impact VTE through their interaction with TFPI2 and, consequently, with TF, a strong blood coagulation initiator, or plasmin, a fibrin-dissolving enzyme. Abbreviations: MEG8, maternally expressed 8; TFPI2AS1, TFPI2 antisense RNA 1; ACO03092.1, long non-coding RNA ACO03092.1; AGAP2-AS1, AGAP2 antisense RNA 1; LINC00473, long intergenic non-protein coding RNA 473; ANRIL, antisense non-coding RNA in the INK4 locus (ANRIL); GUSBP5-AS, GUSB pseudogene 5 antisense; MEG9, maternally expressed gene 9; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; SIRT1-AS, sirtuin1 antisense lncRNA; LINC01123, long intergenic non-protein coding RNA 1123; LINC00659, long intergenic non-coding RNA 00659; CRNDE, colorectal neoplasia differentially expressed; TUG1, taurine upregulated gene 1; NEAT1, nuclear enriched abundant transcript 1 (recently renamed to nuclear paraspeckle assembly transcript 1); XIST, X inactive-specific transcript. LncRNA expression: ↓ downregulated and ↑ upregulated (for MEG9, there are no data on the expression levels). The figure was created with BioRender.com.

Given the influence the haemostatic system has on tumour growth and progression, the high incidence of VTE among OC patients and the negative impact the disease imposes on the prognosis of these patients, VTE-associated lncRNAs may not only influence the pathogenesis of OC-related VTE but might also have a prognostic value in these patients, particularly in an era where liquid biopsies are gaining visibility. Thus, these lncRNAs potentially represent attractive novel biomarkers of OC, which are currently needed to improve the patient's clinical outcome.

Aims

II. Aims

2.1. Main aim

Given the clinical implications, the main objective of the present study was to investigate the impact of lncRNAs underlying VTE pathophysiology on the incidence of OC-related VTE and their prognostic value among patients with EOC regardless of thrombogenesis.

2.2 Specific aims

To accomplish the main aim, the study was designed to target the following tasks:

- [A] Perform a literature review regarding lncRNAs associated with VTE;
- [B] Select the most relevant lncRNAs to be evaluated in the present study;
- [C] Dissect the implications of VTE in OC patients' prognosis;
- [D] Assess the association of the selected lncRNAs with the patients' demographical and clinicopathological features, baseline full blood count and coagulation tests;
- [E] Explore the impact of chemotherapy on the expression levels of these lncRNAs;
- [F] Investigate the role of the evaluated lncRNAs as predictors of OC-related VTE;
- [G] Analyse the prognostic value of these lncRNAs regardless of VTE status.

Methodology

III. Methodology

1. Population description

A retrospective cohort study was performed with adult European-descent patients from the North region of Portugal with a confirmed diagnosis of EOC, admitted from March 2017 to April 2022 to the Clinic of Gynaecology of the Portuguese Institute of Oncology of Porto (IPOP) for first-line treatment. In the study, patients were excluded if they: 1) had other malignant diseases prior to or after OC diagnosis, 2) were pregnant or breastfeeding; 3) had autoimmune diseases and/or were immunosuppressed; 4) had an acute infection at cancer diagnosis; 5) were under anticoagulation due to other diseases rather than VTE and 6) had factor V Leiden and prothrombin G20210A polymorphisms. Applying these criteria, a total of 40 OC patients were enrolled, for whom biological material before the first line of platinum-based chemotherapy was accessible in the biobank of Molecular Oncology and Viral Pathology (GOMPV) research group at IPOP. Most patients had serous carcinoma (33 (82.5%)), followed by clear cell (3 (7.5%)), unusual subtypes (2 (5%)), endometrioid (1 (2.5%)) and mixed EOC (1 (2.5%)).

Disease staging was conducted according to the FIGO staging system updated in 2021 [174]. Considering the existing evidence, cancer-associated VTE was defined as an event occurring six months before to two years after cancer diagnosis [104]. Patients were classified as having VTE based on their medical files. No active screening was conducted since this measure is not yet incorporated into the IPOP clinical routine procedures.

Data concerning the patients' demographical and clinicopathological features, baseline full blood count and baseline coagulation tests were obtained from their medical data files. The median time of follow-up was 24.5 months (minimum = 9.0 months; maximum = 76.0 months). A description of the study cohort characteristics is given in **Table 1**.

Prior to their enrolment, each patient has signed a written informed consent based on the principles of the Helsinki Declaration. This study has also received approval from the ethics committee at the research centre of IPOP (CI-IPOP) (CES. 69/021).

Table 1. Demographic and clinicopathological characterization of OC patients (n = 40)

Variable	n (%)
Age (years)*	63.1 ± 12.5
< 63 years	19 (47.5)
≥ 63 years	21 (52.5)
ECOG at cancer diagnosis	
≤1	36 (90.0)
>1	4 (10.0)
Hormonal status	
Premenopausal	10 (25.0)
Postmenopausal	30 (75.0)
Alcoholism	1 (2.5)
Tobacco consumption	6 (15.0)
Inherited mutations linked to ovarian cancer	
BRCA	4 (10.0)
Other	1 (2.5)
None	35 (87.5)
Histological grade†	
Low	1 (2.5)
High	38 (95.0)
Unknown	1 (2.5)
FIGO stage†	
I/II	10 (25.0)
III/IV	30 (75.0)
First-line treatment	
Surgery plus adjuvant chemotherapy	21 (52.5)
Neoadjuvant chemotherapy plus surgery	1 (2.5)
Neoadjuvant plus surgery plus adjuvant chemotherapy	9 (22.5)
Chemotherapy only	9 (22.5)
Platinum sensitivity‡	29 (72.5)
Maintenance therapy	
PARP inhibitors	13 (32.5)
Bevacizumab	4 (10.0)
Anticoagulation at cancer diagnosis**	2 (5.0)
Platelet antiaggregation at cancer diagnosis	7 (17.5)

* Presented as mean ± standard deviation. † Defined following the FIGO Cancer Report 2021 [175]. ‡ A platinum-sensitive patient was considered as having cancer progression after at least six months after the last dose of platinum-based chemotherapy. ** Included patients who had venous thromboembolism prior to ovarian cancer diagnosis (cancer-related venous thromboembolism). Abbreviations: BRCA, Breast Cancer Gene; ECOG, Eastern Cooperative Oncology Group; FIGO, International Federation of Gynecology and Obstetrics; PARP, Poly (ADP-ribose) polymerase.

2. Sample collection and processing

From each patient, peripheral blood samples were collected in ethylenediamine tetraacetic acid (EDTA)-coated tubes using a standard venipuncture technique. Samples were gathered before and, whenever possible, after the first line of chemotherapy.

Peripheral blood nucleated cells (PBNCs) were isolated by performing ammonium-chloride-potassium (ACK) and phosphate-buffered saline (PBS) washes and subsequent rounds of centrifugation for 10 min at 2500 rpm at room temperature to separate and discard the supernatant. The obtained pellet of PBNCs (without erythrocytes) was further diluted and conserved in TriPure® Isolation Reagent (Roche Applied Science, Germany). Plasma fraction was also isolated from the peripheral blood samples through centrifugation for 5 min at 3000 rpm at room temperature. Both cellular fraction and plasma samples were stored at -80°C until further use.

3. LncRNA selection

After an exhaustive review of the existing literature on lncRNAs associated with thrombogenesis, lncRNAs were selected if they: (1) had an involvement in VTE; (2) had a regulatory role and/or an association with haemostatic key players; (3) were presented in blood circulation and/or expressed by PBNCs, particularly leukocytes and platelets; and (4) had available TaqMan assays for gene expression analysis. Additionally, those with known prognostic value in cancer were prioritized. Applying these criteria, the investigation was centred around the subsequent lncRNAs: MALAT1, TUG1, NEAT1, XIST and MEG8.

4. Total RNA extraction

Total RNA was extracted from the peripheral blood cellular fraction and plasma samples. For the former, the GRS RNA kit - Blood & cultured cells (#GK08.0100) from Grisp Research Resolutions® was used following the manufacturer's instructions with an adjustment. Namely, the duration of the DNase treatment (DNase I set (#GKC01.0100) from Grisp Research Resolutions®) was extended to remove any trace amounts of DNA that could cause interferences in downstream applications.

For total RNA isolation from plasma, first, the samples were centrifugated for 4 min at 2000 rpm at room temperature to prepare platelet-poor plasma. Total RNA was further extracted from the platelet-poor plasma samples using two commercial kits following the manufacturer's instructions: Norgen Plasma/Serum RNA/DNA Purification Mini Kit (Norgen Biotech Corp, Canada; CAT 55000) and MagMax™ mirVana™ Total RNA

Isolation Kit (ThermoFisher Scientific, Waltham, USA; CAT A27828). In all extractions, β -mercaptoethanol was added to the Lysis Buffer to ensure a high-quality RNA isolation. Also, a DNase treatment was incorporated.

RNA concentration and purity were analysed using the NanoDrop Lite spectrophotometer (Thermo Scientific®). Following extraction, RNA samples were stored at -80°C. MagMax™ mirVana™ Total RNA isolation Kit demonstrated a superior performance (high yield of RNA) compared to the Norgen Plasma/Serum RNA/DNA Purification Mini Kit. Thus, RNA samples extracted from platelet-poor plasma using the former were employed in further analysis.

5. cDNA synthesis

RNA samples extracted from the blood cellular fraction (150 ng) and plasma (160 ng) were used as templates to generate complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®) and following the manufacturer's instructions. The conversion was conducted in a Mycycler™ Thermal cycler (Bio-Rad, Hercules, CA, USA) with the recommended cycle conditions: 10 min at 25°C, 120 min at 37°C and 5 min at 85°C. Negative controls (lacking RNA) were incorporated in all reactions as a measure of quality control.

6. Relative quantification of lncRNAs

The expression levels of the lncRNAs were analysed through quantitative real-time polymerase chain reaction (qRT-PCR) using a StepOnePlus™ qPCR system. Data analysis was carried out employing the StepOne software (version 2.3 Applied Biosystems). For quantification of the lncRNAs extracted from the cellular fraction, each PCR reaction was performed using 5 μ L of 2 $\times$ TaqMan® Gene Expression Master mix (Applied Biosystems®), 3.5 μ L of nuclease-free water, 0.5 μ L of 20 $\times$ TaqMan® Gene Expression Assays for *MALAT1* (Hs00273907_s1), *TUG1* (Hs00215501_m1), *XIST* (Hs01079824_m1), *NEAT1* (Hs01008264_s1), *MEG8* (Hs00419701_m1), *GAPDH* (Hs99999905_m1) and *HPRT1* (Hs02800695_m1) and 1.0 μ L of cDNA sample, making a total volume of 10 μ L. For determination of the lncRNAs expression levels in plasma, adjustments were made, namely by using 5 μ L of 2 $\times$ TaqMan® Gene Expression Master mix (Applied Biosystems®), 2.5 μ L of nuclease-free water, 0.5 μ L of the 20 $\times$ TaqMan® Gene Expression Assays and 2 μ L of cDNA sample. For all reactions, the thermal cycling conditions were the following: 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 s at 95°C and 60 s at 60°C. The quantification was conducted in triplicate and negative controls (without cDNA) were included in all reactions for quality control. All

targets and endogenous controls for each sample were amplified in the same plate. Standard deviation (SD) of cycle threshold (Ct) values superior to 0.5 were dismissed. The same baseline and threshold were set for each plate using the analysis software for qRT-PCR from the Thermo Fisher Connect platform (Thermo Fisher Scientific, Waltham, MA, USA) to generate Ct values for all the targets in each sample. *GAPDH* and *HPRT1* were tested as endogenous controls to select the most adequate one for data normalization.

7. Statistical analysis

Data analysis was conducted using the computer software IBM SPSS Statistics for Windows version 29 (IBM Corp., 2020) and GraphPad Prism software version 10.0 (GraphPad Software Inc., La Jolla, CA, USA).

The lncRNAs' normalized relative expression was determined using the Livak method ($2^{-\Delta\Delta C_t}$ method). Among the tested endogenous controls, *HPRT1* was the one with the lowest SD values (i.e., with a more stable expression) and thus it was used to normalize the lncRNAs' expression levels. The interquartile range (IQR) method was applied to identify outliers in the normalized relative expression levels of the lncRNAs, which were dismissed for subsequent analyses. To be noted, as not enough samples derived from plasma fraction had lncRNA amplification for statistical analyses (predominantly for NEAT1, XIST and MEG8), the gene expression data were dismissed. Further analysis was thus focused on the lncRNA expression in PBNCs.

For each lncRNA, four profiles of expression were defined: profile A (low versus (vs.) high expression based on the median value of the normalized relative expression levels), profile B (low vs. intermediate vs. high expression dictated by terciles), profile C (low vs. high expression with the first and second tercile regarded as low expression and the third tercile as high expression) and profile D (low vs. high expression, with the former encompassing the first tercile and the latter the second and third terciles). All the profiles were evaluated in each analysis employing the lncRNA normalized relative expression levels as categorical variable.

The associations of VTE incidence and the prechemotherapy levels of the lncRNAs with patient's demographic and clinicopathological factors, baseline full blood count and coagulation tests were assessed using the Chi-Square test (X^2) and dismissing those with VTE before cancer diagnosis. The list of patient's demographic and clinicopathological factors included: patients' age at OC diagnosis (<63 vs. ≥ 63 years), body mass index (BMI) at diagnosis (<26.0 vs. ≥ 26.0 kg/m²), Eastern Cooperative Oncology Group (ECOG) performance status at diagnosis (≤ 1 vs. >1), hormonal status

(pre- vs.- postmenopausal), alcohol use (no vs. yes), tobacco consumption (no vs. yes), inherited mutations associated with OC (no vs. yes), FIGO stage (I/II vs. III/IV), platinum sensitivity (sensitive vs. resistant), use of PARP inhibitors (no vs. yes), Bevacizumab use (no vs. yes), serum CA-125 levels (<1195 vs. ≥ 1195 U/mL), anticoagulation therapy at cancer diagnosis (no vs. yes), platelet antiaggregation at cancer diagnosis (no vs. yes) and Khorana score (KS) (<2 vs. ≥ 2 and <3 vs. ≥ 3). Data from the baseline full blood count comprised haemoglobin levels (<12.5 vs. ≥ 12.5 U/mL), platelet (<288.0 vs. $\geq 288.0 \times 10^9/L$), leukocytes (<8.2 vs. $\geq 8.2 \times 10^9/L$), neutrophils (<5.1 vs. $\geq 5.1 \times 10^9/L$), monocytes (<0.6 vs. $\geq 0.6 \times 10^9/L$) and lymphocytes counts (<1.6 vs. $\geq 1.6 \times 10^9/L$). The coagulation tests encompassed PT (<14.3 vs. ≥ 14.3 s), aPTT (<26.9 vs. ≥ 26.9 s) and INR (<1.1 vs. ≥ 1.1). For patients' age, categories of the variable were defined based on the mean value given its normal distribution. As for BMI, serum CA-125 levels, baseline full blood count and coagulation tests, the categories were defined based on the median value.

The impact of chemotherapy on the expression levels of the lncRNAs was evaluated using the Wilcoxon matched-pairs signed rank test or paired t-test, depending on the data distribution according to the Shapiro-Wilk test. In this analysis, only patients with samples prior to and after the first line of chemotherapy were considered.

Depending on the data distribution, the Student's t-test or Mann–Whitney U test was employed to evaluate the statistical differences in the prechemotherapy expression levels of the lncRNAs according to OC-related VTE occurrence (without VTE vs. VTE before cancer diagnosis vs. VTE after cancer diagnosis). Alive patients were characterized as not having VTE events only if their follow-up time was at least 2 years. Thus, those with insufficient follow-up time were dismissed in analyses concerning VTE incidence. For confirmation purpose, the Chi-Square test (X^2) was also employed to assess the association between VTE occurrence and the lncRNAs' expression.

To evaluate the impact of VTE and the lncRNAs on patients' prognosis, measures of the clinical outcome included progression-free survival (PFS) and overall survival (OS). The former represents the interval between treatment initiation and either the date of first disease progression or recurrence, related mortality, or the patient's last clinical evaluation. The latter was defined as the time from patient diagnosis until patient death due to all causes or last evaluation. The endpoint definition was made according to Response Evaluation Criteria in Solid Tumours (RECIST) criteria updated in 2009 (RECIST 1.1) [176]. Kaplan-Meier method was employed to generate survival curves, while the probabilities of survival were analysed via the log-rank test. The impact of the evaluated lncRNAs on the risk of disease progression and death were further assessed using the Cox proportional-hazards model, which was adjusted for the most relevant

patient's demographic and clinicopathological factors previously identified by applying the backward Wald method. In those analyses, those with VTE prior to OC diagnosis were dismissed.

Subgroup analyses based on the first treatment (surgery vs. chemotherapy) were conducted in all statistical assessments implicating the lncRNA normalized relative expression levels. Of note, in opposition to those first submitted to surgery, those that were first treated with chemotherapy were treatment-naïve at the time of sample collection.

All tests conducted were two-sided and a 5% level of significance was established.

Results

IV. Results

1. Implications of VTE on patients' prognosis

Among the 30 patients with sufficient follow-up time, 7 (23.3%) developed OC-related VTE, including two patients with VTE prior to and five after OC diagnosis. Regarding the type of VTE, five events (71.4%) were DVT and two (28.6%) were PE. Most of the events (4 (57.1%)) were symptomatic and treated with LMWH (4 (57.1%)), with other options for anticoagulation treatment including DOAC (2 (28.6%)) and vitamin K antagonist (VKA; 1 (14.3%)). Among the patients with VTE before cancer diagnosis, the time interval between VTE and OC diagnosis was 3.5 ± 2.1 months. As for those with VTE after cancer diagnosis, the mean time to VTE was 9.2 ± 8.9 months. Considering both the classical (≥ 3 vs. < 3) and novel KS cut-off (≥ 2 vs. < 2), the score did not present a significant predictive impact on VTE incidence among the OS patients (X^2 , $p > 0.05$). Of note, also no significant association between VTE development and patients' demographical and clinicopathological features, baseline full blood count and coagulation tests was observed (X^2 , $p > 0.05$).

The impact of VTE on the patient's prognosis was investigated. No association between VTE and patients' PFS was observed. Yet, a tendency of a negative impact of VTE on PFS was detected among those with VTE after OC diagnosis (mean PFS of 13.4 ± 2.5 months and 21.9 ± 4.2 months, respectively; log-rank test, $p = 0.171$, **Figure 12a**). On the other hand, venous thrombogenesis was demonstrated to have a negative influence on patients' OS. Specifically, those with the condition had a worse survival time than their counterparts without VTE (mean OS of 26.5 ± 5.2 months and 47.5 months ± 5.9 months, respectively; log-rank test, $p = 0.044$). This negative effect was even more evident when focusing on those with VTE after OC diagnosis (mean OS of 22.2 ± 5.6 months and 47.5 months ± 5.9 months, respectively; log-rank test, $p = 0.028$, **Figure 12b**).

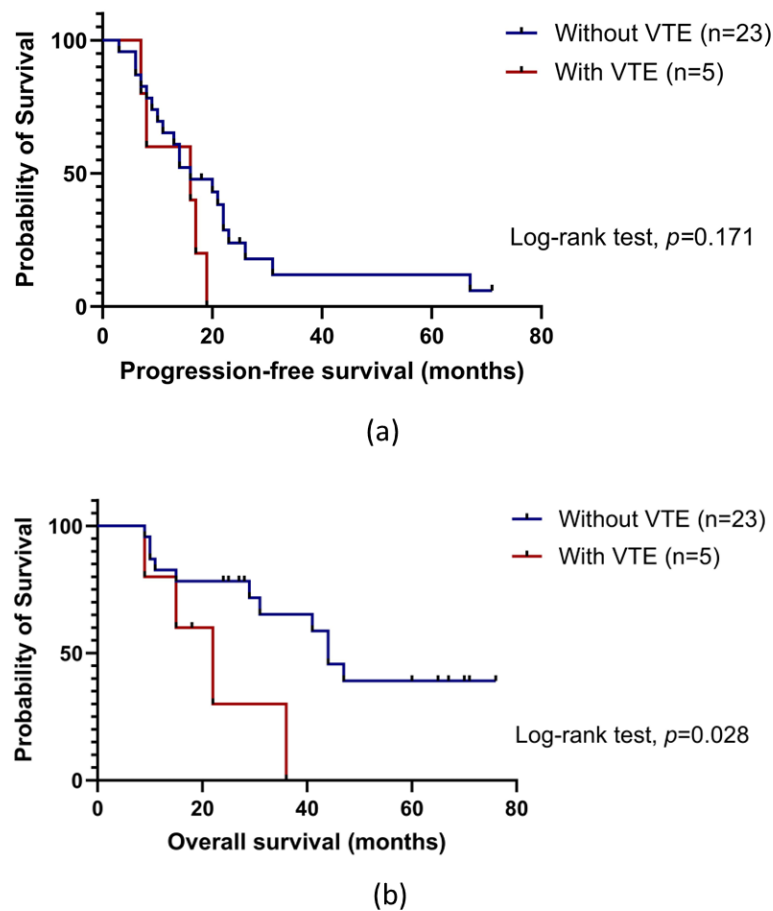


Figure 12. Progression-free survival (a) and overall survival (b) by Kaplan-Meier and log-rank test for ovarian cancer patients ($n=28$), according to venous thromboembolism (VTE) occurrence after cancer diagnosis. A nonsignificant tendency of a negative impact of VTE on the progression-free survival was observed among those with VTE after cancer diagnosis (mean progression-free survival of 13.4 ± 2.5 months and 21.9 ± 4.2 months, respectively; log-rank test, $p=0.171$, Figure 12a). Patients with the condition after OC diagnosis had lower overall survival compared to their counterparts (mean overall survival of 22.2 ± 5.6 months and 47.5 months ± 5.9 months, respectively; log-rank test, $p=0.028$, Figure 12b).

2. LncRNA expression and patients' demographical and clinicopathological features, baseline full blood count and coagulation tests

Associations between the prechemotherapy levels of the lncRNAs in PBNs and the patients' demographical and clinicopathological features, baseline full blood count and baseline coagulation tests were explored dismissing those with VTE before cancer diagnosis. Regarding the former, for MALAT1, TUG1 and NEAT1, no significant associations were observed regardless of the expression profile and the first treatment (X^2 , $p>0.05$). In opposition, prechemotherapy MEG8 levels were significantly associated with KS (<2 vs. ≥ 2) (profile A, X^2 , $p=0.009$; profile B, X^2 , $p=0.048$). For those that had chemotherapy as a first treatment, prechemotherapy MEG8 levels were also significantly

associated with KS (≥ 2 vs. < 2) (profile A, X^2 , $p=0.013$). In both analyses, elevated prechemotherapy MEG8 levels in PBNs were more predominant in the KS <2 group. Moreover, an association was found between XIST expression and CA-125 levels among those initially treated with chemotherapy. Specifically, intermediate and elevated levels of XIST were associated with elevated CA-125 levels (profile B, X^2 , $p=0.047$).

Regarding the baseline full blood count, significant associations were detected for MALAT1, TUG1 and XIST. For MALAT1, irrespective of the primary treatment, its expression was associated with leukocyte (profile A, X^2 , $p=0.041$) and neutrophil (profile A, X^2 , $p=0.041$) counts, where its higher expression was linked to a higher count of these cells. The same lncRNA was also linked to haemoglobin levels, with its higher levels being more predominant among those with higher haemoglobin levels (profile B, X^2 , $p=0.036$; profile C, X^2 , $p=0.028$). Regarding TUG1, irrespective of the first intervention, elevated neutrophil counts are linked to higher levels of this lncRNA (profile B; X^2 , $p=0.048$). For XIST, a significant relationship with haemoglobin levels was found in the surgery group, with its higher expression being associated with higher haemoglobin levels (profile B, X^2 , $p=0.033$). In terms of leukocyte count, a significance was also observed (profile B, X^2 , $p=0.027$), with a specific emphasis on the chemotherapy group (profile B, X^2 , $p=0.037$), where lower levels of XIST were linked to a lower leukocyte count. Similarly, for neutrophil count, a significant association was identified irrespective of the treatment (profile B, X^2 , $p=0.027$) and for the chemotherapy group (profile B, X^2 , $p=0.043$), where lower levels of XIST were also associated with a diminished count of neutrophils.

Concerning the baseline coagulation tests (PT, aPTT and INR), significant associations were detected only for MALAT1 and MEG8. Specifically, higher prechemotherapy MALAT1 levels in PBNs were linked to lower PT times in the chemotherapy group (profile B, X^2 , $p=0.025$). For MEG8, irrespective of the first treatment, higher levels of this lncRNA were associated with a longer PT (profile A, X^2 , $p=0.014$; profile B and D, X^2 , $p<0.001$). When stratified by treatment, this association remained significant for the surgery group (profile A, X^2 , $p=0.038$) and in both chemotherapy and surgery (profile B, X^2 , $p=0.014$ and $p=0.022$, respectively; profile D, X^2 , $p=0.031$ and $p=0.038$, respectively) depending on the expression profile. A significant association was also detected between INR and MEG8 expression, regardless of the treatment, with elevated prechemotherapy levels of MEG8 in PBNs being more predominant among those with a higher INR (profile A and B, X^2 , $p=0.041$ and $p=0.023$; profile D, X^2 , $p=0.030$). Furthermore, the lncRNA expression levels were also associated with aPTT but only in the chemotherapy group, where its elevated levels were related to a higher aPTT (profile D, X^2 , $p=0.044$).

No further significant associations were observed (X^2 , $p>0.05$).

3. Chemotherapy impact on lncRNA expression

For patients whose samples prior to and after the first line of chemotherapy were available ($n=37$), the impact of chemotherapy on the expression levels of the selected lncRNAs was evaluated. Statistically significant differences were detected (Wilcoxon matched-pairs signed rank test, $p<0.05$) between the expression levels before and after chemotherapy for MALAT1, TUG1, NEAT1 and XIST (**Figures 13a** ($p=0.047$), **13b** ($p=0.003$), **13c** ($p=0.005$) and **13d** ($p=0.009$), respectively). In opposition, only a marginally significant difference was observed for MEG8 (**Figure 13e**, $p=0.058$).

A stratified analysis was performed based on the primary treatment (surgery vs. chemotherapy). In the group that underwent chemotherapy as the first intervention ($n=17$), TUG1 levels were significantly decreased after chemotherapy ($p=0.029$). For the other RNAs evaluated, no statistically significant differences were detected in their expression levels (Wilcoxon matched-pairs signed rank test, $p>0.05$). As for those who underwent surgery as the primary treatment ($n=20$), the expression levels of TUG1 and XIST were both significantly decreased after chemotherapy ($p=0.035$ and $p=0.006$, respectively). For the remaining lncRNAs, no differences were observed before and after chemotherapy in the stratified analyses (Wilcoxon matched-pairs signed rank test, $p>0.05$).

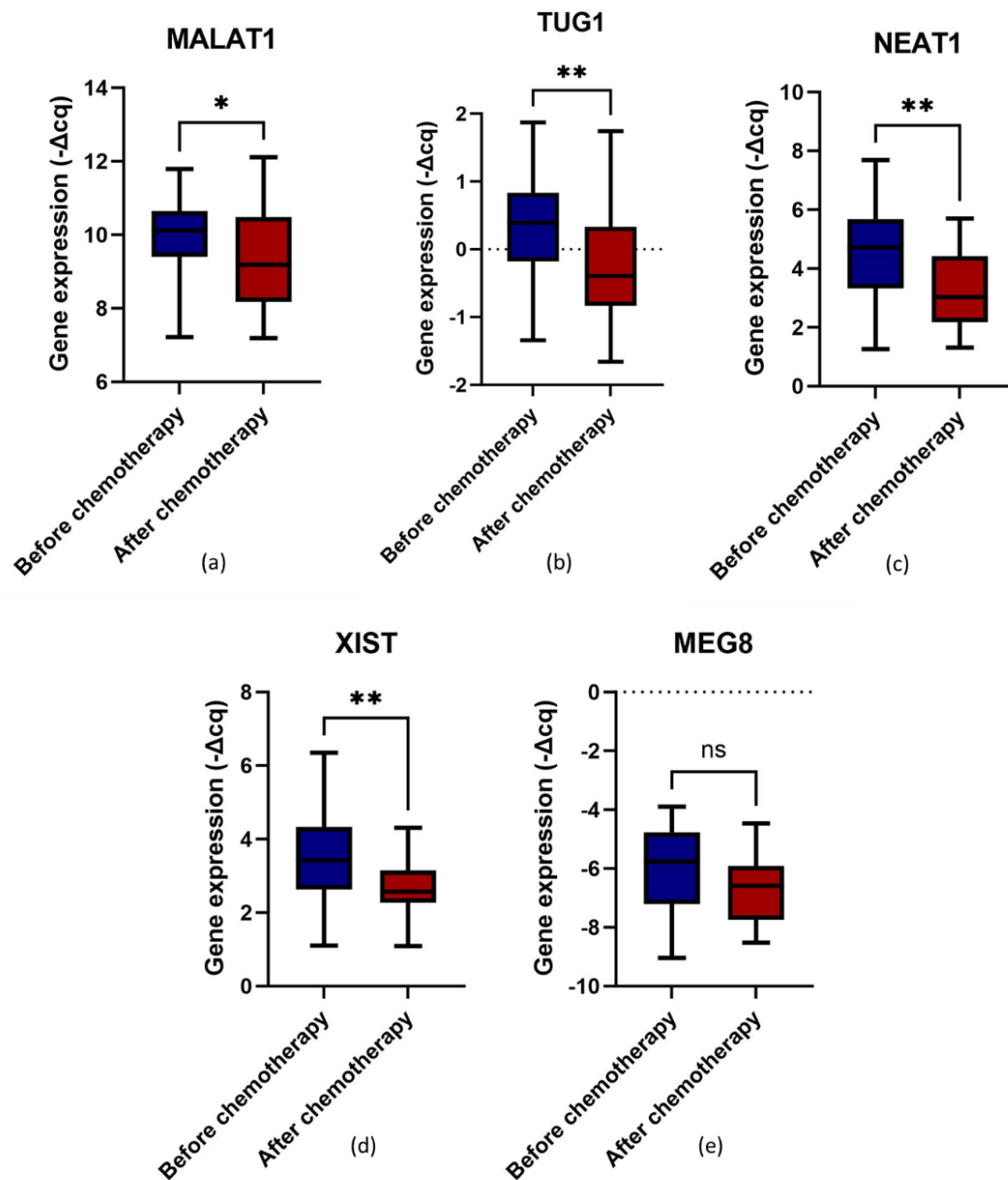


Figure 13. Normalized relative expression levels of lncRNAs ($-\Delta Cq$) in peripheral blood nucleated cells among ovarian cancer patients before and after the first line of chemotherapy: (a) MALAT1 expression; (b) TUG1 expression; (c) NEAT1 expression; (d) XIST expression and (e) MEG8 expression; Wilcoxon matched-pairs signed rank test, * $p < 0.05$; ** $p < 0.01$; ns, non-significant.

4. LncRNA expression and OC-related VTE incidence

The impact of the evaluated lncRNAs on OC-related VTE incidence was investigated. In the analyses, three groups were considered: patients without VTE, those with the condition prior to cancer diagnosis and those with the condition after cancer diagnosis. For MALAT1, TUG1, NEAT1 and XIST, no statistically significant differences were observed between the different groups (Mann-Whitney U test; $p > 0.05$; **Figure 14a, b, c and d**), which was corroborated by the Chi-Square test (X^2) (profile A, B, C and D; X^2 ,

$p>0.05$). Concerning MEG8, its prechemotherapy expression levels were significantly elevated in OC patients who developed a VTE event after cancer diagnosis compared to those without the condition (Mann-Whitney U test; $p=0.037$; **Figure 14e**). This result was further confirmed by the Chi-Square test (X^2) (profile C; X^2 test, $p=0.033$).

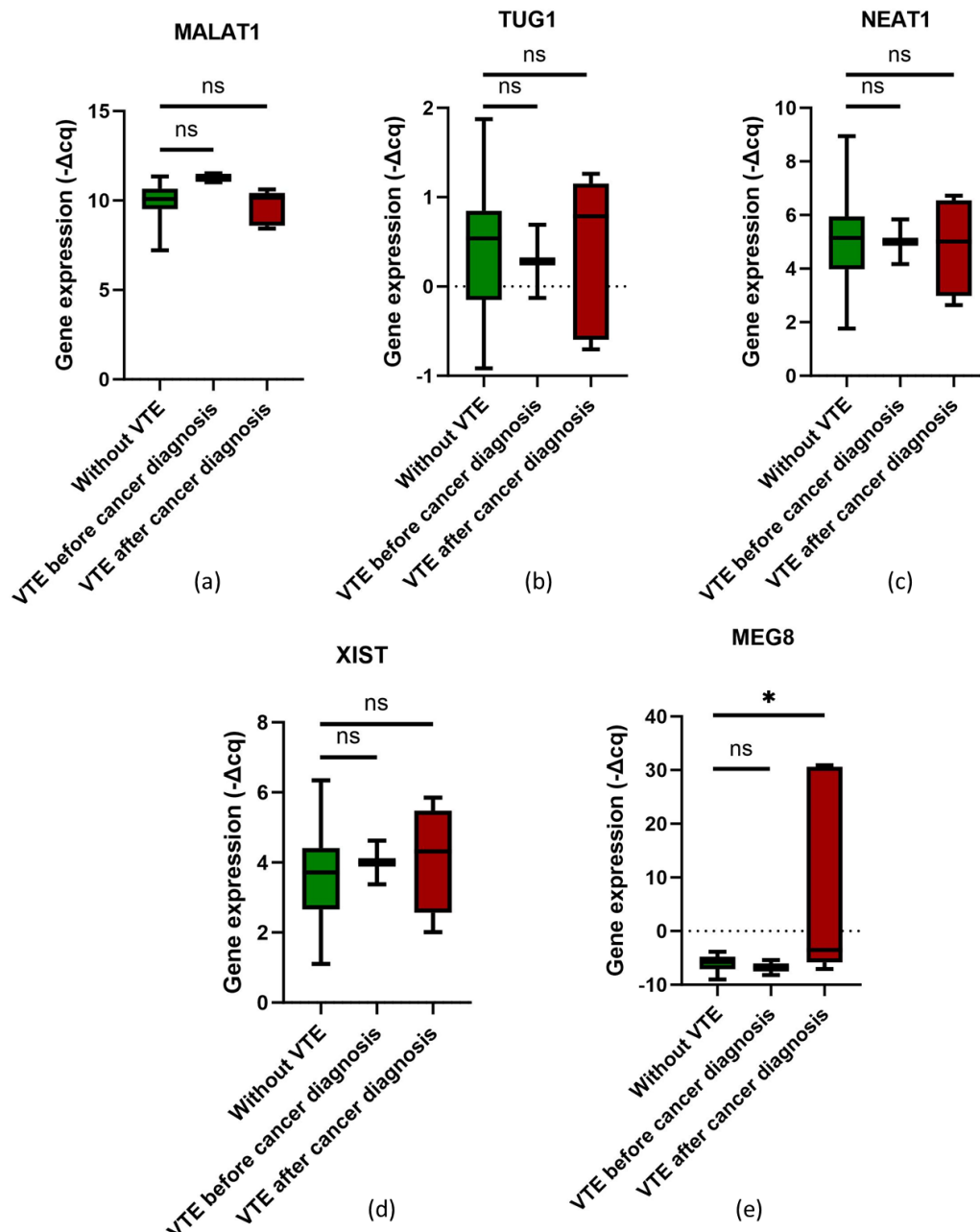


Figure 14. Normalized relative expression levels of lncRNAs (-ΔCq) in peripheral blood nucleated cells among ovarian cancer patients in the context of venous thromboembolism (VTE): (a) MALAT1 expression; (b) TUG1 expression; (c) NEAT1 expression; (d) XIST expression and (e) MEG8 expression; Mann-Whitney U test, * $p<0.05$; ns, non-significant.

5. LncRNA expression and patients' prognosis

The influence of the evaluated lncRNAs on patients' clinical outcome was dissected in univariable and multivariable Cox analyses adjusting for the most relevant patients' prognostic factors. According to a multivariate analysis applying the backward Wald method, surgery (yes vs. no) and platinum sensitivity (sensitive vs. resistant) were shown to be the most relevant predictors of the risk of disease progression, while the latter had a standout performance as a predictor of the risk of death.

For MALAT1, TUG1 and XIST, no impact on the risk of disease progression and death was observed, both in univariable and multivariable Cox analyses, regardless of the expression profile (Cox regression analyses, $p > 0.05$). For MEG8, no significant association with the risk of disease progression was detected in the univariable Cox analysis ($p > 0.05$). However, in a multivariable Cox analysis adjusting for surgery and platinum sensitivity, a significant impact of this lncRNA on the risk of disease progression was observed. Specifically, its low prechemotherapy expression levels were associated with a three-fold increase in the risk of OC progression (profile A, adjusted hazard ratio (aHR)=3.00, 95% confidence interval (95%CI), 1.06-8.51, $p=0.039$; **Table 2**). As for the impact of MEG8 on the risk of death, no significant association was detected in univariable or multivariable Cox analysis adjusting for platinum sensitivity (profile A, aHR=2.70, 95%CI, 0.81-9.02, $p=0.107$; **Table 2**).

Table 2. Multivariable Cox regression analysis on the risk of disease progression and risk of death among ovarian cancer patients (n=29) according to prechemotherapy MEG8 levels in PBNCS.

Variable	aHR	95% CI	p-value	Event
MEG8 (Lower vs. higher expression ¹)	3.00	1.06-8.51	0.039	Risk of disease progression
Surgery (Yes vs. no ¹)	0.003	0.00-0.04	<0.001	
Platinum sensitivity (Sensitive vs. resistant ¹)	0.01	0.00-0.14	<0.001	
MEG8 (Lower vs. higher expression ¹)	2.70	0.81-9.02	0.107	Risk of death
Platinum sensitivity (Sensitive vs. resistant ¹)	0.12	0.04-0.41	<0.001	

Bold values were regarded as statistically significant. ¹ Reference group. Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; PBNCS, peripheral blood nucleated cells.

Concerning NEAT1, no significant association with the risk of disease progression was detected in the univariable and multivariable Cox analysis (profile D, aHR=2.21, 95%CI, 0.77-6.38, $p=0.143$; **Table 3**). As for its impact on the risk of death, a significant association was only detected in the multivariable Cox analysis adjusting for platinum sensitivity. Specifically, its low expression levels prior to chemotherapy were associated with a six-fold increase in the risk of death (profile D, aHR=6.25, 95%CI, 1.60-24.43, $p=0.008$; **Table 3**).

Table 3. Multivariable Cox regression analysis on the risk of disease progression and risk of death among ovarian cancer patients (n=35) according to prechemotherapy NEAT1 levels in PBNs.

Variable	aHR	95% CI	p-value	Event
NEAT1 (Lower vs. higher expression ¹)	2.21	0.77-6.38	0.143	Risk of disease progression
Surgery (Yes vs. no ¹)	0.07	0.02-0.26	<0.001	
Platinum sensitivity (Sensitive vs. resistant ¹)	0.02	0.01-0.10	<0.001	
NEAT1 (Lower vs. higher expression ¹)	6.25	1.60-24.43	0.008	Risk of death
Platinum sensitivity (Sensitive vs. resistant ¹)	0.10	0.03-0.33	<0.001	

Bold values were regarded as statistically significant. ¹ Reference group. Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; PBNs, peripheral blood nucleated cells.

Discussion

V. Discussion

Despite the advancements in the disease management, OC remains the most lethal gynaecological malignancy [177, 178]. Effective disease screening, accurate diagnosis and a multidisciplinary treatment based on a better comprehension of OC are crucial to improve patients' prognosis. Focusing on the treatment setting, new and more adequate prognostic biomarkers are required to better tailor the disease management, particularly given the high heterogeneity that characterizes OC [179]. Traditionally, the study of ovarian tumorigenesis has been focused only on cancer cells [180]. However, to fully understand the disease growth and dissemination and thereby identify prognostic tools, it is essential to explore the surrounding microenvironment and how interactions between tumour cells and stroma promote the disease aggressiveness [181]. The tumour microenvironment comprises cancer-associated fibroblasts, pericytes, immune cells, platelets and endothelial cells, among others, each one playing key roles in tumorigenesis [180]. For instance, tumour cells and elements of the haemostatic system present in the tumour microenvironment are known to establish a bilateral association, with one promoting the other [108]. These interactions underly the pathogenesis of cancer-related VTE, which is a common paraneoplastic event, also representing the main cause of death among oncological patients after the cancer itself [95, 182-184].

Among other solid tumours, OC is one of the most associated with venous thrombogenesis [131-138]. Corroborating a two-way association between thrombosis and OC progression, increased DD levels are reported to be associated with high tumour burden, chemoresistance and poor prognosis, regardless of VTE status [49, 185, 186]. Likewise, high plasma thrombin levels have been shown to negatively impact the OC patients' survival and prognosis, while elevated platelet count is a predictor of the disease recurrence [185, 187]. Also, TF overexpression is linked to aggressive tumours, advanced stages, and decreased survival among OC patients. Interestingly, TF expression seems to vary according to different histological subtypes, with ovarian CCC having the highest expression [188, 189]. Besides, FVII and transcription factor hepatocyte nuclear factor 1-beta (HNF1B), were also shown to be overexpressed in CCC, with the latter regarded as one of the potential links between this subtype and a higher VTE incidence [190]. Ovary tumours also secrete chemokines and cytokines that stimulate platelets, vECs and immune cells to present a procoagulant behaviour [117, 191]. Together, all these players appear to act in a complex network to promote ovarian tumorigenesis allied to immunothrombosis. Concordantly, our research group previously showed that VTE-related genetic polymorphisms have a prognostic value among OC patients, regardless of VTE status [192]. Subsequently, in the last recent years, multiple

lncRNAs have been shown to be implicated in venous thrombus formation and resolution. However, the data on cancer patients is scarce [53]. Thus, given the clinical implications, this study aimed to investigate the impact of VTE-associated lncRNAs on the incidence of OC-related VTE, as well as their prognostic value among OC patients, regardless of thrombogenesis.

In this cohort study, from six months before to two years after cancer diagnosis, the incidence of OC-related VTE was 23.3%, which goes in line with the current evidence, confirming that OC patients have indeed a high prothrombotic risk [131-138]. Furthermore, those with the condition had a worse survival time compared to their counterparts, particularly when focusing on patients with VTE after OC diagnosis (mean OS of 22.2 ± 5.6 months and $47.5 \text{ months} \pm 5.9$ months, respectively; log-rank test, $p=0.028$), which corroborates the existing evidence [190, 193, 194]. As for PFS, no significant association was detected. However, those with VTE after OC diagnosis tend to have a worse PFS than those without (mean PFS of 13.4 ± 2.5 months and 21.9 ± 4.2 months, respectively; log-rank test, $p=0.171$), which is also in line with the literature [193]. Collectively, these findings suggest that VTE significantly deteriorates OC patient's prognosis. As previously mentioned, aside from the VTE itself, deregulated haemostatic components underlying thrombogenesis are known to be exploited by tumour cells to facilitate their progression, which increases the tumour aggressiveness and thrombogenesis, overall deteriorating the patients' prognosis [49]. As such, it is imperative to delve into more effective measures to prevent OC-related VTE among patients at high risk. As previously mentioned, the most studied RAM for forecasting cancer-related VTE is KS [115, 142, 145, 195, 196]. In this study, as expected based on the literature, the score had a poor performance, not being able to predict the incidence of OC-related VTE [137]. Although additional research is needed, specifically in larger cohorts, these preliminary findings reinforce the need for better and more tailored tools to stratify OC patients' risk of VTE development.

Regarding the evaluated lncRNAs, starting with MALAT1 (chromosome 11q13.1), this 8kb lincRNA was first identified in non-small cell lung cancer and in association with lung cancer metastasis [53, 197]. Since then, MALAT1 has been intensively studied, with reports of its deregulation in several conditions such as malignant diseases, Parkinson's disease, diabetic retinopathy and ischaemic stroke [198-203]. Concerning vascular diseases, this ncRNA has been pointed out as a major player due to its downregulation in atherosclerotic plaques and its association with vEC function [67, 204]. Furthermore, there has been a growing interest in understanding the mechanisms through which MALAT1 manages to impact DVT pathogenesis. Inconsistent results, however, led to the hypothesis that this lncRNA may have a dual role in venous thrombosis [53]. On one

hand, via the Wingless-related integration site (Wnt)/ β -catenin signalling pathway, upregulated levels of this lncRNA were suggested to modulate the activity and behaviour of EPCs, decreasing their growth, migration, and survival, thus increasing the risk of thrombogenesis [67, 68]. In another study, MALAT1 modulated HUVEC apoptosis by regulating the miR-383-5p/BCL2-like 11 (BCL2L11) axis in DVT. Specifically, when overexpressed, this lncRNA acted as a decoy to repress the miRNA, which otherwise would target BCL2L11. In turn, higher levels of BCL2L11 reduced cell viability and increased vEC apoptosis, favouring thrombosis [68]. On the other hand, in a more recent study, platelet deficiency of MALAT1 (one of the most abundant lncRNAs in platelets) was reported to enhance platelet adhesion and aggravate thrombus generation through the phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT)/ Glycogen synthase kinase-3 beta (GSK-3 β) signalling pathway [205]. Taken together, the data indicates that MALAT1 may have a cell- and/or context-dependent role in DVT pathways. In the present study, prechemotherapy expression levels of MALAT1 in PBNs, irrespective of the first treatment (surgery vs. chemotherapy), were associated with leukocyte (profile A, X^2 , $p=0.041$) and neutrophil (profile A, X^2 , $p=0.041$) counts, where its higher expression was linked to a higher count of these cells. These results suggest that MALAT1 expression in PBNs is attributed, at least partially, to neutrophils. Indeed, as previously mentioned, these immune cells are known to be implicated in immunothrombosis through the release of NETs (NETosis), which is promoted, for instance, by TF and platelets to increase the prothrombotic cascade [75]. Concordantly, exosomal MALAT1 from HUVECs was previously revealed to stimulate NETosis, triggering a proinflammatory response [206]. Together with the reported upregulated levels of MALAT1 in vECs in the context of thrombogenesis, the data hints that under a proinflammatory and prothrombotic setting (e.g., tumour microenvironment), endogenous or exosomal MALAT1 may promote neutrophils activation, favouring thrombus formation [67, 68]. In line with the role of neutrophils in MALAT1 expression, in this study, the lncRNA expression levels were significantly decreased after chemotherapy (Wilcoxon matched-pairs signed rank test, $p=0.047$). One of the explanations could be that by lessening the tumour burden, chemotherapy might have diminished the cancer stimulatory effect on the PBNs [207]. Moreover, the therapy itself is known to negatively impact leukocyte activity and counts, causing conditions such as neutropenia and leukopenia, which may explain the downregulation of MALAT1 after chemotherapy [208, 209]. Also, chemotherapy can decrease platelet counts and cause vEC dysfunction, altering the signal from these haemostatic key players to leukocytes (i.e., immunothrombosis) [75, 210, 211]. Additionally, higher MALAT1 levels were found to be associated with higher haemoglobin levels (profile B, X^2 , $p=0.036$; profile C, X^2 , $p=0.028$). The current evidence on the link between MALAT1 with haemoglobin is scarce

and conflicting. In myelofibrosis, a study showed that higher plasma levels of MALAT1 were associated with lower haemoglobin levels [212]. In opposition, a study with hepatocellular carcinoma described a positive correlation between serum MALAT1 and haemoglobin levels [213]. Further investigation is thus required to dissect the underlying mechanisms. Indeed, haemoglobin can be a predictive biomarker of cardiovascular diseases, in addition to serving as a prognostic biomarker among cancer patients [142, 214]. For instance, in OC patients, haemoglobin was previously associated with tumour stage, disease residue, chemotherapy completion and patients' survival [214]. Furthermore, in the present study, prechemotherapy expression levels of MALAT1 in PBNs were also found to be associated with lower PT in the group that had chemotherapy as the first treatment, meaning those that were treatment-naïve (profile B, X^2 , $p=0.025$). Although the coagulation tests did not present a predictive ability concerning VTE incidence (most likely due to the low number of patients with abnormal values), this finding suggests that, at OC diagnosis, MALAT1 expression in PBNs might be linked to haemostatic abnormalities, particularly in the extrinsic coagulation pathway [112, 208, 209, 215]. However, and despite the literature available regarding MALAT1 in the setting of VTE, no significant association with OC-related VTE was found, which could be due to the context-dependent role of this lncRNA and the limited cohort size. As such, further studies are needed. As for the implications of MALAT1 in OC pathways regardless of VTE, this lncRNA is reported to be usually upregulated, which is associated with advanced disease stages, high tumour burden and a poor prognosis [216-220]. *In vitro* studies have shown that MALAT1 overexpression is linked to several tumour hallmarks, namely apoptosis evasion, sustained proliferation, angiogenesis, invasion and metastasis (particularly given its association with metalloproteinases and its implications in epithelial-mesenchymal transition (EMT)) [217, 218, 220-223]. Likewise, MALAT1 has been associated with chemoresistance. Specifically, the knockdown of this lncRNA was shown to attenuate cisplatin resistance and promote apoptosis in cisplatin-resistant OC cells [224, 225]. Overall, MALAT1 is deemed an important contributor to ovarian tumorigenesis. However, contrary to the expected, no significant associations of MALAT1 with OS and PFS were detected in this study. In addition to the small cohort size, this might be attributable to the fact that the expression levels of this lncRNA were evaluated in blood components rather than in tumour tissues, in contrast to most of the previously mentioned studies. Hence, more studies with larger cohorts are necessary to assess the implications of MALAT1 expressed by PBNs in OC-related VTE and cancer progression.

Regarding TUG1 (chromosome 22q12.2), this 7.1 kb lncRNA was first identified as upregulated in developing retinal cells and in response to taurine (2-aminoethanesulfonic acid) [53, 226, 227]. This lncRNA is implicated in several malignant diseases and

obesity-related diseases [228, 229]. As for VTE, TUG1 was shown to have a poor expression in the femoral vein tissue of DVT mice. In opposition, the lncRNA overexpression, by downregulating miR-92a-3p and subsequently upregulating the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) pathway (known to regulate angiogenesis), was able to accelerate the proliferation, migration, and tube-forming abilities of EPCs, while decreasing their apoptosis and the thrombus size. Collectively, TUG1, by serving as a decoy, seems to exert a protective effect against DVT [85]. In the current study, elevated prechemotherapy levels of TUG1 in PBNs, irrespective of the primary treatment, were linked to elevated neutrophil counts (profile B; X^2 , $p=0.048$). Indeed, TUG1 has been reported to directly facilitate the transcription of leucine-rich alpha-2-glycoprotein 1 (LRG1), which is a modulator of angiogenesis and inflammation, constitutively expressed by neutrophils. Interestingly, the few existing data indicate that LRG1 promotes the extravasation and activation of neutrophils, stimulating NETosis, which as mentioned before, is a process implicated in immunothrombosis [230, 231]. Furthermore, similarly to MALAT1, TUG1 expression levels in PBNs are decreased after the first line of chemotherapy (Wilcoxon matched-pairs signed rank test, $p=0.003$). Once again, this can be explained by the role of chemotherapy in decreasing the cancer burden and thus the stimulatory signal from tumour cells to the microenvironment, as well its impact on leukocytes (and neutrophils specifically), platelets and vECs, overall leading to a lower expression of TUG1. Regarding baseline haemoglobin levels and the coagulation tests, no significant associations involving TUG1 were observed. As for VTE incidence among OC patients, the lncRNA also did not present any significant impact. Overall, TUG1 expression in PBNs does not seem to influence venous thrombogenesis in the setting of OC. Nonetheless, future investigation is required to validate this finding. As for TUG1 implications in OC pathways regardless of VTE, the current data suggests that this lncRNA is upregulated in OC tissues, associating with tumour stage, grade and lymph node metastasis [232, 233]. The lncRNA knockdown is responsible to inhibit tumour activity while inducing apoptosis and reversing EMT [232-234]. Interestingly, TUG1 knockdown has also been shown to inhibit angiogenesis by suppressing secretion of LRG1 [235]. On the contrary, a previous study showed a detrimental effect of TUG1 with its lower levels being associated with advanced disease stages, cisplatin resistance and reduced survival [236]. In summary, there are several described roles regarding TUG1, which however seem to be conflicting or context-dependent. In the present study, this lncRNA did not show any impact on the risk of disease progression or death. All in all, given the reported roles of TUG1 in VTE and OC, and considering the small cohort size, further studies are warranted to elucidate its roles in both conditions.

The lincRNA NEAT1 (chromosome 11q13.1) is well-studied in the context of cancer, playing also pivotal roles in neurodegenerative diseases, such as Parkinson's, Alzheimer's, Huntington's disease and Amyotrophic lateral sclerosis [88, 237, 238]. Regarding VTE, the first and only study that aimed to unravel the role of NEAT1 was published in 2023. The serum levels of this lncRNA were found to be upregulated in DVT patients compared to healthy individuals. Moreover, NEAT1 was positively associated with vasoconstrictors and factors involved in fibrinolysis and coagulation. In HUVECs, this lncRNA was responsible to inhibit the secretion of adhesion factors, also restraining their proliferation and migration, while promoting their apoptosis. As a result, NEAT1 was stated as being involved in endothelial dysfunction and coagulation/fibrinolysis imbalances [173, 239]. In this study, no statistically significant associations were found between prechemotherapy NEAT1 expression levels in PBNs and the baseline full blood count. According to the literature, this lncRNA regulates immune cell functions and although with a low immune cell specificity, NEAT1 seems to be mainly expressed by monocytes and lymphocytes [240, 241]. Indeed, while cell counts can impact the expression levels of a gene within a sample, the gene expression activity is often the primary contributor, which could explain the absent association between NEAT1 levels and leukocytes counts [242]. Furthermore, decreased levels of this lncRNA were observed after the first line of chemotherapy (Wilcoxon matched-pairs signed rank test, $p=0.005$), which could be once again explained by the negative impact of chemotherapy on tumour burden, leukocytes, platelets and vECs (particularly the former), as previously mentioned. As for its impact on thrombogenesis, NEAT1 did not present a significant association with the baseline coagulation tests neither OC-related VTE incidence. Of note, as previously mentioned, the study that linked NEAT1 to VTE, was conducted analysing the expression of the lncRNA in serum and HUVECs [173]. Additional investigation with a larger cohort size is needed to unravel the role of NEAT1 expression in PBNs in thrombogenesis among OC patients. With respect to ovarian tumorigenesis, a previous study demonstrated that NEAT1 was upregulated in OC compared to non-neoplastic tissues. Also, in the same study, the lncRNA expression levels were positively associated with prognostic factors such as FIGO stage, particularly distant metastasis, and tumour grade. Overall, higher levels of this lncRNA were associated with worse prognosis, with NEAT1 being considered an independent prognosis factor of OC [239]. Concordantly, the lncRNA is documented as being overexpressed and associated with worse prognosis in other solid tumours, including lung, colorectal, oesophageal and prostate cancers [88, 243-247]. However, in the present study, diminished prechemotherapy NEAT1 expression levels in PBNs were significantly associated with an increased risk of mortality adjusting for platinum sensitivity (profile D, aHR=6.25, 95%CI, 1.60-24.43, $p=0.008$). Of note, the present study evaluated the expression of

NEAT1 in blood components, rather than in tumour tissues, in contrast to most studies conducted on NEAT1 implications in cancer pathways, which may explain this (apparently contradictory) result. Inclusively, low levels of NEAT1 were previously reported to have a detrimental role in promyelocytic leukaemia (PML), leading to impaired myeloid differentiation [248]. Under normal circumstances, myeloid differentiation is the process by which hematopoietic stem cells transform into mature blood cells of the myeloid lineage, including erythrocytes, platelets and leukocytes [249]. However, in acute promyelocytic leukaemia (APL), a subtype of acute myeloid leukaemia, this process is deregulated. The condition APL is defined by a chromosomal translocation that leads to the fusion of the genes *PML* and *retinoic acid receptor α* (*RAR α*). This gene fusion results in the production of the PML-RAR α oncoprotein, which blocks the promyelocytic differentiation stage causing an abnormal accumulation of promyelocytes in the peripheral blood [250, 251]. All-trans retinoic acid (ATRA) is a medication used to treat this condition, which reverts this process for proper myeloid differentiation. However, PML-RAR α can additionally repress NEAT1 in PML cells hindering the restoration of the normal differentiation process by ATRA [248]. Consequently, this disrupted differentiation as result of lower NEAT1 expression leads to an excessive accumulation of promyelocytes, potentially resulting in coagulation abnormalities due to, for instance, reduced levels of platelets [252]. In OC, NEAT1-enriched extracellular vesicles derived from M2-polarized tumour-associated macrophages have been previously demonstrated to favour the immune evasion via the miR-101-3p/ zinc finger E-box binding homeobox 1 (ZEB1)/ programmed death-ligand 1 (PD-L1) axis [253]. Whether these two seemingly isolated mechanisms are interrelated and underlying the negative effect of low NEAT1 expression in PBNs needs to be explored. Of note, no significant association between the lncRNA expression and PFS was detected. All in all, further studies with larger cohort sizes are warranted to conclusively establish the role of NEAT1 in VTE and OC.

The lincRNA XIST (chromosome Xq13.2) is one of the most studied lncRNAs, known to be required for X-chromosomal inactivation during early development for dosage compensation [254]. In addition, this lincRNA is also associated with preeclampsia and multiple cancers [255, 256]. Concerning VTE, XIST was found to be highly expressed in the plasma of DVT patients, possibly acting as a decoy by sponging miR-103a-3p, which in turn, targets high-mobility group box 1 (HMGB1) [257]. Concordantly, miR-103a-3p expression was found to be reduced in patients with unprovoked VTE [258]. In DVT, lower levels of this miRNA inhibited normal functions of EPCs, such as migration and angiogenesis [259]. On the other hand, higher miR-103a-3p expression levels together with lower levels of chemokine 12 (CXCL12) inhibited the development of DVT, which is

in line with previous data [260]. As for HMGB1, its levels are highly expressed in patients with same condition. This nuclear protein is one of the main regulators of the coagulation cascade, known also to induce inflammatory reactions and play a part in the apoptosis. In thrombosis, HMGB1 has a pro-thrombotic effect as it promotes both the activation and aggregation of platelets [257, 261]. Also via the XIST/miR-103a-3p/HMGB1 axis, XIST may regulate the expression of TF in HUVECs induced by IL-1 β , as well as increase cell viability and hinder apoptosis by inhibiting the reactive oxygen species (ROS)/nuclear factor κ B (NF- κ B) signalling pathway [257, 262-264]. In the present study, intermediate and elevated prechemotherapy expression levels of XIST in PBNs were associated with elevated leukocyte (profile B, X^2 , $p=0.027$) and neutrophil (profile B, X^2 , $p=0.027$) counts, with a specific emphasis on the chemotherapy group (profile B, X^2 , $p=0.037$ and profile B, X^2 , $p=0.043$, respectively). Like MALAT1 and TUG1, the results suggest that the expression of XIST in PBNs was attributed, at least partially, to neutrophil counts. Concordantly, this lncRNA has been previously implicated in neutrophil-associated biological processes, including NETosis [265, 266]. Concerning chemotherapy's impact on XIST expression levels, a significant association was found with chemotherapy being responsible for the decrease in its levels (Wilcoxon matched-pairs signed rank test, $p=0.009$) as observed for MALAT1, TUG1 and NEAT1. Furthermore, a significant relationship with haemoglobin levels was found in the surgery group, with intermediate and elevated levels of XIST associating with higher haemoglobin levels (profile B, X^2 , $p=0.033$). However, the existing data on this association is scarce. Regarding haemostatic abnormalities assessed by baseline coagulation tests and the incidence of OC-related VTE, no significant associations with XIST were found. Nevertheless, the evidence on its implications in VTE pathways addresses the expression of the lncRNA in plasma [266]. Thus, additional investigation with a larger cohort size is needed to unravel the role of XIST expression in PBNs in thrombogenesis among OC patients. In relation to OC progression, there are some contradictory findings in the literature regarding this lncRNA. A study showed that XIST is highly expressed in EOC tissues and cell lines, being strongly associated with tumour grade, FIGO stage and distant metastasis, and representing an independent prognostic factor among EOC patients. Concordantly, the downregulation of XIST inhibited OC proliferation, migration and invasion [266-268]. In opposition, its overexpression has been associated with longer survival in OC patients, being downregulated in advanced stages, compared to early stages of the disease [269, 270]. Also, the upregulation levels of XIST were reported to increase apoptosis, inhibit proliferation, invasion, and tumour growth *in vivo*, and increase the chemosensitivity of OC cells to cisplatin, thus playing a significant anticancer role [271, 272]. Overall, XIST has conflicting roles in the setting of OC. In this present study, no impact of XIST on the risk of disease progression or death was found.

However, a significant association was detected between XIST and CA-125 levels within the chemotherapy group. Specifically, intermediate and elevated levels of the lncRNA were associated with elevated levels of the OC marker (profile B, X^2 , $p=0.047$). According to the literature, high CA-125 levels correlate with high tumour burden and advanced OC stages, translating into a shorter OS [273]. With elevated XIST expression levels in PBNs being associated with elevated CA-125, it is plausible to suggest that the lncRNA might also increase with tumour burden. However, as mentioned before, the available literature has conflicting findings regarding the role of XIST in ovarian tumorigenesis, and the implications of the lncRNA expression in PBNs in OC progression is yet unclear. Thus, further studies are needed.

The lncRNA MEG8 (chromosome 14q32.31) is known to be dysregulated in several disorders, such as lung, ovarian and colorectal cancers, as well as gestational diabetes mellitus, diabetic nephropathy, and ischemic heart disease [274, 275]. Contrary to the other lncRNAs evaluated in this study, MEG8 expression levels in PBNs were not associated with the baseline full blood count, nor they were impacted by the first line of chemotherapy. This suggests that the expression level of this lncRNA is mainly attributed to the gene expression activity in PBNs rather than the cell quantity, which is sustained even with chemotherapy. Furthermore, in this study, prechemotherapy MEG8 levels were significantly associated with KS (≥ 2 vs. < 2), irrespective of the first treatment (profile A, X^2 , $p=0.009$; profile B, X^2 , $p=0.048$) and among those that had chemotherapy as a first intervention (profile A, X^2 , $p=0.013$). In both scenarios, elevated MEG8 levels were significantly associated with lower KS. In opposition, the lncRNA expression was not found to be individually associated with neither of the KS parameters (prechemotherapy BMI, haemoglobin levels and platelet and leukocyte counts) [115]. Also, it was established an association between elevated MEG8 levels and prolonged PT, aPTT and INR, which indicates its potential association with anomalies of the haemostatic system, involving both the intrinsic and extrinsic pathways [112, 215]. Concordantly, the lncRNA was significantly elevated among OC patients who later experienced a VTE event compared to those who remained VTE-free (Mann-Whitney test, $p=0.037$; X^2 test, $p=0.033$). As mentioned before, elevated MEG8 levels were also significantly associated with lower KS. This seemingly conflicting result can be attributed to the poor performance of KS in predicting OC-related VTE incidence. Inclusively, among OC patients, those classified as having low VTE risk ($KS < 3$ or $KS < 2$, depending on the cut-off) often end up suffering from the condition, according to different studies [196]. In contrast with the other evaluated lncRNAs, MEG8 has not yet been directly linked to VTE in previous studies. Instead, it is reported to be a scaffold lncRNA repressing the expression of TFPI2 [53, 275]. This haemostatic protein is expressed in cells such as vECs, monocytes or macrophages, playing dual roles: as an anticoagulant by targeting the TF coagulation

pathway and as an antifibrinolytic protein by inhibiting plasmin. Collectively, TFPI2 seems to play a context-dependent role in VTE pathways, influencing both thrombus formation and its resolution (**Figure 15**) [63, 64, 276, 277]. Therefore, as MEG8 downregulates the expression of TFPI2, which is also expressed among leukocytes, the present study results (i.e., elevated MEG8 levels in PBNCs precedes OC-related VTE) suggest that TFPI2 as an anticoagulant protein, rather than an antifibrinolytic protein, might be the primary mechanism underlying the promoting effect of MEG8 in blood clot formation (**Figure 15a**). As such, due to MEG8 inhibition of TFPI2 expression, the active TF coagulation pathway ends up contributing to OC-related VTE occurrence. Following its context-dependent role, elevated serum TFPI2 levels were reported to be a diagnostic marker of VTE among OC patients [278, 279]. This hints that, in opposition to blood clot formation, MEG8 levels may be downregulated at thrombus resolution, leading to TFPI2 upregulation, which in turn inhibits plasmin, impairing thrombus resolution (**Figure 15b1**). In sum, elevated prechemotherapy MEG8 levels in PBNCs could be a predictor of a later OC-related VTE occurrence. As for the role of this lncRNA in OC progression, in the present study, lower MEG8 expression levels prior to chemotherapy were linked to a three-fold increase in the risk of disease progression adjusted for surgery and platinum sensitivity (profile A, aHR=3.00, 95%CI, 1.06-8.51, $p=0.039$). In accordance, in a study utilizing integrated bioinformatics analysis, the authors showed that diminished MEG8 levels in OC tissue were associated with a poorer prognosis [280]. This goes in line with the observation that, in opposition to other tumours, TFPI2 is overexpressed in ovarian CCC (the EOC subtype most associated with thrombogenesis), though its precise role and the underlying mechanisms remain unclear [281-284]. Furthermore, low MEG8 levels in vECs are known to impair the endothelial function, which in turn, in addition to promoting VTE, also plays a role in cancer by amplifying the tumour inflammatory signalling and consequently promoting tumorigenesis (**Figure 15b2**) [83, 275, 285]. As for the implications of MEG8 expression in PBNCs in ovarian tumorigenesis, the data is scarce. Nevertheless, MEG8 has been previously linked to immune infiltration in OC [280]. Additionally, lower levels of MEG8 may lead to an overexpression of TFPI2, which as previously mentioned has an inhibitory activity towards plasmin [64]. In turn, this profibrinolytic protein is also implicated in tumour growth and dissemination via degradation of the extracellular matrix, influencing tumour metastasis [286]. Of note, no significant association between MEG8 expression and OS was detected. However, further studies with larger cohorts are needed for clarification. Collectively, an intricate interplay revolving around MEG8 appears to impact both the risk of VTE development and the prognosis of OC patients, regardless of thrombogenesis.

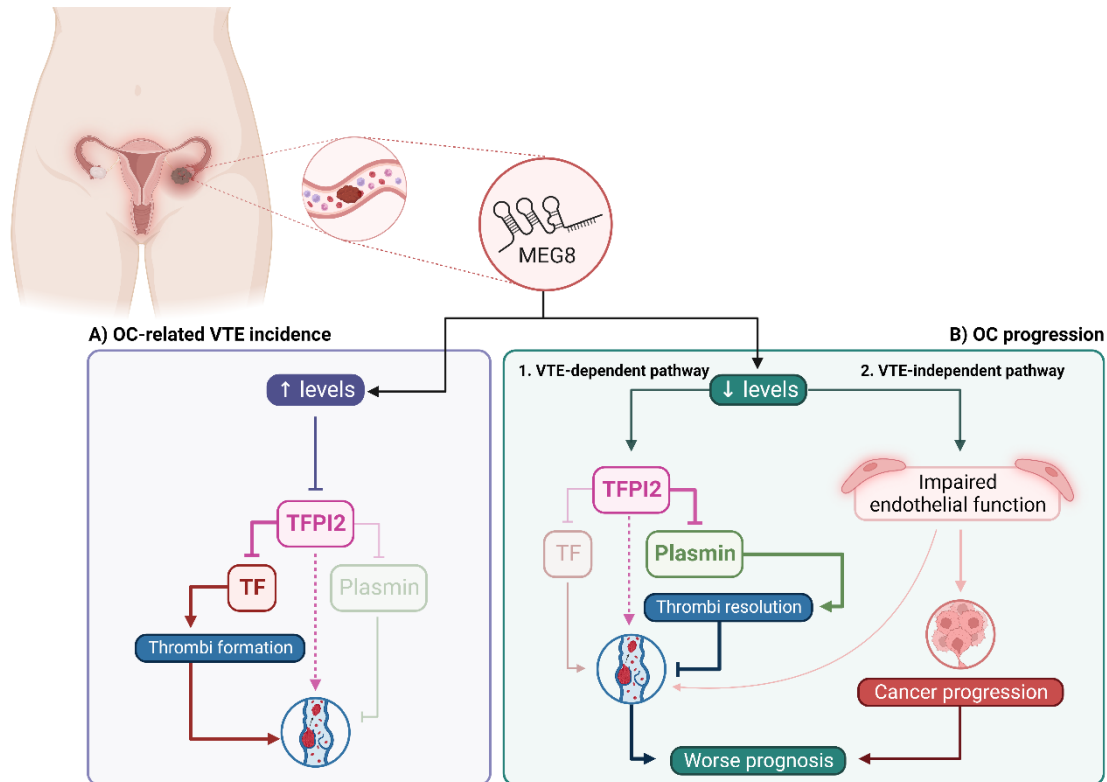


Figure 15. Hypothesis of MEG8 roles in a) OC-related VTE incidence and b) OC progression

Low levels of MEG8 are known to impair endothelial function and elevate TFPI2 expression [275]. TFPI2, in turn, has inhibitory activity towards TF and plasmin [64]. While the former initiates the coagulation cascade, the latter primarily contributes to blood clot removal [63, 64]. **a)** In this study, expression levels of MEG8 were elevated in OC patients who developed a VTE event after cancer diagnosis. The underlying hypothetical mechanism relies on the fact that TFPI2 inhibition towards TF might be more significant at thrombi formation. Specifically, as MEG8 inhibits TFPI2, the latter, when unavailable, cannot inhibit TF, which consequently is able to initiate the coagulation cascade leading to VTE. **b)** Low expression levels of MEG8 were linked to an increase in the risk of disease progression, which can be explained by VTE-dependent and independent pathways. **b1)** A possible VTE-dependent hypothesis could be that TFPI2 inhibits plasmin, preventing it to execute its profibrinolytic functions, hampering VTE resolution and promoting OC progression given the plasmin roles in the remodelling of extracellular matrix [64, 286]. **b2)** Other possible mechanism consists in a VTE-independent pathway where impaired endothelial function promotes cancer progression, aggravating OC patient's prognosis [285]. Parallely, impaired endothelial function contributes also to VTE [83]. Dashed arrows represent the final result through which TFPI2 impacts VTE development. MEG8 expression: ↓ downregulated and ↑ upregulated. Transparency indicates the possible less relevant TFPI2-inhibitory activity in the respective pathway. Abbreviations: MEG8, maternally expressed 8; OC, ovarian cancer; VTE, venous thromboembolism; TFPI2, tissue factor pathway inhibitor. The figure was created with BioRender.com.

Despite the promising results, the present study had limitations that need to be acknowledged. Firstly, its retrospective design had impeded the gathering of relevant data. For instance, it would be important to assess the lncRNA expression also close to VTE occurrence to evaluate the diagnostic capability of these ncRNAs. This would be particularly for MEG8 given its apparent context-dependent role. Second, VTE was not actively screened, which may have led to a potential underestimation of asymptomatic

VTE events. This oversight may have inclusively contributed to the poor performance of KS in this study. Moreover, there was an absence of a control group comprising healthy participants and VTE patients without cancer, which hindered the comparison of the lncRNA expression in health and disease contexts. Additionally, the sample size was small, limiting the statistical power of the study and potentially impairing the generalization of the findings to a broader population. Lastly, it was not possible to evaluate the lncRNAs expression in plasma, which could be attributed to either the method's lack of sufficient sensitivity and/or the potential poor quality of the plasma samples.

Regarding the strengths of this study, firstly, the population under evaluation was relatively homogeneous, which enhances the internal validity of our findings. Second, all the major risk factors related to VTE occurrence were accounted for. Moreover, while previous studies were predominantly centred around aberrant lncRNA expression in OC tissue samples, this study was focused on deregulated lncRNAs in PBNCs, demonstrating the potential clinical relevance of liquid biopsies in OC prognosis. Furthermore, this study represents a pioneering effort in shedding light on the unexplored territory of VTE-related lncRNAs within the context of CAT, since, up to date, no studies have been conducted on the role of these lncRNAs among cancer patients.

Conclusions

VI. Conclusions

OC stands out as the most lethal gynaecological malignancy [2, 3]. Conversely, VTE is a common paraneoplastic event that ranks as the second leading cause of death among cancer patients, imposing a detrimental impact on their prognosis [95, 182-184]. These two conditions have a close relationship, in which tumour cells hijack the haemostatic system to drive cancer growth and dissemination [119]. Recently, lncRNAs have been implicated in VTE pathogenesis [53]. However, their roles in cancer-associated VTE development, as well as their prognostic value, are yet unexplored. Understanding how these lncRNAs influence venous thrombogenesis and OC progression may lead to the identification of valuable biomarkers for VTE prediction and OC management, which are both needed to improve the prognosis of the patients. Thus, in this study, the impact of five lncRNAs, including MALAT1, TUG1, NEAT1, XIST, and MEG8, was evaluated in a cohort of 40 OC patients. According to the findings, OC patients have a high prothrombotic risk and VTE negatively impacts their survival. Among the evaluated lncRNAs, MEG8 in PBNs was the only one that was revealed to be a predictor of OC-related VTE, with its association with the baseline coagulation tests reflecting a possible role of this lncRNA in haemostatic abnormalities. This suggests that MEG8 holds the potential to be an attractive tool to predict VTE risk among OC patients using liquid biopsies, thus offering valuable insights into a patient's thromboembolic risk profile. Interestingly, in line with the bilateral relationship between tumour cells and the haemostatic system, this lncRNA also demonstrated to impact the risk of OC progression regardless of VTE. This multifaceted role puts MEG8 as a potential biomarker for both VTE prediction and OC prognosis assessment. Furthermore, NEAT1 in PBNs was associated with the risk of death, emerging as a potential biomarker when assessing the prognosis of OC patients. While these preliminary findings are promising, it is important to reinforce the necessity for further validation studies in larger cohorts and preferably using a multicentric approach for more robust data.

To our current understanding, this investigation is the first to propose lncRNAs in PBNs as potential predictors of VTE in association with OC. By being able to stratify OC patients based on their VTE risk, a more focused screening and justifiable thromboprophylaxis could be established for those at high risk, thereby safely reducing the occurrence of VTE events without adversities (e.g., bleeding disorders). Likewise, these lncRNAs could be used as additional tools to assess the prognosis among OC patients in a non-invasive manner, helping tailor treatment strategies to improve their clinical outcomes. In terms of future perspectives, the adoption of digital polymerase chain reaction (dPCR) could be valuable to evaluate these lncRNAs in liquid biopsies,

given its high sensitivity. This is particularly important when analysing the expression profiles of the selected lncRNAs in plasma and serum samples [287]. Furthermore, performing single-cell RNA sequencing would be also important to gain a better understanding of which individual PBNs are responsible for expressing the assessed lncRNAs [288, 289]. Additionally, the inclusion of a pre-surgery sample for those who undergo surgery as primary treatment is also something to consider. Lastly, it would be imperative to include control groups comprising healthy individuals and VTE subjects without cancer, preferably using a multicentric approach, to augment the robustness and validity of the acquired results, thus offering deeper insights into the evaluated lncRNAs in different pathophysiological settings.

In conclusion, this first study on the matter sheds light on the functional significance of lncRNAs in OC-related VTE and suggests them as potential prognostic biomarkers in OC, regardless of thrombogenesis. More studies in this field and with real-world OC patients are required to clarify the clinical use of lncRNAs as OC biomarkers in an era of liquid biopsies.

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VII. References

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Attachment 1



OPEN Thrombogenesis-associated genetic determinants as predictors of thromboembolism and prognosis in cervical cancer

Beatriz Vieira Neto^{1,2,8,10}, Valéria Tavares^{1,2,3,8,10}, José Brito da Silva⁴, Joana Liz-Pimenta^{2,5}, Inês Soares Marques^{1,6}, Luísa Carvalho⁷, Lurdes Salgado⁷, Deolinda Pereira⁴ & Rui Medeiros^{1,2,3,6,8,9}✉

Venous thromboembolism (VTE) is a leading cause of death among cancer patients. Khorana score (KS) is the most studied tool to predict cancer-related VTE, however, it exerts poor sensitivity. Several single-nucleotide polymorphisms (SNPs) have been associated with VTE risk in the general population, but whether they are predictors of cancer-related VTE is a matter of discussion. Compared to other solid tumours, little is known about VTE in the setting of cervical cancer (CC) and whether thrombogenesis-related polymorphisms could be valuable biomarkers in patients with this neoplasia. This study aims to analyse the effect of VTE occurrence on the prognosis of CC patients, explore the predictive capability of KS and the impact of thrombogenesis-related polymorphisms on CC-related VTE incidence and patients' prognosis regardless of VTE. A profile of eight SNPs was evaluated. A retrospective hospital-based cohort study was conducted with 400 CC patients under chemoradiotherapy. SNP genotyping was carried on by using TaqMan® Allelic Discrimination methodology. Time to VTE occurrence and overall survival were the two measures of clinical outcome evaluated. The results indicated that VTE occurrence (8.5%) had a significant impact on the patient's survival (log-rank test, $P < 0.001$). KS showed poor performance ($KS \geq 3$, χ^2 , $P = 0.191$). *PROCR* rs10747514 and *RGS7* rs2502448 were significantly associated with the risk of CC-related VTE development ($P = 0.021$ and $P = 0.006$, respectively) and represented valuable prognostic biomarkers regardless of VTE ($P = 0.004$ and $P = 0.010$, respectively). Thus, thrombogenesis-related genetic polymorphisms may constitute valuable biomarkers among CC patients allowing a more personalized clinical intervention.

Thrombosis is a common and often deadly cardiovascular disease that arises from the generation of a thrombus in the arterial or venous circulation, often followed by the formation of an embolus (a foreign or abnormal particle circulating in the blood), which can lead to the reduction or even blockage of the blood flow, a condition known as thromboembolism¹. Venous thromboembolism (VTE) encompasses deep venous thrombosis (DVT), its most common manifestation, and pulmonary embolism (PE), a consequence of DVT in most scenarios (~95%)^{2–5}. Like other common and complex diseases, VTE pathophysiology involves both inherited and acquired factors^{2–4}. The disease is thought to have a strong genetic component, with over 50% of the variation of VTE risk being

¹Molecular Oncology and Viral Pathology Group, Research Center of IPO Porto (CI-IPOP)/ Pathology and Laboratory Medicine Dep., Clinical Pathology SV/ RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Centre (Porto.CCC), 4200-072 Porto, Portugal. ²FMUP, Faculty of Medicine, University of Porto, 4200-072 Porto, Portugal. ³CBAS, Abel Salazar Institute for the Biomedical Sciences, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. ⁴Oncology Department, Portuguese Institute of Oncology of Porto (IPOP), 4200-072 Porto, Portugal. ⁵Department of Medical Oncology, Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD), 5000-508 Vila Real, Portugal. ⁶FCUP, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal. ⁷External Radiotherapy Department, Portuguese Institute of Oncology of Porto (IPOP), 4200-072 Porto, Portugal. ⁸Research Department, Portuguese League Against Cancer (NRNorte), 4200-172 Porto, Portugal. ⁹CEBIMED, Faculty of Health Sciences, Fernando Pessoa University, 4200-150 Porto, Portugal. ¹⁰These authors contributed equally: Beatriz Vieira Neto and Valéria Tavares. ✉email: ruimedei@ipoporito.min-saude.pt

attributed to inherited factors⁶. In the last decades, several VTE-related genetic markers have been discovered in the general population, most of them located in the vicinity or within haemostatic genes, as expected. These genetic factors are known to downregulate anticlotting mechanisms and, in opposition, upregulate proclotting ones^{1,3}. The list includes mutations, particularly in genes related to the anticoagulation system (*PROC*, *PROS1* and *SERPINC1*), but mainly genetic polymorphisms, which have a higher relevance at a population level given their high frequency ($\geq 1\%$)⁴. The most studied genetic polymorphisms underlying VTE susceptibility are the single-nucleotide polymorphisms (SNPs) *F5* rs6025 (Factor V Leiden (FVL)) and *F2* rs1799963 (prothrombin G20210A), which are associated with activated protein C (APC) resistance and increased expression levels of prothrombin, respectively, both favouring thrombogenesis^{7–9}. As for the acquired risk factors, the more impacting ones are advanced age, high body mass index (BMI), autoimmune disorders and cancer¹.

Malignancy is an independent risk factor for VTE development. The association between cancer and VTE was first observed by Trousseau in the nineteenth century¹⁰. Cancer-related VTE, or the Trousseau syndrome as it is known, affects over 15% of oncological patients and constitutes the second most frequent cause of death among them^{4,11–15}. Beyond the disease itself, its detrimental effect on the prognosis of cancer patients is thought to be also attributed to the contribution of the deregulated haemostatic mechanisms that underlie the disease pathogenesis, in both cancer progression and aggressiveness^{4,16,17}. Therefore, identifying tools able to predict cancer-related VTE is demanding as it may allow a more tailored prophylaxis. Additionally, it is extremely important to unravel new predictive biomarkers because cancer patients are more prone to bleeding events, particularly under anticoagulation therapy. In clinical practice, the most studied VTE risk-assessment model is the Khorana score (KS), which encompasses the parameters of tumour site, pre-treatment BMI, platelet, leukocyte and haemoglobin counts, to categorise cancer patients according to VTE risk^{18,19}. Unfortunately, the score performance is poor and the search for predictive biomarkers of cancer-related VTE is still ongoing^{20–24}. Whether genetic determinants related to thrombogenesis in the general population could predict cancer-related VTE is a matter of discussion^{4,24}.

In general, cancer-related VTE is attributed to factors related to the patient (e.g., advanced age), tumour (e.g., primary site, histology and stage) and antineoplastic treatments (e.g., surgery and chemotherapy), which affect blood composition, blood flow and the integrity of blood vessels (Virchow's Triad)^{1,16}. Among solid tumours, gastric and pancreatic cancers are associated with the highest VTE risk, while gynaecological tumours are associated with an intermediate risk, with prostate, breast and skin cancers, among others, constituting the low-risk group²⁴. Compared with other gynaecological cancers, such as ovarian and endometrial tumours, the data concerning VTE incidence and its effect on cervical cancer (CC) patients as well as the underlying mechanisms, are not as solidified. According to the scarce data, VTE incidence in these patients appears to be substantial, which is in part attributed to the disease therapeutic management^{25–27}. The standard treatment for CC patients at locally advanced disease stages (IB2 to IVA) consists of concomitant chemoradiotherapy (CCRT), which exposes these patients to an increased risk of thrombotic events^{26,28–30}. In this context, the present study was designed to analyse the incidence of VTE following CC diagnosis and its impact on the outcome of patients with this malignancy from the Northern Region of Portugal, treated with CCRT. Furthermore, the predictive ability of KS was investigated, as well as the predictive and prognostic value (regardless of VTE status) of thrombogenesis-related genetic polymorphisms previously identified in the general population.

Materials and methods

Study cohort description. A retrospective hospital-based cohort study was conducted with cytological and histologically diagnosed CC patients with European ancestry, admitted to the Clinic of Gynaecology of the Portuguese Institute of Oncology of Porto (IPOP), from February 2002 to October 2009 and from May 2017 to October 2021, for the first-line treatment, from whom biological samples were available in our biobank. Patients were included if they had disease staged as IB2 to IVA and were submitted to CCRT as the first line treatment, with weekly administration of 40 mg/m² of cisplatin during external radiotherapy, and regarding on response followed or not by brachytherapy. Patients were excluded if they were under 18 years old, had done surgery (a known VTE risk factor) at least 6 months before CCRT or were only admitted for a second opinion. A total of 400 CC patients from the north region of Portugal were enrolled. Tumour staging was made according to the International Federation of Gynaecology and Obstetrics (FIGO) staging system³¹.

Patients were classified as having a VTE history in the setting of cancer based on their medical files. No active screening for VTE was made since this measure is not included in the clinical routine procedures at IPOP. As such, there was no accountability for clinically asymptomatic events, which represent the majority of cancer-related VTE events and are also considered to negatively impact a patient's prognosis, although to a lesser extent^{32,33}. Data concerning demographic and clinicopathological factors, as well as the follow-up of the patients, were also obtained from their medical files. The characterization of the study cohort ($n = 400$) according to the VTE status (with VTE vs. without clinical evidence of VTE) is given in Table 1. The mean follow-up was 155.3 months (standard deviation (SD) = 7.6 months).

This study was approved by the ethics committee at IPOP (CES IPO: 287A/014). A written consent according to the principles of the Helsinki Declaration was obtained from each patient before their enrolment in this study.

Sample collection and genomic DNA extraction. Peripheral venous blood samples of each patient were obtained using a standard technique and collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes.

From the blood samples, genomic Deoxyribonucleic Acid (DNA) was extracted using GRS Genomic DNA Kit—Blood & Cultured Cells from Grisp Research Solutions®, as indicated by the manufacturer's instructions.

Variable (no. patients)*	VTE status			P-value
	With VTE (n = 34; 8.5%)	Without clinical evidence of VTE (n = 366; 91.5%)	Total (n = 400)	
Age (400)				
Median	50.0	48.0	49.0	–
≥ 50 years**	17 (50.0)	164 (44.8)	181	0.688
< 50 years**	17 (50.0)	202 (55.2)	219	
Missing data	–	–	–	
Cancer stage (394)				
I and II	22 (64.7)	276 (76.7)	298	0.179
III and IV	12 (35.3)	84 (23.3)	96	
Missing data	–	6	6	
Cancer histology (393)				
Adenocarcinoma and others	8 (24.2)	63 (17.5)	71	0.467
Squamous cell carcinoma	25 (75.8)	297 (82.5)	322	
Missing data	1	6	7	
KS (90)				
< 2	6 (54.6)	49 (62.0)	55	0.883
≥ 2	5 (45.5)	30 (38.0)	35	
0	0 (0.0)	0 (0.0)	0	0.191
1–2	8 (72.7)	72 (91.1)	80	
≥ 3	3 (27.3)	7 (8.9)	10	
Missing data	23	287	310	

Table 1. Demographic and clinicopathological characteristics of CC patients according to VTE status (n = 400). CC cervical cancer, No. number, VTE venous thromboembolism, KS Khorana score. *Number of patients with available data. **Cut-off defined based on the median age of the patients as the variable is not normally distributed (Kolmogorov–Smirnov test, $P < 0.001$).

Polymorphism selection. To cover different aspects of haemostasis, polymorphism selection was made considering genome-wide association studies (GWAS)-identified SNPs linked to VTE susceptibility in the general population, as well as SNPs associated with platelet activation and aggregation (primary haemostasis)^{34–37}. From the identified variants, were prioritized those with reported prognostic value in the context of cancer independently of VTE status. In addition, a minor allele frequency (MAF) of 10% in the Iberian population (Ensembl database, <https://www.ensembl.org/index.html>; accessed on 20th September 2021) was considered to avoid missing SNP genotypes in the cohort study (n = 400). Following these criteria, eight SNPs were selected: *Protein C receptor (PROCR)* rs10747514, *Regulator of G Protein Signalling 7 (RGS7)* rs2502448, *OTU Deubiquitinase 7A (OTUD7A)* rs7164569, *Zinc finger protein, FOG family member 2 (ZFPM2)* rs4734879, *Integrin Subunit Beta 3 (ITGB3)* rs5918, *Glutathione-Disulfide Reductase (GSR)* rs3779647, *Coagulation factor 11 (F11)* rs4253417 and *Contactin 6 (CNTN6)* rs6764623.

Polymorphism genotyping. Polymorphism genotyping was conducted using the TaqMan® Allelic Discrimination methodology in a StepOne Plus Real-time Polymerase Chain Reaction (Real-time PCR) system (Applied Biosystems). Each reaction was performed using 2.5 µL of TaqPath™ ProAmp™ Master Mix (1×), 2.375 µL of sterile water, 0.125 µL of TaqMan® Genotyping Assay Mix (assays ID provided in Table 2) and 1.0 µL of genomic DNA, making a total volume of 6 µL. The thermal cycling conditions for DNA amplification were the following: (1) 95 °C for 10 min (activation of Taq DNA Polymerase), (2) 45 cycles of 95 °C for 15 s (denaturation of DNA chains), and (3) 60 °C for 1 min (primers pairing and extension). Data of DNA amplification were analysed using StepOne Software (version 2.3 Applied Biosystems). Negative controls (without DNA) were included in each RT-PCR reaction to prevent false positives. Moreover, to ensure the quality of SNP genotyping, a double sampling for at least 10% of randomly selected DNA samples was performed, with an accuracy of over 99%. Genotyping results were evaluated by two researchers with no previous knowledge concerning VTE status, demographic and clinicopathological data of the patients included in the study.

Statistical analysis. Data analysis was carried on by using the computer software IBM® SPSS® Statistics software package Version: 28.0.0.0. (IBM Corp. Released 2021). Associations of the genetic variants with VTE status and patient's demographic and clinicopathological factors were assessed using the chi-square test (χ^2) for categorical variables, while the student's t-test or the Mann–Whitney U test was employed for continuous variables depending on their distribution (normal and not normal distribution, respectively). The distribution was assessed using the Kolmogorov–Smirnov test given the large cohort size.

Two measures of clinical outcome were considered, namely time to VTE occurrence and overall survival (OS). The former was defined as the period from cancer diagnosis to VTE occurrence (primary endpoint) or patients' last clinical assessment, while the latter was the time from cancer diagnosis to patient death from all

SNP	MAFi (MA)*	Genotype	N (%)	N total (%)	MAFs (MA)	Failed genotyping (%)	TaqMan SNP genotyping assay ID
PROCR rs10747514	22% (A)	GG	131 (48.9)	268 (100)	30% (A)	132 (33.0)	C___1825060_10
		AG	112 (41.8)				
		AA	25 (9.3)				
RGS7 rs2502448	38% (C)	TT	102 (40.0)	255 (100)	39% (C)	145 (36.3)	C__26887460_10
		CT	109 (42.7)				
		CC	44 (17.3)				
OTUD7A rs7164569	34% (G)	AA	122 (32.5)	375 (100)	45% (G)	25 (6.3)	C___1698935_20
		AG	165 (44.0)				
		GG	88 (23.5)				
ZFPM2 rs4734879	34% (G)	AA	123 (45.7)	269 (100)	31% (G)	131 (32.8)	C___1315535_10
		AG	124 (46.1)				
		GG	22 (8.2)				
ITGB3 rs5918	14% (C)	TT	205 (67.9)	302 (100)	18% (C)	98 (24.5)	C___818008_30
		CT	86 (28.5)				
		CC	11 (3.6)				
GSR rs3779647	47% (C)	TT	90 (27.8)	324 (100)	46% (C)	76 (19.0)	C__25472374_10
		CT	167 (51.5)				
		CC	67 (20.7)				
F11 rs4253417	42% (C)	TT	96 (32.8)	293 (100)	43% (C)	107 (26.8)	C__32291269_10
		CT	141 (48.1)				
		CC	56 (19.1)				
CNTN6 rs6764623	25% (C)	AA	143 (57.9)	247 (100)	24% (C)	153 (38.3)	C__26850683_10
		AC	89 (36.0)				
		CC	15 (6.1)				

Table 2. Genotype distribution of thrombogenesis-related SNPs in a cohort of 400 CC patients. SNP single-nucleotide polymorphism, MAFi minor allele frequency in the Iberian population, MAFs minor allele frequency in the study cohort, MA minor allele, ID identification number. *According to the *Ensembl* database.

causes (secondary endpoint) or last clinical assessment. Survival curves were obtained using the Kaplan–Meier method, while the probabilities of survival were analysed using the log-rank test. After an initial comparison between the survival curves under the additive genetic model, the most suitable genetic model (additive, recessive or dominant) for each genetic polymorphism was selected. In terms of survival, stratified analyses by VTE status (with vs. without clinical evidence of VTE), FIGO stage (III/IV vs. I/II) and age (<50 vs. ≥50 years) were conducted for polymorphisms with at least a marginal association considering the entire cohort ($P < 0.100$). In addition to the log-rank test, univariate Cox regression analyses were also performed to estimate the hazard ratio (HR) values regarding the 10-year risk of VTE occurrence and the 10-year risk of death associated with the relevant genetic polymorphisms. Multivariate Cox regression analyses were also conducted adjusting for FIGO stage (III/IV vs. I/II) and age (<50 vs. ≥50 years), which are known prognostic factors in CC^{38,39}. All tests conducted were two-sided and a 5% level of significance was established.

Ethics approval. This study was approved by the ethics committee at IPOP (CES IPO:287A/014). A written consent according to the principles of the Helsinki Declaration was obtained from each patient before their enrolment in this study.

Results

Genotype distribution of the thrombogenesis-related genetic variants. The genotype distribution of each selected SNP and the respective TaqMan assay are represented in Table 2. Overall, except for RGS7 rs2502448, there were no significant statistical differences between the different genotypes of each variant and the patient's demographic and clinicopathological characteristics (data not shown), namely FIGO stage (III/IV vs. I/II), age (<50 years vs. ≥50 years) and histological subtype (adenocarcinoma/others vs. squamous cell carcinoma) ($P > 0.05$). The distribution of RGS7 rs2502448 genotypes among the different histological subtypes was statistically different (χ^2 , $P = 0.019$). Namely, most patients with squamous cell carcinoma had the variant C allele (63.55%; $n = 203$), while most of those with other histological subtypes had the TT genotype (56.25%; $n = 48$). Nevertheless, in this study, cancer histology was not a predictor of CC-related VTE nor a prognostic factor ($P > 0.05$).

Cancer-associated-VTE: impact of VTE on the clinical outcome of cervical cancer patients. In the study, 34 patients (8.5%) presented symptomatic VTE, the mean time to the disease occurrence being

37.2 months after initiation of cancer management (SD=52.5 months). VTE patients had a lower 10-year OS compared to the ones without clinical evidence of the disease (mean 10-year OS of 60.9 months (SD=8.8 months) and 92.3 months (SD=2.3 months), respectively; log-rank test, $P<0.001$; Fig. 1). This result was corroborated by univariate Cox regression analysis (VTE vs. without clinical evidence of VTE; HR 2.522; 95% confidence interval (CI), 1.530–4.159; $P<0.001$).

From the entire cohort, only 90 patients had the necessary data available for KS determination, (tumour site, pre-treatment platelet and leukocyte count, haemoglobin level and BMI). Following the original cut-off scores (KS ≥ 3 : high risk; 1–2: intermediate risk; 0: low risk), 80 out of the 90 patients were considered as having intermediate risk and 10 were high risk (Table 1). VTE incidence was 10% and 30% among the former and the latter, respectively (χ^2 , $P=0.191$). Considering the new recommended cut-off scores (KS ≥ 2 , high risk; 0–1, low risk), 55 out of the 90 patients were considered as having low risk and 35 were high risk (Table 1). The disease frequency was 14% and 22% among the former and the latter, respectively (χ^2 , $P=0.883$). Considering only the original cut-offs, which had better performance, KS was found to be only marginally associated with time to VTE occurrence ($P=0.078$). Stratified analyses according to FIGO stage (III/IV vs. I/II) and patient's age (<50 vs. ≥ 50 years) also did not reveal a significant predictive impact on the score. Nevertheless, KS showed a prognostic value among the 90 patients (log-rank test, $P=0.046$). Namely, patients with a score ≥ 3 had a lower 10-year OS compared to the ones with a score <3 (mean 10-year OS of 33.4 months (SD=5.9 months) and 48.2 months (SD=2.0 months), respectively). As it was only possible to determine the score for 90 patients and given its poor performance, further analyses integrating the score were not conducted.

Thrombogenesis-related genetic variants: impact on VTE occurrence and clinical outcome. Considering the entire cohort of CC patients, for *ZFPM2* rs4734879, *OTUD7A* rs7164569, *ITGB3* rs5918, *GSR* rs3779647, *F11* rs4253417 and *CNTN6* rs6764623, no statistically significant association with the time to VTE occurrence and patient's prognosis was observed ($P>0.05$). In opposition, *PROCR* rs10747514 and *RGS7* rs2502448 polymorphisms were shown to have a significant impact.

The polymorphism *PROCR* rs10747514 presented a marginal association with VTE development (AA vs. AG/GG; χ^2 , $P=0.101$). When analysing the time to a VTE event, the patients with the AA genotype (A being the minor allele; Table 2) exhibited a mean time of 100.8 months (SD=8.8 months), whereas the G allele carriers presented a mean time of 113.7 months (SD=1.7 months) under a recessive genetic model (AA vs. AG/GG; log-rank test, $P=0.021$; Fig. 2a). The prothrombotic effect of the AA genotype was confirmed by univariate Cox regression analysis (AA vs. AG/GG; HR 3.463; 95% CI 1.128–10.369; $P=0.030$). Furthermore, the multivariate analysis also corroborated the predictive impact of rs10747514 (Table 3). Specifically, carriers of the AA genotype had a fourfold increase in the 10-year risk of VTE occurrence compared to G allele genotypes adjusted for FIGO stage (AA vs. AG/GG; adjusted HR (aHR) 4.273; 95% CI 1.342–13.601; $P=0.014$; Table 3). The variant *PROCR* rs10747514 was also shown to have a potential prognostic value among CC patients (AA vs. AG/GG; log-rank test, $P=0.040$; Fig. 2b). Specifically, patients with the AA genotype presented a mean 10-year OS of 73.7 months (SD=10.8 months), whereas carriers of G allele genotypes exhibited 95.3 months (SD=2.8 months), suggesting a detrimental impact of the AA genotype. By stratifying the analysis according to the patients' VTE status (with vs. without clinical evidence of VTE), a significant impact of *PROCR* rs10747514 (Fig. 2c) on patients' 10-year OS was observed among the group of patients without clinical evidence of VTE (log-rank test, $P=0.004$; Fig. 2c) but not among those with VTE (AA vs. AG/GG; log-rank test, $P=0.087$). In the group without clinical evidence of VTE, the carriers of the AA genotype had a lower 10-year OS compared to the G allele genotypes carriers,

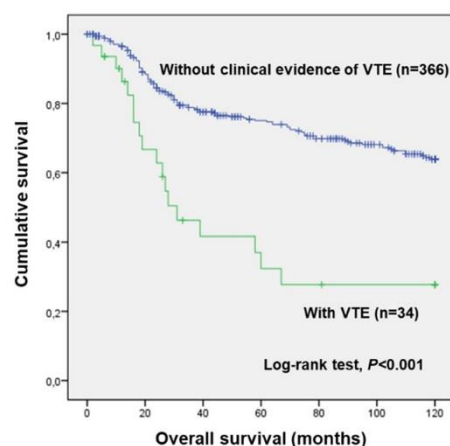


Figure 1. 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for CC patients ($n=400$), according to VTE status. Patients with VTE had a lower 10-year OS compared to the ones without clinical evidence of VTE ($P<0.001$). Specifically, VTE patients had a mean 10-year OS of 60.9 months (SD=8.8 months), while their counterparts presented a mean 10-year OS of 92.3 months (SD=2.3 months).

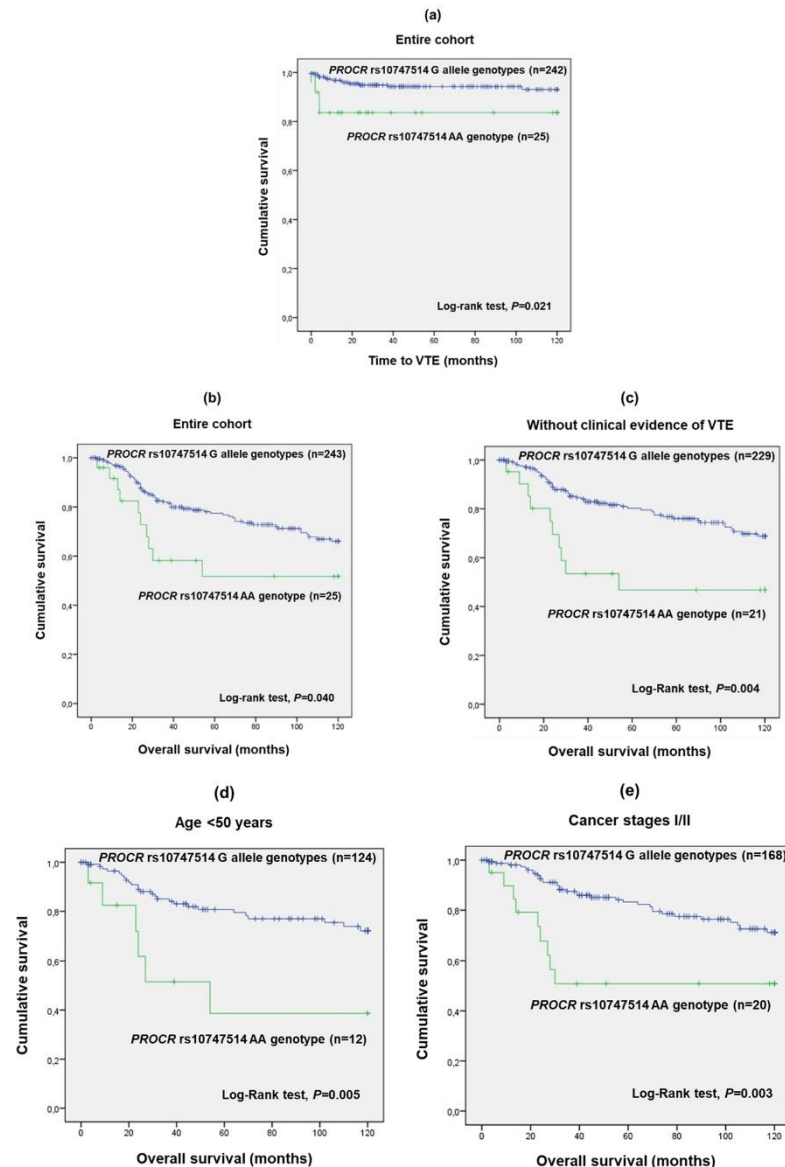


Figure 2. (a) Time to VTE occurrence by Kaplan–Meier and Log-rank test for CC patients, according to *PROCR* rs10747514 (n = 267). Patients with the AA genotype presented a lower time to VTE development compared to the G allele carriers (mean time to VTE of 113.7 months (SD = 8.8 months) and 100.8 months (SD = 1.7 months), respectively; log-rank test, $P=0.021$). (b) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for CC patients, according to *PROCR* rs10747514 (n = 268). Patients with the AA genotype presented a lower 10-year OS compared to the G allele carriers (mean 10-year OS of 73.7 months (SD = 10.8 months) and 95.3 months (SD = 2.8 months), respectively; log-rank test, $P=0.040$). (c) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for CC patients without clinical evidence of VTE, according to *PROCR* rs10747514 genotypes (n = 250). Patients with the AA genotype had a lower 10-year OS compared to patients with the G allele (mean 10-year OS of 68.8 months (SD = 11.4 months) and 98.2 months (SD = 2.8 months), respectively; log-rank test, $P=0.004$). (d) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for young CC patients (< 50 years) without clinical evidence of VTE, according to *PROCR* rs10747514 genotypes (n = 136). Patients with the AA genotype had a lower 10-year OS compared to patients with the G allele (mean 10-year OS of 62.1 (SD = 15.4 months) and 99.2 (SD = 3.8 months) respectively; log-rank test, $P=0.005$). (e) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for CC patients at FIGO I/II stages and without clinical evidence of VTE, according to *PROCR* rs10747514 genotypes (n = 188). Patients with the AA genotype had a lower 10-year OS compared to carriers of G allele genotypes (mean OS of 70.6 months (SD = 11.9 months) and 101.0 months (SD = 3.0 months), respectively; log-rank test, $P=0.003$).

therefore corroborating a detrimental effect of the AA genotype (mean OS of 68.8 months (SD = 11.4 months) and 98.2 months (SD = 2.8 months), respectively; AA vs. AG/GG; log-rank test, $P = 0.004$; Fig. 2c). Considering solely the patients without clinical evidence of VTE, stratified analyses according to age (< 50 vs. ≥ 50 years) and FIGO stage (III/IV vs. I/II) were performed (Fig. 2d,e, respectively). The polymorphism was found to have a significant impact on the 10-year OS of only young patients (< 50 years) (AA vs. AG/GG; log-rank test, $P = 0.005$; Fig. 2d). In terms of the FIGO stage, a statistically significant impact on the 10-year OS was only observed for patients at FIGO I/II stages (AA vs. AG/GG; log-rank test, $P = 0.003$; Fig. 2e). In line with the previous results, univariate Cox regression analysis confirmed the prognostic value of *PROCR* rs10747514 among those without clinical evidence of VTE (AA vs. AG/GG; HR 2.578; 95% CI 1.308–5.083; $P = 0.006$). Furthermore, according to multivariate analysis, those with the AA genotype had also a threefold increase in the 10-year risk of death compared to G allele genotypes when adjusted for FIGO stage (AA vs. AG/GG; aHR 2.612; 95% CI 1.322–5.161; $P = 0.006$; Table 4).

The polymorphism *RGS7* rs2502448 presented a significant association with VTE development (CT/CC vs. TT; χ^2 , $P = 0.009$). The patients with the C allele (minor allele; Table 2) presented a mean time to VTE of 108.5 months (SD = 2.9 months), contrasting with the 118.8 months (SD = 1.2 months) exhibited by TT genotype carriers under a dominant genetic model (CT/CC vs. TT; log-rank test, $P = 0.006$; Fig. 3a). This result suggested a prothrombotic effect of the C allele, which was corroborated by univariate Cox regression analysis (CT/CC vs. TT; HR 10.062; 95% CI 1.329–76.179; $P = 0.025$). Likewise, multivariate analysis showed that the patients with C allele genotypes had a tenfold increase in the 10-year risk of VTE occurrence compared to the TT genotype adjusted for FIGO stage (CT/CC vs. TT; aHR 10.672; 95% CI 1.409–80.813; $P = 0.022$; Table 3). The variant *RGS7* rs2502448 was also shown to have a potential prognostic value among CC patients given the marginal association (CT/CC vs. TT; log-rank test, $P = 0.086$; Fig. 3b). Specifically, C allele carriers presented a mean 10-year OS of 97.1 months (SD = 3.5 months), while the ones with the TT genotype exhibited a mean OS of 86.0 months (SD = 4.9 months), indicating a protective effect of the C allele. By stratifying the analysis according to the patients' VTE status, a significant impact of the variant (Fig. 3c) on patients' 10-year OS was observed only among those without clinical evidence of VTE (log-rank test, $P = 0.010$; Fig. 3c). In this group, carriers of the C allele had a higher 10-year OS compared to patients with the TT genotype, confirming the protective effect of the C allele (mean OS of 102.2 months (SD = 3.4 months) and 85.7 months (SD = 4.9 months), respectively; CT/CC vs. TT; log-rank test, $P = 0.010$; Fig. 3c).

Considering solely those without clinical evidence of VTE, stratified analyses according to age (< 50 vs. ≥ 50 years) and FIGO stage (III/IV vs. I/II) were performed (Fig. 3d,e, respectively). The polymorphism rs2502448 was found to have a statistically significant impact on the 10-year OS of only young patients (CT/CC vs. TT; log-rank test, $P = 0.019$; Fig. 3d). Additionally, regarding the FIGO stage, a significant impact of rs2502448 on the 10-year OS was only observed for patients at FIGO I/II stages (CT/CC vs. TT; log-rank test, $P = 0.005$; Fig. 3e). In line with the previous results, univariate Cox regression analysis confirmed the prognostic value of *RGS7* rs2502448 among the patients without clinical evidence of VTE. Specifically, C allele carriers had a 50% reduction in the 10-year risk of death compared to the ones with the TT genotype (CT/CC vs. TT; HR 0.513; 95% CI 0.305–0.862; $P = 0.012$). Likewise, according to multivariate analysis, the C allele was also associated with a 50% reduction in the 10-year risk of death compared to the TT genotype when adjusted for FIGO stage (CT/CC vs. TT; aHR 0.490; 95% CI 0.288–0.835; $P = 0.009$; Table 4).

Given the observed predictive and prognostic value of *PROCR* rs10747514 and *RGS7* rs2502448, multivariate analyses considering both polymorphisms were conducted adjusted for FIGO stage (III/IV vs. I/II) and age (< 50 vs. ≥ 50 years) (Table 5). The two polymorphisms had an additive impact on CC-related VTE and patient survival.

Discussion

Cancer is a well-known major risk factor for VTE. A close interplay between tumour cells and the haemostatic components has been well-recognized, with thrombogenesis paralleling tumour progression and aggressive behaviour^{1,2,25,40}. In concordance, beyond being highly incident, VTE imposes a negative impact on the prognosis of oncological patients²⁴. As previously mentioned, data concerning VTE incidence and effect in CC patients, as well as the underlying mechanisms, is not as solidified^{25–27}. Based on the somewhat scarce evidence, the frequency of venous thrombogenesis in these patients can reach up to 30% depending on multiple factors, including the type of therapeutic intervention²⁵. Nevertheless, even in VTE absence, cancer patients often present a blood hypercoagulation state, which reflects the deregulated activity of certain haemostatic components involved in coagulation activation. As such, regardless of VTE status, haemostatic components present in the tumour microenvironment might favour cervical tumorigenesis, consequently deteriorating the patients' prognosis^{25,27,35}.

In this cohort study, 34 patients (8.5% of 400) presented clinical evidence of VTE in the setting of malignancy. However, as patients were not actively assessed for VTE presence, events among the ones without clinical evidence of the disease cannot be excluded. Nevertheless, the VTE incidence reported in the present study is comparable to the ones stated in the literature for CC patients under chemoradiotherapy (3%⁴¹, 4%⁴², 6%⁴³, 8%⁴⁴ and 15%³⁰). According to the results, VTE patients had a lower 10-year OS compared to their counterparts (log-rank test, $P < 0.001$). More specifically, those with VTE presented almost a threefold increase in the risk of death compared to the ones without clinical evidence of the disease (HR 2.522; 95% CI 1.530–4.159; $P < 0.001$), suggesting that VTE significantly deteriorates CC patients' prognosis (log-rank test, $P < 0.001$). Indeed, as previously stated, thromboembolism is known to negatively affect the prognosis of cancer patients. Furthermore, beyond the disease itself, tumour cells are known to hijack haemostatic components present in the surrounding microenvironment to fuel their progression towards metastasis, meanwhile disturbing the haemostatic imbalance

Variable	Time to VTE					
	Initial model			Final model*		
	aHR	95% CI	P-value	aHR	95% CI	P-value
<i>PROCR</i> rs10747514 (AA vs. AG/GG ¹)	4.282	1.335–13.737	0.014	4.273	1.342–13.601	0.014
FIGO stage (III/IV vs. I/II ¹)	2.736	1.005–7.447	0.049	2.740	1.011–7.426	0.048
Age (< 50 vs. ≥ 50 years ¹)	1.001	0.961–1.042	0.975	–	–	–
Variable	Time to VTE					
	Initial model			Final model*		
	aHR	95% CI	P-value	aHR	95% CI	P-value
<i>RGS7</i> rs2502448 (CT/CC vs. TT ¹)	10.486	1.384–79.433	0.023	10.672	1.409–80.813	0.022
FIGO stage (III/IV vs. I/II ¹)	2.887	1.072–7.777	0.036	2.818	1.048–7.579	0.040
Age (< 50 vs. ≥ 50 years ¹)	1.468	0.532–4.048	0.459	–	–	–

Table 3. Multivariate analysis (backward Wald method) on the VTE risk of CC patients considering the polymorphisms *PROCR* rs10747514 (n = 262) and *RGS7* rs2502448 (n = 249). VTE venous thromboembolism, CC cervical cancer, aHR adjusted hazard ratio, CI confidence interval. ¹Reference group. *After applying the backward Wald method. Bold values were considered statistically significant.

Variable	10-year risk of death					
	Initial model			Final model*		
	aHR	95% CI	P-value	aHR	95% CI	P-value
<i>PROCR</i> rs10747514 (AA vs. AG/GG ¹)	2.846	1.421–5.703	0.003	2.612	1.322–5.161	0.006
FIGO stage (III/IV vs. I/II ¹)	1.600	0.890–2.878	0.116	1.593	0.888–2.859	0.118
Age (< 50 vs. ≥ 50 years ¹)	0.998	0.976–1.020	0.866	–	–	–
Variable	10-year risk of death					
	Initial model			Final model*		
	aHR	95% CI	P-value	aHR	95% CI	P-value
<i>RGS7</i> rs2502448 (CT/CC vs. TT ¹)	0.496	0.291–0.846	0.010	0.490	0.288–0.835	0.009
FIGO stage (III/IV vs. I/II ¹)	1.166	0.622–2.185	0.631	–	–	–
Age (< 50 vs. ≥ 50 years ¹)	0.835	0.492–1.416	0.503	0.822	0.487–1.388	0.463

Table 4. Multivariate analysis (backward Wald method) on the risk of death of CC patients considering *PROCR* rs10747514 (n = 228) genotypes and *RGS7* rs2502448 (n = 228) genotypes. VTE venous thromboembolism, CC cervical cancer, aHR adjusted hazard ratio, CI confidence interval. ¹Reference group. *After applying the backward Wald method. Bold values were considered statistically significant.

towards thrombogenesis. Given the negative impact of VTE on the prognosis of CC patients, better thromboprophylaxis measures need to be explored, which also demands an improvement of VTE risk prediction models.

The most studied tool for the prediction of cancer-related VTE is KS^{18,19}. In this study, the score had poor performance. While KS ≥ 2 was not found to be associated with increased VTE risk (χ^2 , $P = 0.883$), KS ≥ 3 was only marginally associated with the disease (χ^2 , $P = 0.191$) and time to VTE occurrence (≥ 3 vs. < 3 , log-rank test, $P = 0.078$). The patients with KS ≥ 3 (high-risk) had a VTE incidence of 30%, while the ones with KS = 1–2 (intermediate risk) had a disease incidence of 10%, which is somewhat comparable to the results of a previous study among patients with gynaecological tumours⁴⁵. Also following the literature, the original cut-off score (≥ 3) seems to be more appropriate to determine VTE risk among these patients⁴⁶. Although out of the scope of this study, KS ≥ 3 was found to predict a worse prognosis (≥ 3 vs. < 3 , log-rank test, $P = 0.046$). Overall, data on the discriminatory performance of KS among cancer patients is inconsistent^{20,47,48}. Further investigation is, however, required to validate these findings among CC patients, particularly in larger cohorts and considering other treatments.

As for the polymorphisms, starting with rs10747514, this intronic variant located in *PROCR* leads to the substitution of a guanine (G) for an adenine (A)⁴⁹. The gene *PROCR* located in chromosome 20 encodes for the human endothelial protein C receptor (EPCR), a transmembrane glycoprotein predominantly present in the membrane of endothelial cells, being engaged in haemostasis^{50–53}. Specifically, this protein is implicated in the protein C (PC) anticoagulant pathway by acting as the cellular receptor of PC, a serine protease with anticoagulant activity^{52–54}. During haemostasis triggering, circulating PC binds to EPCR, leading to the generation of APC. Once dissociated from EPCR, APC together with its cofactor protein S (PS) inhibits the activated coagulation factor V (FVa) and the activated coagulation factor VIII (FVIIIa), consequently downregulating thrombin generation and preventing venous thrombogenesis^{53,55}. As for *PROCR* rs10747514, its functional consequence is unclear. Indeed, intronic variants may affect either gene expression, for instance by disrupting a transcription factor binding site or changing the protein function [196, 197]. Thus, it is plausible that *PROCR* rs10747514 might

affect EPCR expression and/or the protein activity with further implications in VTE development. In this study, the *PROCR* rs10747514 AA genotype was associated with a lower time to VTE occurrence compared to the G allele, suggesting that the AA genotype has a prothrombotic effect among CC patients (log-rank test, $P = 0.021$). This effect was corroborated by both univariate (AA vs. AG/GG; HR 3.463; 95% CI 1.128–10.369; $P = 0.030$) and multivariate Cox regression analysis adjusted for FIGO stage (AA vs. AG/GG; aHR 4.273; 95% CI 1.342–13.601; $P = 0.014$). Namely, patients carrying the AA genotype had a fourfold increase in the 10-year risk of VTE compared to their counterparts. Together with the literature, it was hypothesized that the AA genotype might either lead to lower EPCR levels and/or activity or to a higher shedding of the receptor from the endothelial membrane (an effect reported for another *PROCR* polymorphism), thus impairing EPCR anticoagulant effect^{56,57}.

Beyond EPCR's implication in the haemostatic pathway, this protein has also been suggested to interfere with carcinogenesis^{58,59}. Indeed, EPCR through coagulation-independent mechanisms plays an important role in several tumorigenic-related processes, including apoptosis, angiogenesis, inflammation, cell migration and proliferation^{52,55,60}. For instance, the interaction of APC with EPCR and protease-activated receptors (PAR) allows the upregulation of the antiapoptotic protein B-cell lymphoma 2 (BCL-2), as well as the downregulation of p53, Bax and caspases 3, 8 and 9, therefore promoting cell survival⁵⁵. Altogether, growing evidence strongly suggests a tumour-promoting effect of EPCR^{52,55,58,60–62}. Concordantly, in this study, *PROCR* rs10747514 was found to have a prognostic value among CC patients, with the AA genotype exhibiting a detrimental impact on the 10-year OS of the patients (AA vs. AG/GG; log-rank test, $P = 0.040$). When stratifying the analysis according to VTE status, a significant prognostic impact of the *PROCR* rs10747514 was only observed among the group of patients without clinical evidence of VTE occurrence (AA vs. AG/GG; log-rank test, $P = 0.004$). The most likely explanation for the absence of a prognostic value in the context of VTE is the reduced number of VTE patients with available information concerning the polymorphism genotypes ($n = 17$), which may have prevented the detection of a significant association (AA vs. AG/GG; log-rank test, $P = 0.087$). Nevertheless, considering solely the patients without clinical evidence of VTE, the *PROCR* rs10747514 AA genotype was associated with a lower 10-year OS compared to the G allele genotypes in the group of younger (AA vs. AG/GG; log-rank test, $P = 0.005$) and early disease stage CC patients (AA vs. AG/GG; log-rank test, $P = 0.003$), confirming the negative impact of the AA genotype. Indeed, the patient's age and cancer stage are well-known factors related to thrombogenesis¹. Specifically, age combined with sex hormonal changes is reported to influence the expression levels of proteins involved in the coagulation system⁶³. Namely, activation of the PC pathway is suppressed with ageing, which is thought to be one of the mechanisms underlying age-associated thrombosis⁶⁴. Therefore, assuming that younger patients have a higher *PROCR* expression, the AA genotype might impose an additive effect by increasing EPCR activity, consequently favouring cancer pathways. As for the cancer stage, most genetic polymorphisms associated with thrombogenesis seem to be only relevant before metastasis. These variants might contribute to metastatic dissemination, but once it happens other biological mechanisms might be more preponderant in cancer progression¹. Univariate (AA vs. AG/GG; HR 2.578; 95% CI 1.308–5.083; $P = 0.006$) and multivariate Cox regression analysis adjusted for FIGO stage (AA vs. AG/GG; aHR 2.612; 95% CI 1.322–5.161; $P = 0.006$) confirmed the prognostic value of *PROCR* rs10747514 in the cohort of CC patients without clinical evidence of VTE. Namely, patients carrying the AA genotype had a threefold increase in 10-year risk of death compared to their counterparts in both analyses. Altogether, it was hypothesized that the AA genotype might be associated with higher EPCR levels and/or activity, thereby promoting tumorigenesis and deteriorating the CC patients' clinical outcome. However, the prothrombotic effect of the AA genotype observed in this study (AA vs. AG/GG; aHR 4.273; 95% CI 1.342–13.601; $P = 0.014$) seems to oppose this hypothesis. Although future functional studies are required to elucidate the underlying biological processes, one possible explanation could be an increased EPCR expression and/or activity paralleling with PC trapping by EPCR, thus impairing the anticoagulation pathway while promoting cancer progression through coagulation-independent mechanisms in individuals without symptomatic VTE. Overall, *PROCR* rs10747514 may be a useful biomarker to predict VTE among CC patients and assess their prognosis even in the absence of venous thrombogenesis.

The intronic variant rs2502448 located in *RGS7* results in a substitution of a thymine (T) to a cytosine (C). The gene *RGS7* is located in chromosome 1 and encodes for the Regulator of G-protein signalling 7 (*RGS7*)⁴⁹. This protein belongs to the multifunctional superfamily of regulators of G-protein signalling (RGS) responsible for controlling the signalling cascades of G-protein, an enzyme engaged in cell signalling transduction. The G-protein activity is actively controlled by RGSs (including *RGS7*) and G protein-coupled receptors (GPCRs). While the former inhibits G-protein signalling through the hydrolysis of guanosine triphosphate (GTP), the latter stimulates GTP-binding leading to G-protein activation^{65–68}. Among its several functions, *RGS7* has been previously described to play a role in platelet function, with some genetic polymorphisms within *RGS7* demonstrated to be associated with a risk for venous thrombosis and ischemic stroke^{66,67,69–71}. As GPCRs are crucial for platelet activity, *RGS7*, an antagonist of GPCR signalling transduction, might prevent platelet aggregation, consequently reducing the risk of venous thrombogenesis^{65,68,72}. Although its functional consequence is unclear, as an intronic variant, *RGS7* rs2502448 might alter *RGS7* expression and/or protein functionality, favouring VTE development. Thus, considering that the *RGS7* rs2502448 C allele was previously associated with higher platelet reactivity under acetylsalicylic acid (ASA; also known as aspirin) treatment (odds ratio (OR) 3.45; CI 1.82–6.53), and given the potential *RGS7* negative regulation of platelet activation and aggregation, the C allele might be linked to lower protein expression and/or activity, favouring venous thrombogenesis^{36,71}. Concordantly, in this study, the *RGS7* rs2502448 C allele was associated with a lower time to VTE, indicating a prothrombotic effect of this allele among CC patients (log-rank test, $P = 0.006$). This observation was further confirmed by both univariate (CT/CC vs. TT; HR 10.062; 95% CI 1.329–76.179; $P = 0.025$) and multivariate Cox regression analysis adjusted for FIGO stage (CT/CC vs. TT; aHR 10.672; 95% CI 1.409–80.813; $P = 0.022$). Specifically, patients carrying the C allele genotypes had a tenfold increase in the 10-year risk of VTE compared to their counterparts.

Figure 3. (a) Time to VTE occurrence by Kaplan–Meier and Log-rank test for CC patients, according to *RGS7* rs2502448 genotypes (n = 254). Patients with the C allele had a lower time to VTE occurrence compared to patients with the TT genotype (mean time to VTE of 118.8 months (SD = 2.9 months) and 108.5 months (SD = 1.2 months), respectively; log-rank test, $P = 0.006$). (b) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for CC patients, according to *RGS7* rs2502448 (n = 255). Patients with the C allele had a higher 10-year OS compared to patients with the TT genotype (mean 10-year OS of 97.1 months (SD = 3.5 months) and 86.0 months (SD = 4.9 months), respectively; log-rank test, $P = 0.086$). (c) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for CC patients without clinical evidence of VTE, according to *RGS7* rs2502448 genotypes (n = 238). Patients with the C allele genotypes had a higher 10-year OS compared to patients with the TT genotype (mean 10-year OS of 102.2 months (SD = 3.4 months) and 85.7 months (SD = 4.9 months), respectively; log-rank test, $P = 0.010$). (d) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for young CC patients (< 50 years) and without clinical evidence of VTE, according to *RGS7* rs2502448 genotypes (n = 128). Patients with the C allele had a higher 10-year OS compared to TT genotype carriers (mean OS of 105.2 months (SD = 4.4 months) and 84.4 months (SD = 6.7 months), respectively; log-rank test, $P = 0.019$). (e) 10-year overall survival (OS) by Kaplan–Meier and Log-rank test for CC patients at FIGO I/II disease stages and without clinical evidence of VTE, according to *RGS7* rs2502448 genotypes (n = 182). Patients with the C allele genotypes had a higher 10-year OS compared to patients with the TT genotype (mean 10-year OS of 104.7 months (SD = 3.6 months) and 85.9 months (SD = 5.4 months), respectively; log-rank test, $P = 0.005$).

Beyond haemostasis, RGS play several roles, many of those involved in malignancy. RGS expression levels have been reported to be deregulated in a tissue-specific manner, particularly RGS1, mediating cellular processes such as survival, proliferation, and migration^{73–76}. Inclusively, genetic variants in RGS genes have been found to have a prognostic value among oncological patients^{76,77}. Little is known, however, about the role of *RGS7* in tumorigenesis, particularly in the context of CC since RGS most likely acts in a tissue-specific manner⁷⁶. In the present study, *RGS7* rs2502448 was marginally associated with the 10-year OS of the CC patients, with the C allele exhibiting a benefit impact (CT/CC vs. TT; log-rank test, $P = 0.086$). Based on the previously mentioned hypothesis that the C allele might be linked to lower *RGS7* expression and/or activity, thereby favouring venous thrombogenesis, *RGS7* may exert oncogenic roles, which would explain the beneficial impact associated with the *RGS7* rs2502448 C allele. Nevertheless, additional studies are required to explore the role of *RGS7* in cervical tumorigenesis. Like *PROCR* rs10747514, a significant impact of *RGS7* rs2502448 on patients' 10-year OS was only observed among the group of patients without clinical evidence of VTE (CT/CC vs. TT; log-rank test, $P = 0.010$). Among these patients, the *RGS7* rs2502448 C allele was associated with a higher 10-year OS compared to the TT genotypes in the group of younger (CT/CC vs. TT; log-rank test, $P = 0.019$) and early disease stage CC patients (CT/CC vs. TT; log-rank test, $P = 0.005$), corroborating the beneficial impact of C allele. Indeed, age-associated changes in the expression level of RGS proteins have been reported^{78,79}. Thus, it was hypothesized that younger patients may have a higher expression of *RGS7*, meaning that in these patients the polymorphism rs2502448 may have an additive impact. As for the influence of the cancer stage, as previously mentioned, genetic polymorphisms associated with thrombogenesis are thought to be more impacting in a premetastatic context, favouring events towards metastasis. Concordantly, *RGS7* rs2502448 was previously found to be associated with the 5-year OS of epithelial ovarian cancer (EOC) patients at early disease stages (log-rank test, $P = 0.035$) but not at advanced stages, suggesting a potential role of this variant in metastasis regardless of tumour type³⁶. The prognostic value of *RGS7* rs2502448 in patients without clinical evidence of VTE was corroborated by both univariate (CT/CC vs. TT; HR 0.513; 95% CI 0.305–0.862; $P = 0.012$) and multivariate Cox regression analysis adjusted for FIGO stage (CT/CC vs. TT; aHR 0.490; 95% CI 0.288–0.835; $P = 0.009$). Namely, patients carrying the C allele had a 50% reduction in the 10-year risk of death compared to their counterparts in both analyses. Altogether, it was hypothesized that the C allele may lead to lower expression levels of *RGS7*, thus having a beneficial effect on CC patients' clinical outcomes, assuming that the protein has oncogenic roles. Our research group previously found that, among EOC patients, the rs2502448 C allele was also associated with a higher 5-year OS compared to the TT genotype (log-rank test, $P = 0.035$), also leading to the hypothesis of lower protein levels associated with the C allele³⁶. Overall, like *PROCR* rs10747514, *RGS7* rs2502448 seems to be a valuable tool to predict VTE among CC patients and assess their prognosis even in the absence of the disease.

The remaining evaluated polymorphisms were not associated with VTE development or the patient's prognosis ($P > 0.05$). Nevertheless, as this is a preliminary study, additional investigation with a larger cohort is required to validate this study's findings. Briefly, the intronic variant rs4734879 located within *ZFPM2* is thought to be associated with the expression levels of *Vascular endothelial growth factor A* (*VEGFA*) and other haemostatic components implicated in thrombogenesis^{49,80–83}. Overall, in the context of cancer-related VTE, this variant has not been validated yet, at least to the best of our knowledge⁴. In a previous study conducted by our research group, *ZFPM2* rs4734879 was found to be a prognostic factor among EOC patients, which was attributed to its potential influence in tumour neo-angiogenesis³⁵. The synonymous variant rs7164569 located in *OTUD7A* might be associated with the downregulation of Nuclear Factor Kappa B (NF- κ B), which positively regulates platelet activity^{49,84}. Since *OTUD7A* acts towards NF- κ B inhibition, this deubiquitinase might impair platelet responses decreasing the risk of thrombogenesis^{85–89}. However, no study has yet validated the role of rs7164569 in cancer-related VTE⁴. As the NF- κ B signalling pathway is implicated in several processes associated with tumorigenesis, *OTUD7A* by inhibiting it may suppress these cellular processes, suggesting that this deubiquitinase may impose a protective effect in cancer progression^{87,88}. To the best of our knowledge, however, the variant rs7164569 has not yet been linked to the prognosis of oncological patients³⁵. The missense variant *ITGB3* rs5918 has been extensively studied given its putative impact on platelet responses and venous thrombogenesis in the general population^{37,49,90}. Until now, however, no study has yet demonstrated the role of this polymorphism

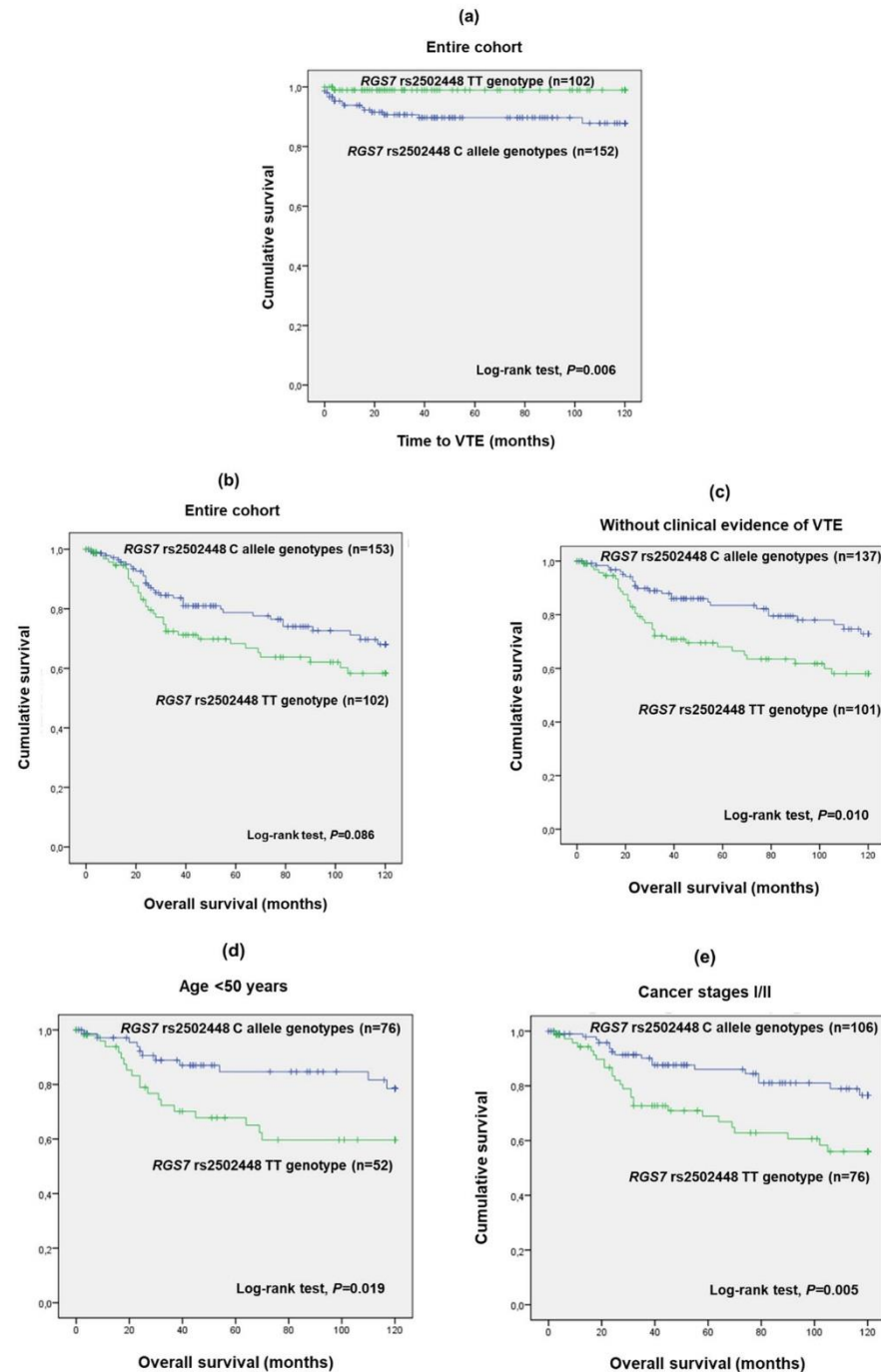


Fig. 4. Impact of RGS7 rs2502448 genotype on survival. (a) Entire cohort: Time to VTE (months). (b) Entire cohort: Overall survival (months). (c) Without clinical evidence of VTE: Overall survival (months). (d) Age <50 years: Overall survival (months). (e) Cancer stages I/II: Overall survival (months). Log-rank test P -values are provided for each plot.

in cancer-related VTE⁴. Beyond platelet activation, which itself promotes cancer progression since platelets are engaged in many processes towards tumour dissemination, GP IIb/IIIa is implicated in several signalling pathways involved in tumour progression. Concordantly, the variant showed a prognostic value among EOC patients³⁶. The intronic variant GSR rs3779647 was previously associated with platelet activation, however, to the best of our knowledge, this genetic variant has not been linked to cancer-related VTE^{4,49,71,91}. The polymorphism was previously associated with EOC patients' survival, which is thought to be due to the contribution of GSR to resistance to platinum-based chemotherapy through its role in glutathione metabolism^{36,92}. The intronic variant rs4253417 locates within the *F11* gene that encodes for the coagulation factor XI (FXI), which is implicated in the intrinsic coagulation pathway culminating with thrombin generation and consequently fibrin deposition^{49,93,94}.

Variable	Initial model			Final model*			Event
	aHR	95% CI	P-value	aHR	95% CI	P-value	
<i>PROCR</i> rs10747514 (AA vs. AG/GG ¹)	3.994	1.112–14.342	0.034	3.852	1.071–13.860	0.039	10-year risk of VTE
<i>RGS7</i> rs2502448 (CT/CC vs. TT ¹)	9.884	1.299–75.212	0.027	9.818	1.289–74.746	0.027	
FIGO stage (III/IV vs. I/II ¹)	3.200	1.153–8.880	0.025	3.223	1.156–8.989	0.025	
Age (< 50 vs. ≥ 50 years ¹)	1.418	0.503–4.003	0.509	–	–	–	
<i>PROCR</i> rs10747514 (AA vs. AG/GG ¹)	3.830	1.752–8.373	0.001	3.628	1.683–7.822	0.001	10-year risk of death
<i>RGS7</i> rs2502448 (TT vs. CT/CC ¹)	2.349	1.331–4.147	0.003	2.370	1.346–4.171	0.003	
FIGO stage (III/IV vs. I/II ¹)	1.367	0.719–2.599	0.341	–	–	–	
Age (< 50 vs. ≥ 50 years ¹)	0.992	0.570–1.724	0.977	–	–	–	

Table 5. Multivariate analysis (backward Wald method) on the 10-year risk of VTE (n = 237) and the 10-year risk of death (n = 217) of CC patients. VTE venous thromboembolism, CC cervical cancer, aHR adjusted hazard ratio, CI confidence interval. ¹Reference group. *After applying the backward Wald method. Bold values were considered statistically significant.

The variant rs4253417 was found to be associated with FXI circulating levels but has not been validated in the context of malignancy^{4,95}. No direct role of FXI in tumorigenesis is known, however, it may indirectly influence cancer pathways by modulating the activity and/or levels of downstream coagulation proteins^{96–105}. The intergenic variant rs6764623 locates close to *CNTN6*, which encodes for a protein that seems to trigger the Notch pathway, which mediates inflammatory responses implicated in cardiovascular disorders^{49,106}. Although unclear, the polymorphism is thought to regulate gene expression through cis and trans effects, potentially influencing *CNTN6* levels³⁵. The variant has been associated with a thrombotic risk in the general population, however, the same has not yet been demonstrated among oncological patients^{4,85}. Also, rs6764623 was shown to have a prognostic value among EOC patients, which could be due to the multiple roles of the Notch signalling pathway in carcinogenesis³⁵.

The most notable limitation of the study was its retrospective nature, which prevented the collection of relevant data. Specifically, as it is well known, several factors modulate the risk for VTE development, including, but not limited to, other comorbidities (for instance, haematological diseases), pregnancy, breastfeeding, use of oral contraception and a history of VTE, which were not accounted for in this study due to lack of information for most patients¹. Furthermore, data concerning the use of anticoagulants or antiaggregating agents prior to cancer diagnosis were also unavailable and given that no active screening of VTE is included in the clinical routine procedures, asymptomatic VTE events are not accounted for, which could partially explain the poor performance of KS. Inclusively, as VTE was not actively screened, the distribution of patients with and without VTE could be different, which represents an intrinsic limitation in this study. Another important data missing was the specific cause of death among these patients, which prevented the assessment of cancer specific survival, as well as a possible VTE-related mortality. Likewise, information on the discontinuation of antineoplastic treatment due to VTE, which may impact the patients' survival, is also missing. Lastly, the temporal gap regarding the patients' recruitment (February 2002 to October 2009 and May 2017 to October 2021) due to the requirement of biological samples may have led to selection bias.

Conclusion and future perspectives

Even when there is a successful antithrombotic treatment, VTE is associated with a spectrum of complications that deteriorate cancer patients' prognosis¹⁰⁷. Thus, the primary effort of investigation on cancer-related VTE should be focusing on the disease prevention. Altogether, the results of the present study corroborate the detrimental effect VTE has on the survival of CC patients and suggest that the genetic variants *PROCR* rs10747514 and *RGS7* rs2502448 could be useful predictive biomarkers of thrombogenesis in these patients, in addition to serving as potential tools for prognosis assessment regardless of VTE status. In opposition, the KS was only marginally associated with CC-related VTE, showcasing a poor performance. Overall, the present study highlights the need for a more tailored thromboprophylaxis among oncological patients and better and more personalized cancer management considering the role of haemostatic components in tumorigenesis. In this sense, thrombogenesis-associated genetic polymorphisms might be the bridge to connect both worlds. However, as this is a preliminary study with limitations, additional investigation in larger cohorts and considering other relevant factors are required to validate the results. Prospective studies with active screening of VTE are also a must to better explore the role of *PROCR* rs10747514 and *RGS7* rs2502448 and assess the performance of KS among CC patients. Likewise, it would be relevant to compare the predictive ability of the thrombogenesis-related polymorphisms with the KS and investigate potential drug targets that could be used for both thromboprophylaxis and CC treatment.

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Author contributions

All authors had a significant contribution to the study. Conceptualisation, R.M. and V.T.; Funding acquisition, V.T.; Investigation, B.V.N., V.T., J.B.d.S., L.C., L.S. and R.M.; Supervision, D.P. and R.M.; Writing of original draft, B.V.N., V.T. and I.S.M.; manuscript review and editing, B.V.N., V.T., J.B.d.S., J.L.-P., I.S.M., L.C., L.S., D.P. and R.M.

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Additional information

Correspondence and requests for materials should be addressed to R.M.

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Attachment 2



Review

Long Non-Coding RNAs in Venous Thromboembolism: Where Do We Stand?

Inês Soares Marques ^{1,2}, Valéria Tavares ^{1,3,4}, Beatriz Vieira Neto ^{1,5}, Inês N. R. Mota ^{1,2}, Deolinda Pereira ⁶ and Rui Medeiros ^{1,3,4,5,7,*}

- ¹ Molecular Oncology and Viral Pathology Group, Research Center of IPO Porto (CI-IPOP)/Pathology and Laboratory Medicine Dep., Clinical Pathology SV/RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Centre (Porto.CCC), 4200-072 Porto, Portugal; ines.soares@ipoporto.min-saude.pt (I.S.M.); valeria.tavares@ipoporto.min-saude.pt (V.T.); i37300@ipoporto.min-saude.pt (B.V.N.); ines.mota@ipoporto.min-saude.pt (I.N.R.M.)
- ² Faculty of Sciences of University of Porto (FCUP), 4169-007 Porto, Portugal
- ³ Faculty of Medicine of University of Porto (FMUP), 4200-072 Porto, Portugal
- ⁴ Abel Salazar Institute for the Biomedical Sciences (ICBAS), University of Porto, 4050-313 Porto, Portugal
- ⁵ Research Department, Portuguese League Against Cancer (NRNorte), 4200-172 Porto, Portugal
- ⁶ Oncology Department, Portuguese Institute of Oncology of Porto (IPOP), 4200-072 Porto, Portugal; dpereira@ipoporto.min-saude.pt
- ⁷ Faculty of Health Sciences, Fernando Pessoa University, 4200-150 Porto, Portugal
- * Correspondence: ruimedei@ipoporto.min-saude.pt; Tel.: +351-22-508-4000

Abstract: Venous thromboembolism (VTE), a common condition in Western countries, is a cardiovascular disorder that arises due to haemostatic irregularities, which lead to thrombus generation inside veins. Even with successful treatment, the resulting disease spectrum of complications considerably affects the patient's quality of life, potentially leading to death. Cumulative data indicate that long non-coding RNAs (lncRNAs) may have a role in VTE pathogenesis. However, the clinical usefulness of these RNAs as biomarkers and potential therapeutic targets for VTE management is yet unclear. Thus, this article reviewed the emerging evidence on lncRNAs associated with VTE and with the activity of the coagulation system, which has a central role in disease pathogenesis. Until now, ten lncRNAs have been implicated in VTE pathogenesis, among which MALAT1 is the one with more evidence. Meanwhile, five lncRNAs have been reported to affect the expression of TFPI2, an important anticoagulant protein, but none with a described role in VTE development. More investigation in this field is needed as lncRNAs may help dissect VTE pathways, aiding in disease prediction, prevention and treatment.

Keywords: RNA; long non-coding; venous thromboembolism; deep vein thrombosis; tissue-factor-pathway inhibitor 2



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1. Introduction

The blood circulatory system (or the cardiovascular system) is responsible for transporting and delivering various compounds, including nutrients and gases, being also engaged in removing waste products [1]. As such, ensuring the integrity of the blood vessels in this complex network is vital, a function carried out by the haemostatic system, which allows continuous blood circulation [2].

Haemostasis is a complex and dynamic process that rapidly responds to vascular injury. Several biochemical and cellular processes are triggered following vascular damage to form a blood clot, a process known as blood coagulation or blood clotting. This mechanism allows for the stopping of blood loss, restores haemostasis and initiates vascular repair [3]. Haemostasis is, however, under tight regulation to limit blood coagulation to sites of vascular damage, preventing haemorrhage and the formation of unnecessary blood clots

known as thrombi. The generation of a thrombus (i.e., thrombosis) can be life-threatening as it might restrict or even block the blood circulation inside the vessels, preventing the transportation of compounds and exchange of gases [4,5]. Thus, proclotting and anticlotting mechanisms that regulate haemostasis must be balanced to avoid excessive bleeding and blood clotting. To maintain the haemostatic balance, four key players—vascular endothelial cells (vECs), platelets, coagulation cascade, and fibrinolytic system—interact in an intricate and tightly controlled manner (Figure 1). The damage to blood vessels activates platelets, which adhere to the exposed subendothelial matrix. Simultaneously, the coagulation cascade, a chain of enzymatic reactions that culminate in fibrin generation, is triggered to stabilize the platelet plug with fibrin and form a blood clot at the injury site to stop the bleeding [3,6]. Subsequently, the fibrinolytic system is responsible for clot lysis (i.e., fibrinolysis), which prevents thrombosis and restores blood flow. The vECs, in turn, express and/or release a set of proteins that modulate the platelet activity, the coagulation cascade and fibrinolysis towards haemostasis restoration [6]. As expected, when one of these four components is malfunctioning, the haemostatic balance can be disturbed, leading to bleeding or thrombotic disorders [5].

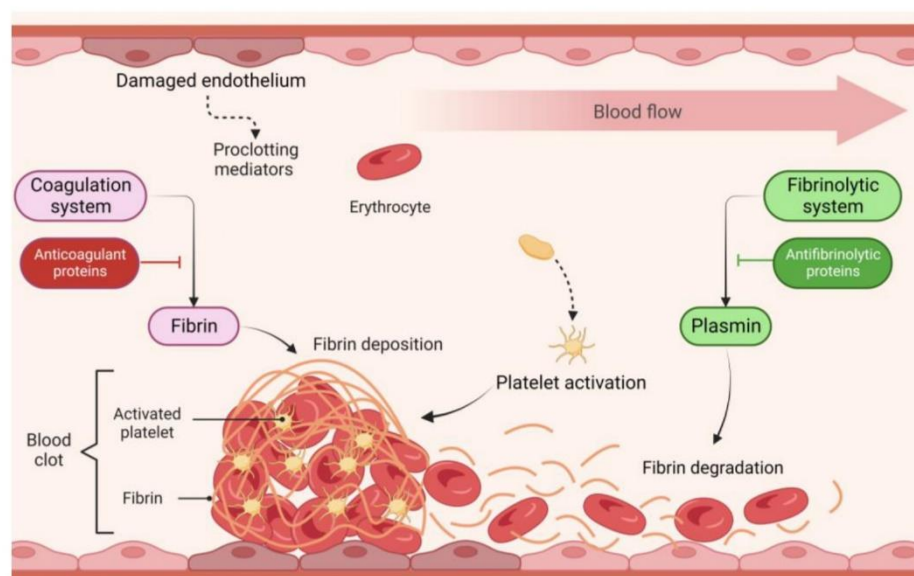


Figure 1. Interplay between the components of the haemostatic system. The figure was created with [BioRender.com](https://www.biorender.com) (accessed on 10 July 2023).

Deep venous thrombosis (DVT) and pulmonary embolism (PE) are two thrombotic events that collectively are known as venous thromboembolism (VTE). The former occurs when a thrombus forms within a deep vein (usually leg veins), leading to abnormal swelling and ulceration [7–10]. As for PE, this is a frequent complication of DVT that occurs when the thrombus migrates to the lungs, representing the most fatal VTE manifestation. Worldwide, every year VTE affects nearly ten million people [10]. If not treated properly, this cardiovascular disorder can be lethal, resulting in approximately 15% of mortality within the first three months following diagnosis [8–10]. However, even with successful treatment, the disease is also associated with a spectrum of potential complications that lead to substantial morbidity, namely VTE recurrent events, post-thrombotic syndrome, pulmonary hypertension, major bleeding under anticoagulation therapy and long-term disability [11,12]. Currently, anticoagulation therapy is the standard therapeutic approach for VTE, which, however, has proven to not always be efficient in the removal of the existing thrombus. Thus, it is crucial to dissect the molecular pathways involved in

VTE pathogenesis to identify novel biomarkers and improve risk stratification, disease prevention and treatment [7,12,13].

Like other common diseases in the general population, VTE has a complex pathophysiology with both acquired and genetic risk determinants modulating the disease's susceptibility. Advanced age, physical trauma, immobilization and active cancer constitute the most common acquired risk factors [11,14,15]. As for genetic factors, Factor V Leiden gene polymorphism (F5 rs6025), prothrombin gene polymorphism 20210 (F2 rs1799963), mutations causing inherited deficiencies of antithrombin, protein C and protein S, as well as the antiphospholipid antibody syndrome, account for about 7% to 22% of the elderly population attributable risk [14,15]. These factors can trigger what is known as Virchow's triad, which encompasses three conditions that prompt VTE development, namely blood hypercoagulability, stasis of blood flow within the vessels (i.e., venous stasis) and vascular endothelial damage [16].

The most clinically used biomarker for VTE risk prediction and diagnosis is D-dimer (DD), which is a product resultant from the breakdown of cross-linked fibrin (i.e., fibrinolysis) [17]. In addition to the first VTE events, DD is also associated with the rate of recurrent events and mortality [18]. This thrombotic biomarker was first identified in the 1980s and has since been used in clinical practice for the diagnosis of VTE, particularly given its long half-life [17,19]. However, DD levels are significantly elevated in several other conditions, such as inflammation, stroke, long hospitalization periods and individuals' advanced age, being unspecific of VTE presence [17,19,20]. To improve the specificity in detection, since DD alone is insufficient to confirm a diagnosis of VTE, elevated DD levels are followed by other diagnostic methods, including duplex ultrasonography for DVT and/or computed tomography for PE diagnosis [21,22]. Other suggested VTE biomarkers include markers for thrombophilia, for instance, soluble P-selectin and C-reactive protein (CRP). However, these biomarkers are still under evaluation and can lack specificity and/or sensitivity [23,24]. Thus, novel and trustworthy biomarkers are required to reduce the time of diagnosis and consequently improve the morbidity and mortality rates associated with VTE. Recently, emerging evidence suggested a role of long non-coding RNAs (lncRNAs) in the disease's pathogenesis. This class of non-coding RNAs (ncRNAs) has the potential to expand our knowledge about VTE, and they represent attractive novel biomarkers of this disorder. Thus, in this narrative review, the discussion focus was the role of lncRNAs in VTE development and their implications in the haemostatic system, aiming to assess their potential clinical application as biomarkers and/or therapeutic targets in VTE management. To do so, a PubMed search without time restriction was performed using combinations of the terms "long non-coding RNA", "lncRNA", "venous thromboembolism", "deep vein thrombosis", "deep venous thrombosis", "pulmonary embolism", "thrombosis", "haemostasis", "coagulation", "tissue factor" and "tissue factor pathway inhibitor" anywhere in the article. Relevant publications were also found by cross-referencing the bibliographic references of the retrieved papers. The identified scientific articles were published in the last six years (from 2017 to 2023).

2. LncRNAs: A Novel Epigenetic Regulator in VTE

LncRNAs are ncRNAs longer than 200 nucleotides with the particularity of forming secondary structures, which enables them to bind to other biomolecules such as DNA, RNA and even proteins, thus affecting gene expression at different levels [25–28]. Another feature of these ncRNAs is the fact that they can function in the nucleus, where they are transcribed, but also at the cytoplasm, with most of them operating in both. In fact, lncRNAs can move between the two compartments in response to signals, even though the underlying mechanisms are unclear [29].

Similarly to messenger RNAs (mRNAs), lncRNAs are also transcribed by RNA Polymerase II and are often capped and polyadenylated, also presenting evolutionarily conserved patterns and a high level of tissue specificity [28]. According to the NONCODE database (version 6.0) (<http://www.noncode.org>, accessed on 24 May 2023), there are

96,411 human lncRNA genes [30]. Based on their genomic position, orientation and relative location to nearby protein-coding genes, these ncRNAs can be classified as intergenic, sense, antisense, intronic and bi-directional (Figure 2) [29]. Specifically, intergenic lncRNAs (lincRNAs), as the name indicates, do not overlap with any other genes [31]. As for those that do overlap, they can be sense or antisense depending on whether they possess sequence elements that will pair with other RNAs in the same or the opposite direction, respectively [32,33]. Intronic lncRNAs are located within the introns of other genes, while bi-directional lncRNAs correspond to those that are transcribed from the same region in the genome as another gene but in the opposite direction [29]. In addition to linear lncRNAs, another class is circular lncRNAs, which are formed by back-splicing of coding and non-coding transcripts [34].

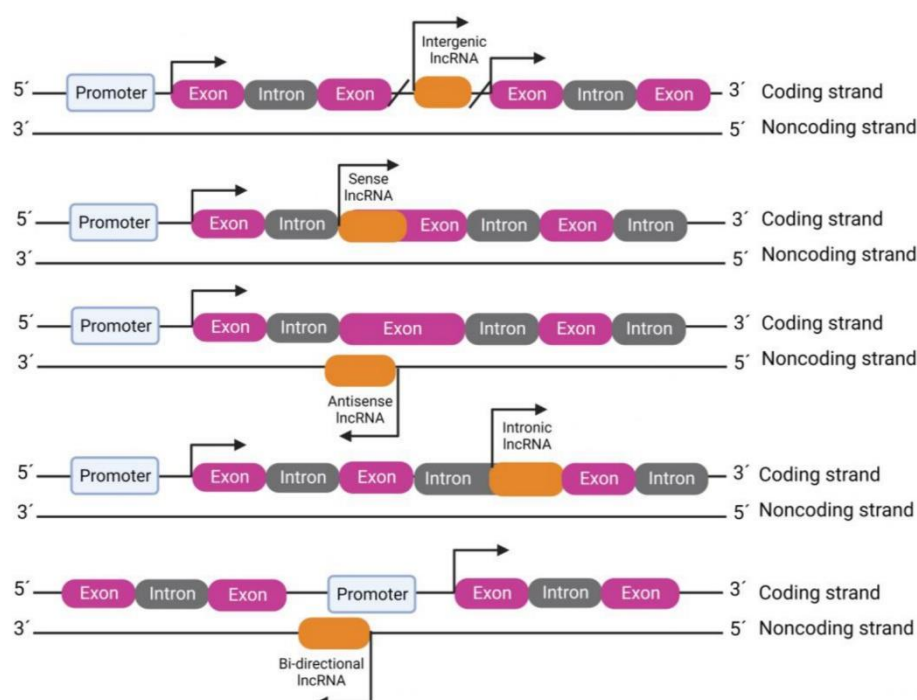


Figure 2. Genomic localization of lncRNAs. The figure was created with [BioRender.com](https://www.biorender.com) (accessed on 23 July 2023).

Despite the inconsistency in classification, the roles of lncRNAs in gene expression regulation can be divided into four main non-exclusive archetypes: signalling, decoy, guide and scaffold functions (Figure 3) [26]. As signalling molecules, lncRNAs can operate as spatiotemporal markers, reflecting the biological effects of transcription factors or signalling pathways. As decoys, lncRNAs can sequester transcription factors and other proteins into nuclear subdomains or away from chromatin. Decoy lncRNAs can also operate as competitive endogenous RNAs (ceRNAs), functioning as microRNA (miRNA or miR) sponges, consequently influencing the expression of miRNAs' targets (mRNAs). On the other hand, guide lncRNAs can recruit RNA-binding proteins to target genes, while scaffolds may bring together several proteins to build complexes with specific biological roles, influencing transcription. Of note, lncRNAs localized in the nucleus can modify chromatin, activate or repress transcription of target genes by interacting with DNA sequences or transcription factors, while cytoplasmic lncRNAs are mostly associated with mRNA stability and translation, promoting or repressing protein synthesis [28,35,36]. Specifically, in the nucleus, lncRNAs are known to interact with DNA methyltransferases or demethylases, influencing the addition (methylation) or removal of methyl groups (demethylation) on

specific genomic regions. Consequently, DNA methylation/demethylation can then lead to chromatin structure modifications [37]. Furthermore, lncRNAs can also directly mediate histone modifications of target genes. By recruiting histone modification complexes, such as histone methyltransferases or demethylases, to target gene promotor-associated binding sites, lncRNAs can induce changes in promotor histone modification patterns. These complexes then add or remove specific histone marks, such as methylation, acetylation or other modifications, on the histone tails. These modifications result in either a more open or closed chromatin conformation at the target gene promotor [38]. Both mechanisms, DNA methylation/demethylation and histone modifications, affect the accessibility of genes to the transcriptional machinery [37,38]. The versatility in these functions (chromatin regulation, transcriptional activation and repression, and RNA and protein modifications) make lncRNAs important players in normal physiology and in the development of a variety of diseases, such as cancer, cardiovascular and neurodegenerative diseases, in which their dysregulated levels have been consistently observed [8,13,28,35,39–46].

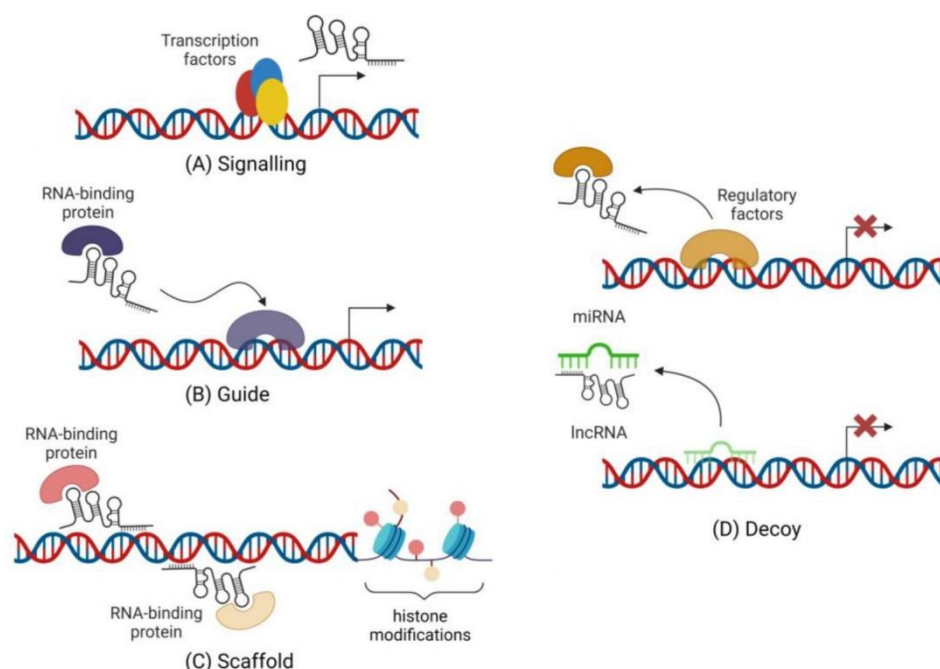


Figure 3. Four archetypes of lncRNAs functions. The figure was created with [BioRender.com](https://www.biorender.com) (accessed on 23 July 2023).

The first report of a putative implication of lncRNAs in cardiovascular diseases came from genome-wide association studies (GWAS). These studies independently identified single-nucleotide polymorphisms (SNPs) located in a *locus* close to the protein-coding genes CDKN2A and CDKN2B as related to susceptibility to coronary artery disease [47,48]. This *locus*, which is in human chromosome 9p21, is adjacent to the lncRNA named antisense non-coding RNA in the INK4 *locus* (ANRIL), indicating a potential role of this lncRNA in coronary artery disease, which was later corroborated by subsequent studies [49]. Recently, reports have also suggested that the expression of lncRNAs is deregulated in VTE. Interestingly, most of the reported lncRNAs were linked to the activity of vECs. As Virchow's triad postulates, endothelial injury is a major risk factor for thrombogenesis [50]. The vECs play important roles in the maintenance of haemostasis and thrombus development [51]. In fact, several studies have confirmed the close relationship between vECs and the occurrence of DVT [52]. Under physiological conditions, these cells act as a barrier, preventing coagulation factors and platelets from activating and forming blood clots. Additionally, they secrete anticoagulant factors such as tissue factor pathway inhibitor (TFPI), nitric oxide

(NO) and prostacyclin. They also exert an anticoagulant activity by expressing molecules such as heparin-like molecules, as well as thrombomodulin (TM) and endothelial protein C receptor (EPCR), both implicated in the protein C anticoagulant pathway that leads to the inactivation coagulation factors Va (FVa) and VIIIa (FVIIIa). Besides the anticoagulant activity, vECs also favour fibrinolysis via tissue-type plasminogen activator (tPA) production, which by converting plasminogen to plasmin, dissolves fibrin clots [53]. Contrarily, under pathological conditions (for instance, inflammation), vECs can express adhesion molecules such as P-selectin, which facilitates blood clot formation. Inclusively, under these conditions, these cells can become dysfunctional, which compromises their antithrombotic properties, increasing VTE risk [54–57].

In addition to mature vECs, two cell populations are of relevance when it comes to dissecting the endothelium biology, namely the endothelial progenitor cells (EPCs) and human umbilical vein endothelial cells (HUVECs) (Figure 4). The former are multipotent cells derived from bone marrow that circulate in the peripheral blood and can differentiate into mature vECs when needed [13,52]. Inclusively, studies have already shown that EPCs have the potential to prevent thrombus propagation and recurrence and to promote endothelial regeneration, revascularization and vein wall remodelling [52,58]. When there is a vascular injury, a series of mechanisms are triggered to repair the damage. One of these mechanisms is angiogenesis, during which new vessels are formed from pre-existing ones, allowing the restoration of normal vascular function and thrombus resolution [53,59]. During this process, several angiogenic factors are released, such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs), which together promote angiogenesis and stimulate the proliferation and migration of vECs to the injury site to help in the repair process [57]. Additionally, vECs can bind to platelets and inhibit their role in thrombus formation [60]. Taken together, these cells might be a promising therapeutic approach for patients for whom DVT treatments are unsuccessful [7]. As for HUVECs, they are primary cells present in the vein of the umbilical cord. These cells are commonly used as a model system for studying the biology of the endothelium and its response to environmental cues, angiogenesis and the development of cardiovascular diseases [61,62].

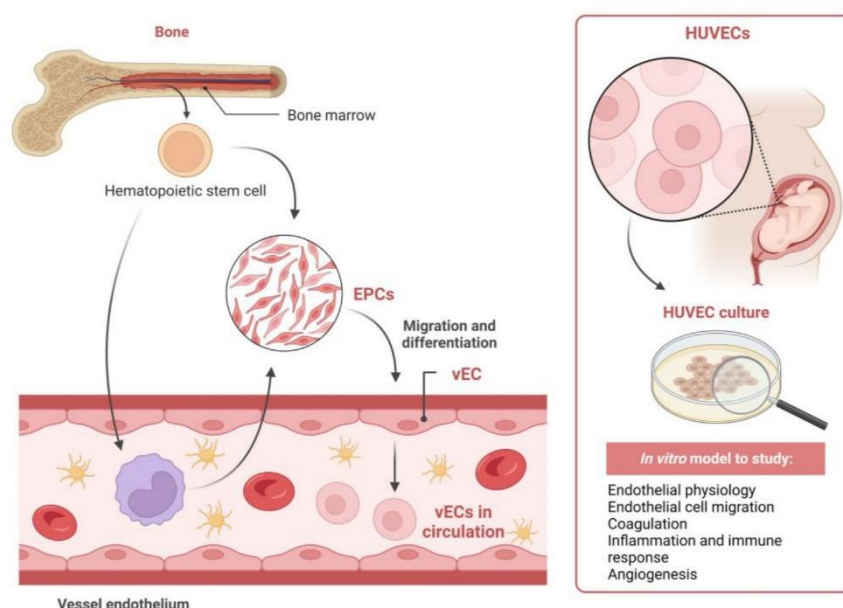


Figure 4. Biology of vascular endothelial cells (vECs). Abbreviations: EPC, endothelial progenitor cell; HUVEC, human umbilical vein endothelial cell. The figure was created with [BioRender.com](https://www.biorender.com) (accessed on 23 July 2023).

To the best of our knowledge, a total of 13 studies from 2017 to 2023 were performed, pinpointing a total of ten lncRNAs implicated in venous thrombosis (Table 1).

2.1. ANRIL

ANRIL, also known as CDKN2B antisense RNA 1 (CDKN2B-AS1), is a lncRNA first identified in patients with hereditary melanoma and neural system tumour. Moreover, ANRIL is implicated in other diseases, such as atherosclerosis and cancer [63–66]. This antisense lncRNA is highly expressed in the HUVEC autophagy model. Although its roles in thrombosis are not entirely understood, ANRIL is suggested to modulate TM expression [53,67]. By cooperating with protein C and thrombin activable fibrinolysis inhibitor (TAFI), TM is important in the maintenance of the endothelial microenvironment. More specifically, TM exhibits anticoagulant and anti-inflammatory properties. Also, it enhances fibrinolysis and improves the endothelium barrier function, overall inhibiting thrombosis [53,68]. Zeng et al. [67] reported that ANRIL is highly expressed among patients with thrombosis. As having a “decoy” archetype, the authors indicated that this lncRNA might act as a ceRNA, sponging miR-99a and miR-449a in the thrombus. This mechanism consequently promotes the activation of beclin-1 while significantly increasing the levels of TM. Through these mechanisms, the authors suggested that ANRIL could promote angiogenesis and thrombosis, which seems to be conflicted. As this lncRNA was evaluated in the presence of thrombi (thrombosis patients versus healthy individuals), the data should be analysed with caution since the lncRNA might be related to thrombus resolution rather than thrombus formation. This would explain the TM upregulation. Another limitation of the study is the fact that the authors fail to differentiate the different types of thrombotic events considered in the study. To be noted, ANRIL has also been associated with different manifestations of arterial thrombosis [69,70]. Overall, more studies are needed to clarify the role of ANRIL in venous thrombosis.

2.2. GUSBP5-AS

The recently identified lncRNA GUSB pseudogene 5 antisense (GUSBP5-AS) was shown to be upregulated in EPCs among DVT patients [7,13]. Acting as a decoy, this ncRNA is reported to sponge miR-223-3p, which targets forkhead box protein O1 (FOXO1). This leads to the activation of the AKT pathway, which enhances fibroblast growth factor 2 (FGF2), matrix metalloproteinase-2/9 (MMP2/9) and F-actin expression, promoting angiogenesis, EPC migration and invasion while reducing the apoptosis of these cells. As previously mentioned, these mechanisms are important for thrombus resolution and recanalization, as well as to improve the home ability of EPCs to thrombosis sites. Taken together, GUSBP5-AS seems to promote thrombus resolution and, thus, might help pinpoint a new potential therapeutic approach for DVT [7].

2.3. MEG9

The lncRNA maternally expressed gene 9 (MEG9) is suggested to protect the vasculature from DNA damage, according to an abstract. While DNA-damaging agents are known to induce the expression of this lncRNA, vascular growth factors have the opposite effect. The knockdown of MEG9 inhibits proapoptotic proteins, increases vEC death and decreases sprouting angiogenesis, implying that MEG9 might have a regulatory role in thrombosis since its inhibition in vECs accelerated fibrin formation [71]. Even though the available data about this lncRNA remains scarce, this abstract, together with other findings, indicates that MEG9 in vECs might impact pathways associated with inflammation and thrombosis [71–74]. More studies are required to explore the role of this lncRNA in VTE pathogenesis.

2.4. MALAT1

The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), as the name indicates, was first identified in non-small cell lung cancer (NSCLC) and in

association with lung cancer metastasis [75]. Since then, this lincRNA has been intensively studied, with reports of its deregulation in several conditions, such as cancer and Parkinson's disease [9,76–81]. Concerning vascular disease pathogenesis, MALAT1 has been pointed out as a major player due to its downregulation in atherosclerotic plaques and its association with vEC function [8,82]. There has been a growing interest in understanding the mechanisms through which MALAT1 manages to impact DVT. The performed studies had, however, inconsistent results, which led to the hypothesis that MALAT1 may have a dual role in thrombosis. On the one hand, this lncRNA was found to be upregulated in DVT through regulation of the activity and behaviour of EPCs [8,9]. Concordantly, in the same study, silenced MALAT1 enhanced the growth, migration and survival of EPCs. Those changes were reversed when the Wnt/ β -catenin signalling pathway was inhibited, suggesting that this pathway is a potential downstream target of MALAT1 in DVT. Thus, the MALAT1/Wnt/ β -catenin axis might be a promising novel therapeutic target for DVT treatment [8]. In another study, MALAT1 modulated HUVEC apoptosis by regulating the miR-383-5p/BCL2-like 11 (BCL2L11) axis in DVT. When overexpressed, this lncRNA repressed miR-383-5p, which otherwise would target BCL2L11, suggesting this ncRNA as a decoy. In turn, higher levels of BCL2L11 reduced cell viability and increased vEC apoptosis. Therefore, the MALAT1/miR-383-5p/BCL2L11 axis might also be effective for DVT management [9]. A more recent study investigated the mechanisms by which MALAT1 affects platelet activity and thrombus formation, as this is one of the most abundant lncRNAs in platelets. According to the results, platelet deficiency of MALAT1 was reported to enhance platelet adhesion and aggravate thrombus generation through the phosphoinositide 3-kinase (PI3K)/AKT/Glycogen synthase kinase-3 beta (GSK-3 β) signalling pathway, which contradicts the previous findings [83]. Taken together, the data indicate that MALAT1 might have a context-dependent role in DVT pathways. Additional studies are needed to better comprehend its implications in thrombogenesis, as this lncRNA seems to be a promising biomarker and therapeutic target for DVT.

2.5. SIRT1-AS

Sirt1 antisense lncRNA (Sirt1-AS) was first identified in 2014 and is implicated in myogenesis via the regulation of silent information regulator 1 (Sirt1) expression [84]. Sirt1 is a NAD⁺-dependent lysine deacetylase (member of the sirtuins family) activated in response to cellular stress. This enzyme is known to inhibit the development of several diseases, such as pulmonary fibrosis and cerebral ischemia, including also thrombotic events, by regulating the activity of platelets and vECs [85–88]. Specifically, sirt1-AS can bind and overlap with the 3' untranslated region (3'-UTR) of Sirt1 mRNA, forming a lncRNA-mRNA duplex, acting as a guide able to increase Sirt1 stability and expression. By promoting Sirt1 expression, Sirt1-AS can suppress the development of endothelial ageing and alleviate the formation of thrombus by improving the viability and proliferation of HUVECs [37]. Concordantly, in patients with DVT, Sirt1-AS is reported to be downregulated, which may affect Sirt1 abundance leading to an increased risk of thrombotic events [37,89,90]. Overall, the current evidence suggests that Sirt1-AS could be a potential DVT biomarker [37].

2.6. LINC01123

Long intergenic non-protein coding RNA 1123 (LINC01123) is known to act as an oncogene [91–93]. This lincRNA is also reported to play a role in carotid atherosclerosis by promoting cell proliferation and migration [94]. In a recent study, LINC01123 was shown to have a relevant role in lower extremity DVT (LEDVT) in rats via the miR-125a-3p/interleukin-1 receptor type 1 (IL1R1) axis. By sponging miR-125a-3p, which targets IL1R1, LINC01123 is associated with a rise in the length and weight of the thrombus, as well as higher levels of pro-inflammatory cytokines, namely interleukin-6 (IL-6) and interleukin-8 (IL-8). This, in turn, favours inflammatory cell infiltration, thrombosis in the lumen, and collagen fibre hyperplasia, which collectively leads to thrombo-inflammation in

the setting of LEDVT. The findings might give some insights into novel treatment options for patients with this condition [95].

2.7. TUG1

LncRNA taurine upregulated gene 1 (TUG1) was first identified as upregulated in developing retinal cells and in response to taurine (2-aminoethanesulfonic acid) [96]. This lncRNA is also implicated in other conditions, namely colorectal cancer and obesity-related diseases [97,98]. More recently, TUG1 was shown to have a poor expression in DVT mice. However, by downregulating miR-92a-3p and subsequently upregulating the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) pathway (known to regulate angiogenesis), TUG1 overexpression was able to accelerate the proliferation, migration and tube-forming abilities of EPCs, while decreasing their apoptosis and the thrombus size. Collectively, this lncRNA, by serving as a decoy, seems to exert a protective effect against DVT and provide femoral vein pathological damage. Thus, the TUG1/miR-92a-3p/HMGCR cascade might open new perspectives to treat DVT [58].

2.8. XIST

The lncRNA X inactive-specific transcript (XIST) is one of the most studied lncRNAs, known to be required for X-chromosomal inactivation during early development for dosage compensation [99]. In addition, XIST is also associated with preeclampsia and cancer [100,101]. This lncRNA was found to be highly expressed in the plasma of DVT patients, possibly acting as a decoy by sponging miR-103a-3p, which in turn, targets high-mobility group box 1 (HMGB1) [102]. Concordantly, miR-103a-3p expression was found to be reduced in patients with unprovoked VTE [103]. In DVT, lower levels of this microRNA inhibited normal functions of EPCs, such as migration and angiogenesis [104]. On the other hand, higher miR-103a-3p expression levels, together with lower levels of chemokine 12 (CXCL12), inhibited the development of LEDVT, which is in line with previous data [105]. As for HMGB1, its levels are highly expressed in DVT patients. This nuclear protein is one of the main regulators of the coagulation cascade, also known to induce inflammatory reactions and play a part in apoptosis. In thrombosis, HMGB1 has a pro-thrombotic effect as it promotes both the activation and aggregation of platelets [102,106]. Also, via the XIST/miR-103a-3p/HMGB1 axis, XIST may regulate the expression of tissue factor (TF, also known as thromboplastin or coagulation factor III (FIII)) in HUVECs induced by IL-1 β , as well as increase cell viability and hinder apoptosis by inhibiting the reactive oxygen species (ROS)/nuclear factor κ B (NF- κ B) signalling pathway [102,107–109].

2.9. LINC00659 and UXT-AS1

LINC00659, a novel lncRNA, was found highly expressed for the first time in colorectal cancer with implications in cancer cell growth and apoptosis, possibly through the PI3K-AKT pathway [110]. More recently, this lncRNA was implicated in gastric cancer [111,112]. High altitude is known to activate the coagulation system, increasing the risk of developing VTE, although there is still a lack of evidence regarding this matter. However, a pro-thrombotic phenotype often occurs, mainly in travellers and mountaineers, possibly due to the hypoxia state present at high altitudes, which increases platelet aggregation and triggers blood coagulation [113–117]. In this context, Jha et al. [114] aimed to compare the lncRNA expression profiles in the peripheral blood of human patients with DVT at high altitudes with control subjects without DVT. According to the results, LINC00659 and UXT antisense RNA 1 (UXT-AS1) were upregulated in DVT patients, possibly because both of these lncRNAs sponge miR-15 and miR-143, which otherwise would inhibit the expression of the pro-thrombotic genes *serpin family E member 1* (SERPINE1) and *hypoxia-inducible factor 1 subunit alpha* (HIF1A). The former encodes for plasminogen activator inhibitor 1 (PAI-1), a key inhibitor of fibrinolysis, by blocking the activation of plasminogen to plasmin [118]. On the other hand, HIF1 (encoded by HIF1A) regulates the activity of the NLR family pyrin domain containing 3 (NLRP3) inflammasome, a protein complex involved in the

formation of blood clots under hypoxia [119]. To be noted, as UXT-AS1 has no described role in VTE other than DVT associated with high altitude, this lncRNA does not fit the scope of this review. In another study, aiming to dissect the role of LINC00659 in vECs, the lncRNA was also reported to target and downregulate miR-525-5p, which, in turn, is known to downregulate BCL2 associated X, apoptosis regulator (Bax), a member of the Bcl-2 family of proteins linked to apoptosis, suggesting its role as a decoy. Via this mechanism, LINC00659 could inhibit the proliferation and cell viability of HUVECs and promote their apoptosis. Thus, the authors revealed a potential mechanism through which LINC00659 might impact DVT and indicate the LINC00659/miR-525-5p/Bax axis as a potential new direction for the diagnosis and treatment of DVT [120]. In a more recent study aimed to unravel the role of LINC00659 in LEDVT, the lncRNA, in addition to being elevated both in inferior vena cava tissues and EPCs of patients with LEDVT, also inhibited proliferation and migration of EPCs, as well as angiogenesis [121]. As an explanation, the authors hypothesized that LINC00659 might bind to the promoter of eukaryotic translation initiation factor 4A3 (EIF4A3) to upregulate its expression, suggesting that this ncRNA acts as a guide to promote EIF4A3 expression. In turn, EIF4A3 represses fibroblast growth factor 1 (FGF1) expression and facilitates its methylation through the recruitment of DNA methyltransferases 3A (DNAMT3A) to its promoter region. As FGF1 promotes EPCs viability and neovascularization in addition to its role in cell proliferation, migration, invasion and angiogenesis, by repressing this protein, LINC00659 might increase the risk for LEDVT development [122,123].

2.10. CRNDE

Long non-coding RNA Colorectal neoplasia differentially expressed (CRNDE) was initially found to be upregulated in colorectal cancer tissues [124]. More recent studies have indicated that this lncRNA is also implicated in other malignancies, in addition to regulating vascular smooth muscle cell behaviour and inflammation and apoptosis in alcoholic liver disease [125–128]. A recent study found that CRNDE and the prenylcysteine oxidase 1 (PCYOX1) were upregulated in the blood of DVT mice [129]. The protein PCYOX1 is an enzyme involved in the breakdown of prenylated proteins found in vascular and blood cells. Deficiency of PCYOX1 is suggested to cause platelet hypo-reactivity [130,131]. Thus, acting as a decoy and through the sequestering of miR-181a-5p (targets PCYOX1), CRNDE might lead to PCYOX1 upregulation, exacerbating thrombus formation and vascular inflammatory injury in DVT [129].

Table 1. LncRNAs with reported roles in VTE pathogenesis.

LncRNA	Study	LncRNA Location ¹	LncRNA Expression in VTE	Sample/Compartment or Study Model	LncRNA Target	Mechanism of Action	LncRNA Function in VTE
ANRIL	Zeng et al. (China, 2019) [67]	9p21.3	↑	HUVECs and rats	↓miR-99a ↓miR-449a	Increases beclin-1 expression via miR-99a and miR-449a sponging and upregulates thrombomodulin	Promotes thrombosis
GUSBP5-AS	Sun et al. (China, 2020) [7]	4q31.21	↑	Human EPCs and mice	↓miR-223-3p	Sponges miR-223-3p, which targets FOXO1 and activates the Akt pathway and enhances FGF2, MMP2/9 and F-actin expression	Promotes DVT resolution
MEG9	Espinosa-Diez et al. (EUA, 2020) [73]	14q32.31	n.a	ECs	n.a	Inhibits fibrin formation	Possibly has a protective effect against thrombosis
MALAT1	Du et al. (China, 2020) [8]	11q13.1	↑	Human EPCs	Wnt/β-catenin	Reduces endothelial function	Promotes DVT
	Wang et al. (China, 2022) [9]			HUVECs	↓miR-383-5p/BCL2L11		
	Sun et al. (China, 2022) [83]			CD34+ megakaryocytes and mice	PI3K/AKT/GSK-3β	Inhibits platelet activity and thrombus formation	Inhibits DVT
Sirt1-AS	Lou et al. (China, 2021) [39]	10q21.3	↓	Patients' blood, mice and HUVECs	↑Sirt1	Suppresses the endothelial ageing and alleviates the thrombus formation through Sirt1/FOXO3a axis	Attenuates ageing-related DVT
LincRNA 1123	Yang et al. (2022, China) [95]	2q13	↑	Rats	↓miR-125a-3p	Sponges miR-125a-3p, which targets IL1R1, facilitating thrombus formation and inducing higher levels of IL-6 and IL-8	Promotes LEDVT

Table 1. Cont.

LncRNA	Study	LncRNA Location ¹	LncRNA Expression in VTE	Sample/Compartment or Study Model	LncRNA Target	Mechanism of Action	LncRNA Function in VTE
TUG1	Feng et al. (China, 2022) [58]	22q12.2	↓	Mice EPCs and mice	↓miR-92a-3p	Accelerates proliferation, migration and tube-forming abilities and decreases apoptosis of EPCs and thrombus size through downregulation of miR-91a-3p and upregulation of HMGCR	Protects against DVT
XIST	Cao et al. (China, 2022) [102]	Xq13.2	↑	Human plasma and HUVECs	↓miR-103a-3p	Reduces ECs normal functions through miR-103a-3p sponging	Promotes DVT
LINC00659	Zhang et al. (China, 2023) [121]	20q13.33	↑	Human IVC tissue samples, human EPCs and mice	↑EIF4A3	Upregulates EIF4A3 expression and exacerbates endothelial progenitor cell dysfunction	Promotes LEDVT
	Zhu et al. (China, 2023) [120]		n.a	HUVECs	↓miR-525-5p	Inhibits proliferation and cell viability in HUVECs through downregulation of miR-525-5p, which targets Bax	Possibly promotes DVT, given its role in HUVECs
CRNDE	He et al. (China, 2023) [129]	16q12.2	↑	Mice IVC	↓miR-181a-5p	Competitively bound to and inhibits miR-181a-5p, promoting Pcyox11 expression and aggravating thrombus formation in DVT	Promotes DVT

Abbreviations: DVT, deep vein thrombosis; ECs, endothelial cells; EPCs, endothelial progenitor cells, HUVECs, human umbilical vein endothelial cells; IVC: inferior vena cava; LEDVT; lower extremity deep vein thrombosis; lncRNA; long non-coding ribonucleic acid; n.a, not available; VTE, venous thromboembolism. ¹ According to HGNC: HUGO Gene Nomenclature Committee (<https://www.genenames.org/>, accessed on 20 May 2023) and Ensembl database (<https://www.ensembl.org/index.html>, accessed on 20 May 2023). LncRNA or target expression: ↓ downregulated and ↑ upregulated.

3. lncRNAs Targeting the Coagulation System

The coagulation system plays a leading role in VTE pathogenesis. Inclusively, as previously mentioned, treatment with anticoagulants constitutes the standard therapeutic approach to treat VTE events [132]. The most used anticoagulants are low molecular weight heparin (LMWH), unfractionated heparin (UFH) and direct oral anticoagulants (DOACs). LMWH and UFH bind to antithrombin III to inhibit the activity of several clotting factors, namely thrombin (or activated coagulation factor II (FIIa)) and activated coagulation factor X (FXa). On the other hand, DOACs act by inhibiting either thrombin or FXa, depending on the drug used [133].

The coagulation cascade involves a series of sequential proteolytic activation reactions, with a complex interplay between several proteins named coagulation factors that culminate in fibrin formation. There are three major coagulation pathways: extrinsic (also called the TF coagulation pathway) and intrinsic (contact coagulation pathway), which converge on a common coagulation pathway [134]. Thromboplastin, or TF, is a transmembrane protein located constitutively on the surface of all cells except for endothelial and blood cells. When damage to blood vessels occurs, TF is released into the bloodstream, initiating the extrinsic coagulation pathway. By combining coagulation factor VII (FVII), the complex TF-FVII (extrinsic tenase complex) activates coagulation factor X (FX) [135]. FXa then participates in the common coagulation pathway. On a phospholipid surface, FXa forms a complex with activated coagulation factor V (FVa), known as prothrombinase complex, that converts prothrombin (coagulation factor II (FII)) into thrombin. Next, thrombin transforms fibrinogen (coagulation factor I (FI)) into fibrin (activated FI (FIa)) that deposits at the vascular injury site, forming a network together with platelets to create a blood clot [136]. Parallely, the intrinsic coagulation pathway is initiated by factors released into the surrounding damaged tissue and circulating blood after injury, such as collagen and other negatively charged surfaces. Once activated, coagulation factor XII (FXII) activates coagulation factor XI (FXI), which then activates coagulation factor IX (FIX). In the presence of calcium ions (coagulation factor IV (FIV)), activated FIX (FIXa) forms a complex with coagulation factor VIII (FVIII) that generate FXa [137]. Both extrinsic and intrinsic pathways thus produce FXa, which then participates in the common coagulation pathway leading to thrombin generation and, ultimately, fibrin deposition (Figure 5A) [136]. To be noted, thrombin also activates TAFI, FV, FVIII, FXI and coagulation factor XII (FXIII), creating a positive feedback loop that enhances clot formation. In addition, thrombin also promotes platelet adhesion by inactivating a disintegrin and metalloprotease with thrombospondin type 1 motif (ADAMTS13). On the other hand, this multifunction protein has anticoagulant activity through the activation of protein C, a natural anticoagulant that regulates the blood coagulation system through the inactivation of FVa and FVIIIa [138].

Until now, few studies have been conducted to identify lncRNAs targeting the coagulation system, which were focused on the tissue factor pathway inhibitor 2 (TFPI2). As the name already indicates, TFPI is a serine protease inhibitor that blocks the activity of the extrinsic or TF coagulation pathway [139]. Specifically, under physiological conditions, this natural anticoagulant protein is known to damp coagulation by effectively inhibiting the extrinsic tenase complex and the prothrombinase complex [140,141]. Beyond coagulation disorders, deregulated levels of TFPI were shown in several diseases, such as cancer, diabetes mellitus and renal diseases [142–150]. Tissue factor pathway inhibitor 1 (TFPI1) and TFPI2 are two distinct coagulation inhibitors (encoded by different genes) with the role of maintaining the haemostatic balance [151]. Alternative splicing of TFPI1 leads to the synthesis of two primary isoforms, namely TFPI α and TFPI β . The former is a soluble protein released into the bloodstream by vECs and activated platelets, while the latter is a glycosylphosphatidyl inositol-anchored protein presented at the endothelium surface. Moreover, TFPI α inhibits both extrinsic tenase and prothrombinase, whereas TFPI β blocks the extrinsic tenase complex more effectively, not being able to inhibit the prothrombinase complex [139]. As for TFPI2, it has inhibitory activity towards tenase

complex, FXIa, plasmin and some matrix metalloproteinases [142,149]. Reduced synthesis of TFPI2 has been associated with angiogenesis, inflammation, atherosclerosis and tumour growth and metastasis [152].

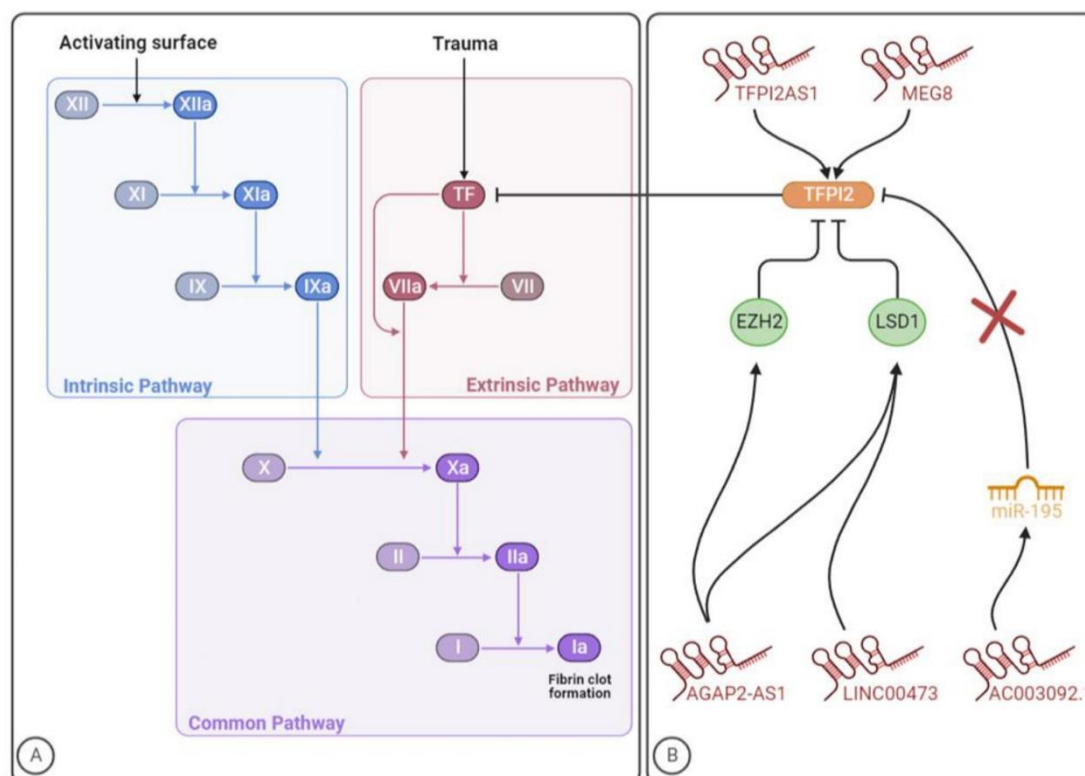


Figure 5. The involvement of lncRNAs in the clotting cascade. (A) Coagulation cascade. (B) lncRNAs that regulate TFPI2 expression/activity. The figure was created with [BioRender.com](https://www.biorender.com) (accessed on 10 July 2023).

To the best of our knowledge, a total of five studies from 2017 to 2022 were performed identifying five lncRNAs with implications in TFPI2 expression and/or activity (Table 2). The mechanisms through which these lncRNAs might impact TFPI2 and, consequently, the coagulation pathway, are summarized in Figure 5B. Given the role of the TF coagulation pathway in VTE development, further investigation should be carried out to explore whether lncRNAs regulating TFPI2 expression might impact the disease pathogenesis and dissect the underlying mechanisms of action.

3.1. TFPI2AS1

TFPI2 antisense RNA 1 (TFPI2AS1) is an antisense lncRNA that is reported to positively regulate TFPI2 expression in NSCLC tissues, acting as a guide. Specifically, TFPI2AS1 is upregulated and inhibits tumour cell proliferation and metastasis by upregulating TFPI2 expression, although the underlying mechanisms are unclear [153]. Beyond cancer-induced hypercoagulability, dysregulation of TF-associated signalling pathways and their constituents play an important role in tumour progression, particularly by impacting angiogenesis and tumour invasion. While TF shows tumour-enhancing characteristics, its natural inhibitors, TFPI1 and TFPI2, are associated with tumour-suppressing properties [154].

3.2. *Linc00473*

Long intergenic ncRNA 00473 (*linc00473*) is a lincRNA that has been consistently linked to cancer cell proliferation, survival and metastasis [155,156]. Preeclampsia is a poorly comprehended pathological condition characterized by high blood pressure (hypertension) and proteinuria in the second half of pregnancy [157]. In a study aiming to explore the role of *linc00473* in preeclampsia, this lincRNA was found to be downregulated in patients' placenta, while in vitro, the lincRNA overexpression stimulated trophoblast proliferation. Acting as a molecular guide, *Linc00473* was found to bind to lysine-specific demethylase 1 (LSD1), responsible for the demethylation of histone H3 lysine 4 dimethylation (H3K4me2) and histone H3 lysine 9 dimethylation (H3K9me2), thus affecting gene expression and inhibiting the expression of TFPI2 [158]. The role of TFPI2 in preeclampsia is, however, still unclear since both its increased and decreased levels have been reported in preeclamptic patients. According to the current evidence, TFPI2 levels might depend on the expression of glypican-3, a TFPI2-binding protein in placental tissue [159].

3.3. *AC003092.1*

Among glioblastoma patients, the lincRNA *AC003092.1* was correlated with increased temozolomide (TMZ) resistance, higher risk of disease relapse and poor prognosis. Even though this study focused on *AC003092.1* as a potential therapeutic target for glioblastoma patients, the conducted investigation indicated that this lincRNA regulates TFPI2 expression through the miR-195/TFPI2 axis in glioblastoma. Specifically, by acting as an endogenous CeRNA and, consequently, as a decoy, *AC003092.1* prevents miR-195 from targeting TFPI2, thereby increasing TFPI2 expression [160].

3.4. *AGAP2-AS1*

AGAP2 antisense RNA 1 (AGAP2-AS1) is a lincRNA that was first found overexpressed in human NSCLC, being also implicated in other malignant diseases such as colorectal cancer and melanoma [161–163]. By interacting with specific RNA-binding proteins, namely enhancers of zeste 2 polycomb repressive complex 2 subunit (EZH2) and LSD1, some lincRNAs can regulate cell phenotypes. The former is a subunit of polycomb repressive complex 2 (PRC2) with catalytic activity, which can suppress gene expression by enhancing histone H3 lysine 27 trimethylation (H3K27me3). As for LSD1, it can repress transcriptional activity through the enzymatic demethylation of histone H3 lysine 4 mono- and dimethylation (H3K4me1/2). In glioblastoma, *AGAP2-AS1* acts as a guide and is suggested to inhibit TFPI2 expression through EZH2 and LSD1 binding [164].

3.5. *MEG8*

Long non-coding maternally expressed 8 (*MEG8*) was shown to be dysregulated in several disorders, such as lung, ovarian and colorectal cancer as well as gestational diabetes mellitus and diabetic nephropathy [165]. A study regarding ischemic heart disease revealed an induction of this gene in patients with this disease. By modulating the expression of TFPI2, which is a known angiogenesis inhibitor, *MEG8* is suggested to regulate the angiogenic sprouting [166,167]. In concordance, experiments with HUVECs showed that TFPI2 was five times more expressed after *MEG8* silencing. The negative regulation of this lincRNA reduces the inhibitory histone modification H3K27me3 at the TFPI2 promoter, therefore acting as a scaffold. Endothelial function is impaired by *MEG8* silencing, suggesting a beneficial role of this lincRNA in preserving cell viability. Knowing that TFPI2 is an angiogenesis inhibitor and the role it plays in the coagulation cascade and extracellular matrix remodelling, the *MEG8*/TFPI2 axis could be a potential therapeutic target for VTE management [166].

Table 2. LncRNAs that regulate TFPI2 expression or activity.

LncRNA	First Author (Country, Year) [Ref]	LncRNA Location ¹	Disease	Sample/Compartment or Study Model	LncRNA Expression in the Disease	LncRNA Targets	LncRNA Role in TFPI2 Expression
TFPI2AS1	Gao et al. (China, 2017) [153]	7q31-q32	NSCLC	NSCLC tissue and cells	↑	TFPI2	↑
Linc00473	Wu et al. (China, 2018) [158]	6q27	Preeclampsia	Placenta tissues and trophoblast cell lines	↓	LSD1/TFPI2	↓
AC003092.1	Xu et al. (China, 2018) [160]	7q21.3	Glioblastoma	Glioblastoma tissue and cells and mice	↓	MiR-195/TFPI2	↑
AGAP2-AS1	Luo et al. (China, 2019) [164]	12q14.1	Glioblastoma	Glioblastoma tissue and cells and mice	↑	EZH2 and LSD1/TFPI2	↓
MEG8	Kremer et al. (2022, The Netherlands) [166]	14q32.31	Ischemic heart disease	Left ventricular tissues and HUVECs	↓	TFPI2	↑

noindentAbbreviations: HUVECs, human umbilical vein endothelial cells; lncRNA; long non-coding ribonucleic acid; NSCLC, non-small cell lung cancer. ¹ According to HGNC (HUGO Gene Nomenclature Committee) (<https://www.genenames.org/>, accessed on 20 May 2023) and Ensembl database (<https://www.ensembl.org/index.html>, accessed on 20 May 2023). LncRNA or TFPI2 expression: ↓ downregulated and ↑ upregulated.

4. Conclusions

Venous thrombogenesis is a common haemostatic disorder associated with substantial morbidity and mortality, particularly in Western countries. Understanding the disease pathophysiology is crucial to improve disease risk stratification, diagnosis and management. Emerging evidence indicates that lncRNAs may have a role in VTE pathogenesis. However, their clinical usefulness as predictive biomarkers for VTE is yet unclear. According to the current review, only a few studies have explored the implications of these ncRNAs in VTE pathways. Moreover, the research repeatability is low, with only MALAT1 and LINC00659 being reported by multiple studies. The lack of external validation in larger cohorts and the limited statistical power of the individual studies are currently the major challenges in this research field. However, as previously mentioned, the first study pinpointing the role of lncRNA in VTE was published in 2019, being that this research field is relatively new. Of note, until now, the studies have only focused on DVT, dismissing a potential role of lncRNAs in PE development. Another limitation of these studies is the fact that the lncRNAs were evaluated in individuals already diagnosed with thrombosis, making it difficult to link these ncRNAs to thrombus formation or thrombus resolution. Meanwhile, multiple lncRNAs have been suggested to modulate the expression of TFPI2, an important anticoagulant. The role of these ncRNAs in VTE pathogenesis should be thus investigated, given the central role of the TF coagulation pathway in thrombosis. Overall, more studies in this field and with real-world VTE patients are required to clarify the clinical use of lncRNAs as VTE biomarkers in an era of minimally invasive methods. As for disease treatment, the use of these ncRNAs is not straightforward. As previously mentioned, the field of lncRNA research is relatively new. A lot of investigation is required to overcome current hurdles before lncRNAs could be translated into effective therapies for VTE patients. The limitations include lncRNAs' susceptibility to degradation by nucleases present in the bloodstream, the possibility of unintended interactions with off-targets, the possible immune responses that might reduce their effectiveness, and the lack of an established, safe and effective delivery route of lncRNAs to the target site [168–170]. Thus, the development of rigorous and more precise investigations using, for instance, animal models should be conducted to better elucidate the roles of these ncRNAs and their therapeutic characteristics and toxicity effects. Using specific lncRNA mimics and inhibitors to change lncRNA expression may be a novel strategy to prevent or treat VTE. In conclusion, this first review on the matter sheds light on the functional significance of lncRNAs in VTE and suggests them as potential biomarkers and therapeutic targets.

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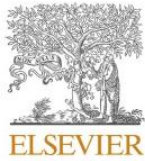
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Review

Cancer-associated thrombosis: What about microRNAs targeting the tissue factor coagulation pathway?



Valéria Tavares^{a,b,c,1}, Beatriz Vieira Neto^{a,d,1}, Inês Soares Marques^{a,e,1}, Joana Assis^f,
Deolinda Pereira^g, Rui Medeiros^{a,b,c,d,h,*}

^a Molecular Oncology and Viral Pathology Group, Research Center of IPO Porto (CI-IPOP) / Pathology and Laboratory Medicine Dep., Clinical Pathology SV/ RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Centre (Porto.CCC), 4200-072 Porto, Portugal

^b Faculty of Medicine of University of Porto (FMUP), 4200-072 Porto, Portugal

^c Abel Salazar Institute for the Biomedical Sciences (ICBAS), University of Porto, 4050-313 Porto, Portugal

^d Research Department, Portuguese League Against Cancer (NRNorte), 4200-172 Porto, Portugal

^e Faculty of Sciences of University of Porto (FCUP), 4169-007 Porto, Portugal

^f Clinical Research Unit, Research Center of IPO Porto (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Centre (Porto.CCC), 4200-072 Porto, Portugal

^g Oncology Department, Portuguese Institute of Oncology of Porto (IPO Porto), 4200-072 Porto, Portugal

^h Faculty of Health Sciences, Fernando Pessoa University, 4200-150 Porto, Portugal

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ABSTRACT

Cancer patients are often diagnosed with venous thromboembolism (VTE), a cardiovascular disease that substantially decreases their quality of life and survival rate. Haemostasis in these patients is deregulated, which is reflected in the common presentation of a blood hypercoagulation state. Despite the inconsistent results, existing evidence suggests that the expression of microRNAs (miRNAs) is deregulated in the context of venous thrombogenesis in the general population. However, few miRNAs are known to be linked to cancer-associated VTE due to the lack of studies with oncological patients. Parallely, coagulation factor III, also known as tissue factor (TF), tissue factor pathway inhibitor 1 (TFPI1) and tissue factor pathway inhibitor 2 (TFPI2) have been proposed to have a central role in cancer-associated VTE and tumour progression. Yet, contrary to what was expected, the role of miRNAs targeting the TF coagulation pathway (or extrinsic coagulation pathway) is poorly explored in cancer-induced thrombogenesis. In this review, in addition to miRNAs implicated in VTE, TF and TFPI1/2-targeting miRNAs were revised. Future studies should clarify the implications of these non-coding RNAs in tumour coagulome.

1. Introduction

Haemostasis is a complex and dynamic biological process that responds quickly and efficiently upon a vascular injury to reduce blood loss and restore vascular integrity, while providing a barrier against infections in cooperation with the immune system [1,2]. Four main players are involved in this process: platelets, endothelial cells, coagulation cascade, and fibrinolytic system. Following endothelium damage, platelets in interaction with endothelial cells form a primary haemostatic plug to limit blood loss (primary haemostasis), while

simultaneously the activated coagulation cascade leads to the formation of fibrin, which deposits at the injury site, consolidating the platelet plug (secondary haemostasis). In parallel, anticoagulant and fibrinolytic proteins prevent the formation of unnecessary blood clots (e.g., thrombus) inside the vasculature by respectively limiting the formation of fibrin and causing its breakdown (tertiary haemostasis) [3]. These haemostatic players are under tight regulation to maintain the haemostatic balance and limit the blood coagulation to where and when it is needed, allowing the blood to be in continuous circulation, preventing haemorrhage and thrombus formation (i.e., thrombosis) [3,4].

* Corresponding author at: Molecular Oncology and Viral Pathology Group, Research Center of IPO Porto (CI-IPOP) / Pathology and Laboratory Medicine Dep., Clinical Pathology SV/ RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Centre (Porto.CCC), 4200-072 Porto, Portugal.

E-mail address: ruimedei@ipporto.min-saude.pt (R. Medeiros).

¹ Authors with the same contribution (joint first authors).

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Thromboembolism is a common cardiovascular disorder characterized by the generation of a thrombus within the arterial or venous circulation. This event is often followed by the formation of an embolus (an abnormal mass, in this case, an unattached fragment of a thrombus, that circulates in the bloodstream), which can potentially block blood vessels (embolism), jeopardizing nutrient transport and gas exchange [4]. Among cancer patients, the clinical manifestation of thromboembolism mainly involves the veins (venous thromboembolism, VTE), which accounts for 20% of the overall VTE incidence [5]. In the general population, particularly in the Western regions, nearly 1 in 12 individuals (~8%) are diagnosed with VTE in their lifetime [6]. In comparison, approximately 15% of cancer patients have at least one episode of the disease [5].

Cancer-associated VTE, also known as Trousseau Syndrome, has two main manifestations, namely deep venous thrombosis (DVT) and pulmonary embolism (PE). This common paraneoplastic event is associated with serious complications, including VTE recurrence, chronic thromboembolic pulmonary hypertension (CTEPH), post-thrombotic syndrome and death [6–9]. Like in the general population, VTE susceptibility among cancer patients is attributed to three key factors: endothelial injury/dysfunction (damage of the endothelium), venous stasis (slowdown or obstruction in the normal blood flow within the veins) and blood hypercoagulability (increased tendency for blood clot formation), which are influenced by acquired and inherited determinants [3]. These three factors together form the Virchow triad proposed in 1856 by Rudolf Virchow as the primary explanation behind VTE pathogenesis [3]. Starting with endothelial injury, conditions such as physical trauma, high blood pressure (hypertension), atherosclerosis or infections, can lead to endothelial dysfunction, shifting the anticoagulant properties of the endothelium towards a procoagulant and pro-inflammatory phenotype [10]. Specifically among oncological patients, tumour-derived factors and therapeutical approaches, such as chemotherapy, are known to lead to endothelial damage [11]. Likewise, venous stasis, caused for instance by extended patient immobility, can lead to the accumulation and constriction of plasma proteins in vascular sites, and cause hypoxia and endothelial dysfunction, resulting in thrombogenesis [11,12]. As for blood hypercoagulability, it is mainly caused by an imbalance in the expression of pro-clotting and anti-

clotting proteins (Fig. 1) due to either genetic (e.g., genetic polymorphisms and/or mutations) and/or environmental stimuli (e.g., trauma, infections and the release of procoagulant and anti-fibrinolytic proteins by tumour cells) [3,11]. Collectively, these three factors promote venous thrombogenesis. Compared with the general population, however, thrombosis in cancer patients seems to be a distinct and more complex pathological entity, most likely due to the contribution of the tumour itself and anti-neoplastic treatment-related factors [13]. Although the exact mechanisms are not entirely understood, the role of the tissue factor (TF) coagulation pathway as the initiator of blood coagulation has consistently been studied in cancer-associated VTE. Cumulating data show that TF is upregulated in most cancer types and its levels are strongly associated with tumour progression [14–20].

Over the years, the pathogenesis of cancer-associated VTE has been dissected with great interest given its growing incidence as the population ages and the prevalence of risk factors increases. The disease has a significant impact on the prognosis of oncological patients, representing the second most common cause of death in this group [5,13,21]. Besides, the treatment of cancer-associated VTE can be a challenge due to potential drug-drug interactions with anti-neoplastic systemic therapies allied to the increased risk of haemorrhage [22]. Given these clinical challenges and the severity of cancer-associated VTE complications, thromboprophylaxis measures are of extreme importance. However, since not all cancer patients can benefit from thromboprophylaxis due to the risk of bleeding events, tools to stratify them based on their risk of developing VTE are required. In the last two decades, several risk assessment models (RAMs) have been proposed to help identify those at increased risk for cancer-associated VTE. The list includes Khorana (the most studied), Onkotev, Vienna, Protecht and Conko-004 scores [23–28]. These RAMs may help direct thromboprophylaxis for high-risk patients while minimizing the risk of bleeding associated with the use of anticoagulant drugs. However, considering the difficulty associated with the validation of these RAMs, more investigation on potential biomarkers of cancer-associated VTE is warranted [29,30].

Among cancer patients, D-dimer (a product from fibrinolysis) is the widely used biomarker for VTE prediction and diagnosis in clinical use [31]. However, this marker is unspecific to VTE, being elevated in other conditions including advanced age, inflammation and long

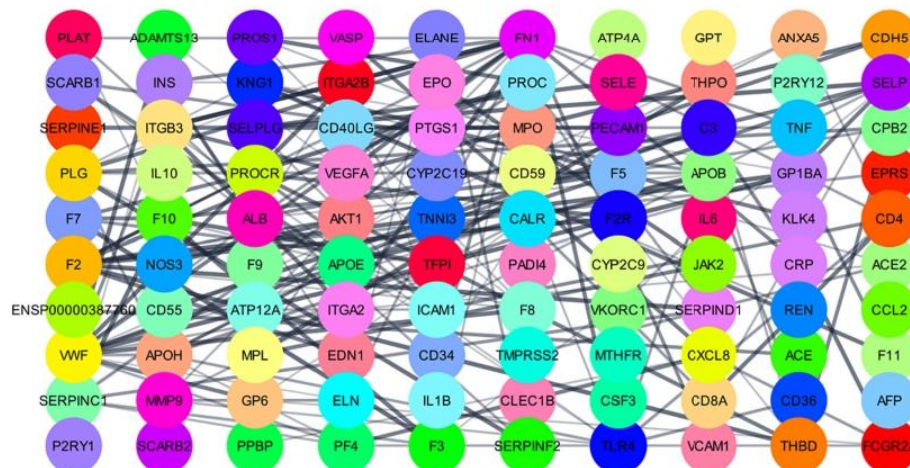


Fig. 1. Top 100 genes involved in thrombosis according to the DISEASES database (100 nodes and 184 edges with significant enrichment, $p = 1.0 \times 10^{-16}$). The protein-protein interaction was generated using STRINGapp disease query from Cytoscape 3.9.1 (accessed on 28 June 2023). Several genes are implicated in the pathogenesis of thrombosis. The list includes genes encoding for platelet-related proteins (e.g., *integrin subunit beta 3* (ITGB3)), coagulation factors (e.g., *coagulation factor 3* (F3)), anticoagulants (e.g., *tissue factor pathway inhibitor* (TFPI)), fibrinolytic modulators (e.g., *plasminogen* (PLG)), pro-inflammatory cytokines (e.g., *interleukin 6* (IL-6)), adhesion proteins (e.g., *vascular cell adhesion molecule 1* (VCAM1)), pro-angiogenic factors (e.g., *vascular endothelial growth factor A* (VEGFA)), metalloproteinases (e.g., *matrix metalloproteinase 9* (MMP9)) and metabolism-related proteins (e.g., *cytochrome P450 family 2 subfamily C member 19* (CYP2C19)).

immobilization periods, all common among cancer patients [31–33]. Although other VTE biomarkers have been suggested, they also lack sensitivity and/or specificity, which is the case of C-reactive protein [34,35]. Meanwhile, microRNAs (miRNAs or miRs) have emerged as epigenetic regulators of venous thrombogenesis not only in the broader population but also among cancer individuals. Inclusively, several miRNAs targeting the TF pathway have been identified. Yet, despite the central role of the pathway, research on these non-coding RNAs in cancer-associated VTE remains poorly explored. Given the implications, this narrative review aims to overview the role of miRNAs in venous thrombosis, further focusing on the impact of TF pathway-targeting miRNAs on cancer-associated VTE. For this purpose, a search in the PubMed database was performed without time restriction and using combinations of the terms “microRNA”, “miRNA”, “miR”, “venous thromboembolism”, “deep vein thrombosis”, “deep venous thrombosis”, “pulmonary embolism”, “thrombosis”, “cancer”, “tumour” and “tissue factor” anywhere in the article. The retrieved scientific articles were published in the last 12 years (from 2011 to March 2023).

2. MiRNAs

MiRNAs are small (18 to 25 nucleotides), single-stranded and highly conserved non-coding RNAs (ncRNAs) that regulate gene expression at a post-transcriptional level. Although other mechanisms have been described, most studies indicate that miRNAs bind to the 3' untranslated region (3' UTR) of target messenger RNAs (mRNAs) leading to translation inhibition and potential mRNA degradation (Fig. 2) [36–38]. Interestingly, a single miRNA can modulate the expression of various genes, influencing several biological systems, each one in a specific manner [39]. Contrariwise, a single gene can also be targeted by different miRNAs [40].

Genes encoding miRNAs can be located in exons, introns and even intergenic regions [41]. The biogenesis of miRNAs encompasses two types of pathways, the canonical and non-canonical, the former being the most common and extensively studied (Fig. 2). Each pathway requires a specific set of nuclear and cytoplasmic proteins, ultimately forming a functional miRNA-induced silencing complex (miRISC) that binds to target mRNAs to mediate gene silencing [36]. Notably, the miRNA biogenesis is under tight spatial and temporal control [42].

Given their ability to collectively regulate the expression of numerous genes (~60%), miRNAs have been proposed as potential biomarkers for several disorders, including cardiovascular and malignant diseases [43–46]. There are multiple advantages to using miRNAs as biomarkers, particularly in an era of liquid biopsies [47]. These ncRNAs are highly stable in biofluids, even when faced with extreme conditions including major temperature and pH variations, several freeze-thaw cycles and RNase treatment. Furthermore, they are easily detected and show high sensitivity [48]. In addition to serving as biomarkers, alone or in combination with others, the ability of miRNAs to target several mRNAs and their associated pathways puts these molecules at the forefront of ncRNA-based therapeutics [39]. In fact, major technological innovations have contributed to the development of miRNA mimics (for miRNA replacement) and inhibitors (anti-miRNA oligonucleotides) to help restore physiological cell functions disrupted by diseases. In addition to improving the transfer of miRNA mimics and inhibitors, these innovations include the reduction of off-target effects and immunogenicity, which are the two major issues surrounding ncRNA-based therapeutics [49].

2.1. Role of MiRNAs in venous thromboembolism

Like other biological processes, haemostasis is thought to be

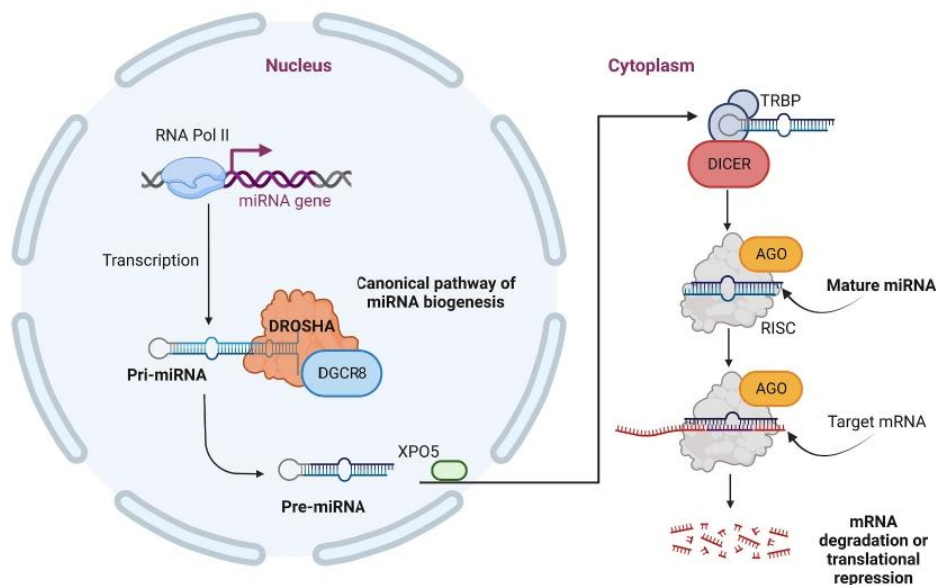


Fig. 2. Canonical pathway of miRNA biogenesis. The figure was created with BioRender.com (accessed on 30 July 2023).

In the canonical pathway of miRNA biogenesis, RNA pol II mediates the transcription of the miRNA gene resulting in a primary miRNA (pri-miRNA), which typically has a stem-loop structure. Subsequently, the pri-miRNA is cleaved by Drosha (a ribonuclease III enzyme) and its co-factor DGCR8, generating a precursor miRNA (pre-miRNA). This hairpin-shaped structure is then transported to the cytoplasm by XPO5. At the cytoplasm, pre-miRNA is cleaved by Dicer (another ribonuclease III enzyme) and TRBP, generating a small RNA duplex consisting of a guide (mature miRNA) and a passenger strand (miRNA*). This structure is further incorporated in RISC, where the guide strand becomes functional, while the passenger one is usually degraded. The multiprotein complex RISC guided by the mature miRNA then binds to target mRNAs for gene silencing. This interaction leads to translational repression or mRNA degradation, the latter requiring a higher complementary degree between the miRNA and the mRNA [42,50].

Abbreviations: AGO, Argonaute; mRNA, messenger RNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; RNA Pol II, RNA polymerase II; TRBP, TAR RNA-binding protein; XPO5, exportin 5.

controlled by miRNAs. Indeed, abnormal expression of haemostasis-related genes due to genetic mutations and polymorphisms is known to influence interindividual susceptibility to VTE. However, there are still non-characterized elements, including miRNAs and long non-coding RNAs (lncRNAs), that also trigger such variations [3,13,51]. MiRNAs exert their effect in the haemostatic system by modulating the expression of haemostatic plasma proteins (mainly synthesized in the liver) or the activity of endothelial cells, platelets and leucocytes [52–54]. The expression level of several haemostatic proteins seems to be regulated by miRNAs, including procoagulants (fibrinogen (coagulation factor I (FI)), coagulation factor XI (FXI) and TF), anticoagulants (tissue factor pathway inhibitor (TFPI), antithrombin and protein S) and fibrinolytic regulators (plasminogen activator inhibitor-1 (PAI-1)) [52,55]. Noteworthy, fibrinogen was the first proposed to be regulated by miRNAs, namely miR-409, miR-18a and miR-29 family (a, b and c) [56].

Given the pivotal role of the haemostatic system in VTE pathogenesis, it is reasonable to anticipate the potential deregulation of miRNAs during venous thrombogenesis. Concordantly, epidemiological studies in the general population have reported several miRNAs as promising

diagnostic and/or prognostic biomarkers of this disease (Table 1).

Considering only studies on first events of VTE (i.e., dismissing recurrent events) in the general population (without focusing on specific traits that may influence the disease pathogenesis) and with miRNA quantification taking place close to VTE diagnosis, a total of 14 epidemiological studies have been conducted (Table 1) [57–70]. All were case-control studies essentially assessing the value of miRNAs as diagnostic biomarkers of DVT, PE or both (VTE). The first study, which was performed by Xiao et al. (2011) [57], revealed that plasma miR-134 was upregulated in acute PE patients compared to healthy individuals and subjects with cardiopulmonary disorders, suggesting that this miRNA could be a valuable diagnostic biomarker. In the subsequent reports, some integrating animal model approaches, a total of 18 deregulated miRNAs in VTE patients were identified. Among those, only miR-126 was reported in more than one study. Rodriguez-Rius et al. (2020) [67] demonstrated that plasma miR-126 levels were elevated among VTE patients compared to healthy controls. Likewise, Rosetti et al. (2021) [68] showed that increased serum levels of this miRNA serve not only as a diagnostic tool but also as a prognostic biomarker of VTE by

Table 1
miRNAs implicated in venous thromboembolism (VTE) in the general population according to epidemiological studies.

Study	Country	Study design	Sample/Methods	Deregulated miRNAs in VTE patients
Xi et al. (2011) [57]	China	Case-control study: 32 subjects with acute PE 32 healthy controls 22 individuals without acute PE (with reported cough, chest pain or dyspnoea)	Plasma miRNA microarray miRNA qRT-PCR	miR-134
Zhou et al. (2016) [58]	China	Case-control study with sex and age matching: 37 subjects with PE 37 healthy controls	Plasma Ready-to-use PCR Panel miRNA qRT-PCR	miR-28-3p
Wang et al. (2016) [59]	Sweden	Case-control study with sex and age matching: 53 subjects with DVT 185 control individuals without DVT	Plasma Ready-to-use PCR Panel miRNA qRT-PCR	miR-424-5p and miR-136-5p
Kessler et al. (2016) [60]	Germany	Case-control study: 30 subjects with acute PE 30 subjects with myocardial infarction 12 healthy controls	Serum miRNA microarray miRNA qRT-PCR	miR-1233
Wang et al. (2017) [61]	China	Case-control study with sex and age matching: 78 subjects with acute PE 70 healthy controls	Plasma miRNA qRT-PCR	miR-27a and miR-27b
Sahu et al. (2017) [62]	India	Case-control study: 20 subjects with VTE 20 healthy controls	Plasma miRNA qRT-PCR	miR-145
Jiang et al. (2018) [63]	China	Case-control study with sex and age matching: 30 subjects with DVT 30 healthy controls	Plasma miRNA qRT-PCR	miR-320a and miR-320b
Liu et al. (2018) [64]	China	Case-control study: 60 subjects with acute PE 50 healthy controls	Plasma miRNA microarray miRNA qRT-PCR	miR-221
Sun et al. (2020) [65]	China	Case-control study: 81 subjects with acute DVT 20 healthy controls	Plasma miRNA qRT-PCR	miR-103a-3p
Zhang et al. (2020) [66]	China	Case-control study with sex and age matching: 36 subjects with DVT 36 healthy controls	PBMC miRNA microarray miRNA qRT-PCR	miR-338-5p
Rodriguez-Rius et al. (2020) [67]	Spain	Case-control study with sex and age matching: 52 subjects with VTE 52 healthy controls	Plasma miRNA qRT-PCR	miR-126-3p, miR-885-5p, miR-194-5p and miR-192-5p
Rosetti et al. (2021) [68]	Italy	Case-control study with sex and age matching: 23 subjects with VTE 23 healthy controls	Serum miRNA qRT-PCR	miR-126 (also a prognostic biomarker of VTE)
Jian et al. (2021) [69]	China	Case-control study with sex and age matching: 47 subjects with VTE 40 healthy controls	Plasma miRNA qRT-PCR	miR-200c-3p
Rahmanpour et al. (2022) [70]	Iran	Case-control study with sex and age matching: 200 subjects with VTE 200 healthy controls	Serum miRNA qRT-PCR	miR-26a

MiRNAs in green: upregulated in DVT, PE or VTE; miRNAs in blue: downregulated in DVT, PE or VTE. The bold miRNAs were identified in multiple studies (miR-126).

Abbreviations: DVT, deep vein thrombosis; PBMC, peripheral blood mononuclear cell; PE, pulmonary embolism; qRT-PCR, quantitative real-time polymerase chain reaction; VTE, venous thromboembolism.

being associated with thrombotic residual at follow-up.

Overall, the role of miRNAs as biomarkers of VTE in the general population is still debatable. The small cohort sizes, lack of subsequent validation and low reproducibility of the findings (likely due to the variability in patients' characteristics, sample collection and laboratory procedures) represent major limitations. Also, whether these miRNAs could be potential therapeutic targets for VTE management needs to be addressed. Notably, the expression of all the miRNAs was evaluated after VTE diagnosis. As such, it is uncertain whether these ncRNAs were related to thrombus formation or resolution, which also needs to be clarified.

Although not the scope of this review, Wang et al. (2019) [71] were the first to report circulating miRNAs as predictors of VTE recurrent events. The list included 12 plasma miRNAs, namely miR-15b-5p, miR-21-5p, miR-26b-5p, miR-27b-3p, miR-30c-5p, miR-103a-3p, miR-106a-5p, miR-197-3p, miR-222-3p, miR-361-5p, miR-532-5p and miR-652-3p. Interestingly, miR-27b and miR-103a were also associated with first manifestations of VTE (Table 1).

2.2. Role of MiRNAs in cancer-associated thrombosis

Considering their mechanistic contribution to VTE pathogenesis, miRNAs have also emerged as putative biomarkers of venous thrombogenesis in the cancer setting [38]. However, to date, only four published epidemiological studies have addressed these molecules as putative predictors of cancer-associated VTE, at least to the best of our knowledge (Table 2).

The first study reporting the implications of miRNAs in cancer-associated VTE was conducted by Kim et al. (2019) [72]. In this case-control study derived from a prospective study (i.e., nested case-control), colorectal cancer patients that developed VTE (cases, $n = 7$) were sex, age, cancer stage and treatment matched with patients with the same neoplasia but without VTE (controls, $n = 14$) in a 1:2 ratio. Among the cases, the expression level of the plasma miRNAs was evaluated prior to VTE development. Through sequencing, a total of nine miRNAs were found to be downregulated among those with VTE. Following this study, Oto et al. (2020) [73] explored the predictive capability of miRNAs in the thrombotic risk of intracranial tumour patients. To do so, the authors performed a prospective study with patients with glioma ($n = 59$) and meningioma ($n = 93$), of whom 31% ($n = 18$)

and 39% ($n = 36$) developed post-surgical PE, respectively. From the original cohort, 50 patients were selected from each tumour type, 34% ($n = 17$) of which developed post-surgical PE. Prior to surgery, the plasma expression level of the miRNAs was measured using the quantitative reverse transcription polymerase chain reaction (qRT-PCR) methodology. According to the results, two distinct profiles of six miRNAs, one for glioma and the other for meningioma, were considered good predictors of post-surgical PE. Remarkably, both profiles outperformed the Khorana score. Of note, miR-126 and miR-222, two of the identified PE-related miRNAs (both upregulated in PE patients), were previously reported to respectively predict VTE first and recurrent events in the general population [68,71]. In the same year, the authors conducted another prospective cohort study but with patients with pancreatic ductal adenocarcinoma ($n = 26$) and distal extrahepatic cholangiocarcinoma ($n = 6$), of whom 10 developed VTE [74]. The plasmatic expression levels of the evaluated miRNAs were quantified at cancer diagnosis and before VTE occurrence. According to the results, nine miRNAs were found to predict VTE development, including miR-103a-3p (upregulated in VTE patients). Noteworthy, this miRNA, as previously mentioned, was linked to primary and recurrent events of VTE in the broader population [65,71]. Lastly, Anijs et al. (2022) [75] intended to explore the predictive capability of miRNAs concerning VTE predisposition among colorectal cancer patients. As such, the authors conducted a retrospective study with 418 patients, of whom 5.5% ($n = 23$) developed a thrombotic event one year before to one year after cancer diagnosis. This subgroup of patients was then matched with controls (non-VTE patients) according to age, sex, tumour stage and histological grade. Following the quantification of miRNA expression levels in tumour tissue through edgeR, 19 miRNAs were pinpointed as predictors of VTE among colorectal cancer patients.

Like the studies on VTE in the general population, there is low reproducibility of results concerning the role of miRNAs as biomarkers of cancer-associated VTE. Specifically, no miRNA was identified in more than one study. Besides, compared with the data on the general population, only three miRNAs were reported to be implicated in VTE among both cancer and noncancer subjects, namely miR-103a, miR-126 and miR-222. In addition to the complexity of the disease pathogenesis, this low reproducibility can be explained, once again, by the small cohort sizes, differences in the population characteristics and the timings of sample collection and processing. Further investigation with larger

Table 2
miRNAs associated with VTE in the cancer setting.

Study	Country	Study design	Sample/Methods	Deregulated miRNAs in patients with cancer-associated VTE
Kim et al. (2019) [72]	United States of America	Prospective study with sex and age matching: 7 CCR patients with VTE 14 CCR patients without VTE	Plasma miRNA microarray	miR-4451, miR-942-3p, miR-8063, miR-3132, miR-3118, miR-105-5p, miR-891a-5p, miR-200a-5p and miR-6832-3p.
Oto et al. (2020) [73]	Spain/Italy	Prospective cohort study: 34 glioma and meningioma patients with post-surgical incidental PE 66 glioma and meningioma patients without post-surgical incidental PE	Plasma miRNA qRT-PCR	Glioma: miR-363-3p, miR-22-5p, miR-93-3p miR-451a, miR-140-3p and miR-222-3p (main analyses). Meningioma: miR-331-3p, miR-29a-3p, miR-23a-3p, miR-23b-3p, miR-126-5p and miR-660-5p.
Oto et al. (2020) [74]	Spain/Denmark	Prospective cohort study with sex and age matching: 10 PDAC and DECC patients with VTE 22 PDAC and DECC patients without VTE	Plasma miRNA qRT-PCR	miR-486-5p, miR-32-5p, miR-106b-5p, miR-144-3p, miR-144-5p, miR-103a-3p, miR-19a-3p, miR-30e-3p and miR-326 (main analyses)
Anijs et al. (2022) [75]	Netherlands	Prospective study: 17 CCR patients with VTE 18 CCR patients without VTE	CCR tissue miRNA microarray	miR-184, miR-509-3-5p, miR-1307-5p, miR-514-3p, miR-203a-3p, miR-3652, miR-92b-5p, miR-10394-5p, miR-10394-3p, miR-3648, miR-10401-5p, miR-10396b-5p, miR-663a and miR-664b-5p (main analyses).

MiRNAs in green: upregulated in DVT, PE or VTE; miRNAs in blue: downregulated in DVT, PE or VTE.

Abbreviations: CCR, colorectal cancer; DECC, Distal Extrahepatic Cholangiocarcinoma; PDAC, Pancreatic Ductal Adenocarcinoma; PE, pulmonary embolism; qRT-PCR, quantitative real-time polymerase chain reaction; VTE, venous thromboembolism.

cohorts and external validation is required to dissect the role of miRNAs in the pathogenesis of cancer-associated VTE. Furthermore, a possible strategy could be to focus on miRNAs with established roles in the haemostatic system, particularly the coagulation cascade, given its central role in venous thrombogenesis.

3. Tissue factor coagulation pathway and cancer-associated thrombosis

3.1. Involvement of tissue factor in cancer

The transmembrane glycoprotein TF, also known as CD124, coagulation factor III (F3) or thromboplastin, is mainly recognized as the key initiator of the extrinsic coagulation pathway (Fig. 3). While constitutively expressed by extravascular tissue cells (epithelial cells, pericytes, fibroblasts, and smooth cells), the protein expression can be transiently induced in monocytes and vascular endothelial cells under pathological conditions, such as infection [14]. At a tissue level, this 47-kDa protein is mostly expressed in the heart, brain, lungs, and kidney, which are highly vascularized organs. The TF is released into the bloodstream upon vascular injury, acting as a haemostatic envelope [76]. Once exposed to the circulating proteins, this glycoprotein associates with coagulation factor VII (FVII) to form the extrinsic tenase complex. This TF-activated FVII (FVIIa) complex then converts coagulation factor X (FX) to its activated form (FXa), which subsequently triggers a series of events towards fibrin deposition at the injury site [20,77].

Like in the general population, TF plays a relevant part in cancer-

associated VTE development. Tumour cells are known to constitutively express and release TF, alone or within microparticles, or even induce its production by normal cells in the tumour microenvironment, particularly monocytes and endothelial cells [80]. This is the best-characterized mechanism through which these cells initiate prothrombotic cascades. Interestingly, besides being pivotal in blood coagulation, TF also plays multifaceted roles in cancer progression [14]. Specifically, this coagulation initiator is reported to contribute to angiogenesis, tumour cell proliferation and survival, invasion, cancer stemness, immune evasion and metastasis via coagulation-dependent and independent mechanisms [5,76,81]. Most tumour types, particularly the most aggressive ones, have been found to overexpress TF driven by oncogenic events (Fig. 4), which is associated with poor prognosis [82–85]. Inclusively, tisotumab vedotin (Tivdak™), a TF-specific human antibody-drug conjugate, has been designed to manage TF-expressing tumours [86]. In 2021, after the results of innovaTV-201 (NCT02001623) and innovaTV-204 (NCT03438396) clinical trials, the US Food and Drugs Administration (FDA) approved tisotumab vedotin for the treatment of metastatic or recurrent cervical cancer patients with disease progression under or after chemotherapy [86–88]. Preliminary data indicate that patients with other tumours may benefit from the expansion of the therapeutic application of tisotumab vedotin, including bladder, ovarian, lung, prostate and pancreatic cancers [87].

Human TF is encoded by *F3* (chromosome 1p21.3), which possesses six exons. The gene can produce two protein isoforms: the full-length TF (47 kDa) as the product of all exons and the alternatively spliced isoform TF (26 kDa), with the latter having lower procoagulant activity [76,89].

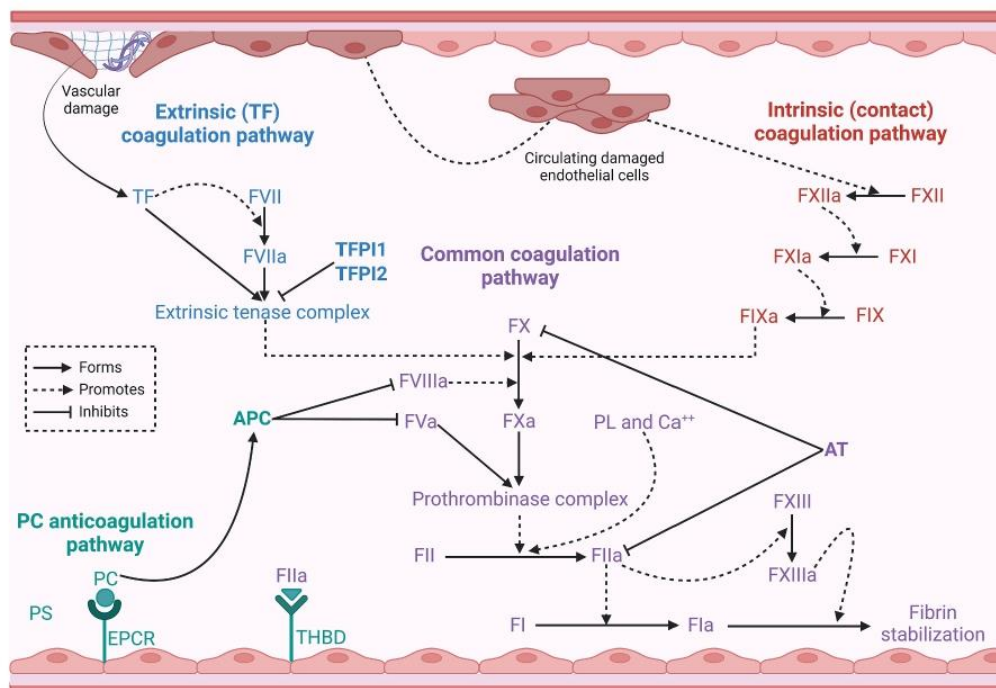


Fig. 3. Pathways of blood coagulation according to the waterfall/cascade model. The figure was created with BioRender.com (accessed on 10 July 2023).

The coagulation cascade involves three major pathways: extrinsic (or TF coagulation pathway), intrinsic (or contact coagulation pathway) and common coagulation pathways. Upon endothelium damage, TF is released into the bloodstream initiating the extrinsic pathway. The complex TF-FVII (extrinsic tenase complex) activates FX, which then participates in the common pathway. Meanwhile, negatively charged surfaces at the injury site (such as damaged endothelial cells) trigger the intrinsic pathway activating FXII, which leads to a series of reactions that culminate in FXa generation. Both extrinsic and intrinsic pathways thus converge on a common coagulation pathway heading to FIIa (thrombin) generation and consequently (FIIa) fibrin deposition. Anticoagulant proteins (TFPI, PS, PC, EPCR, THBD and AT) repress fibrin synthesis by inactivating coagulation factors [78,79].

Abbreviations: a, activated; APC, activated protein C; AT, antithrombin; Ca⁺⁺, calcium ions; EPCR, endothelial protein C receptor; F, factor; PC, protein C; PL, phospholipids; PS, protein S; TF, tissue factor; TFPI, tissue factor pathway inhibitor; THBD, thrombomodulin.

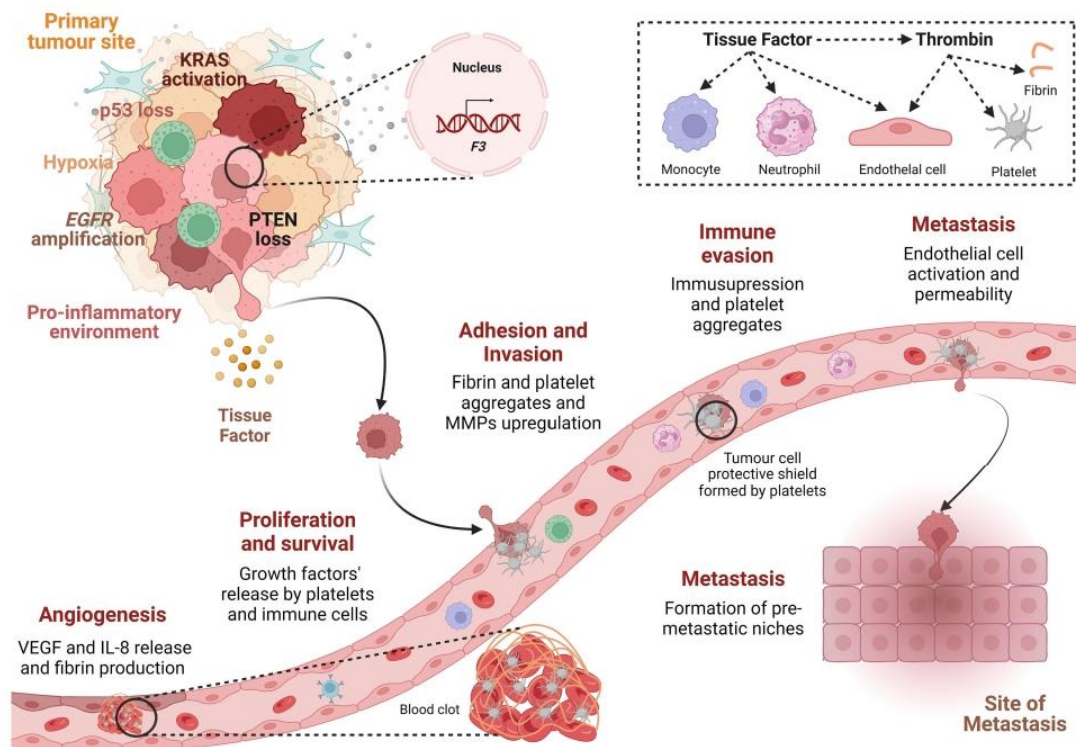


Fig. 4. Simplistic representation of the roles of tissue factor in tumour progression. The figure was created with [BioRender.com](https://www.biorender.com/) (accessed on 31 July 2023).

Tumour overexpression of tissue factor results from oncogenic events (such as KRAS activation, p53 and PTEN loss and EGFR amplification), being the expression further enhanced in a pro-inflammatory and hypoxic microenvironment. This coagulation factor plays multifaceted roles to modify the tumour microenvironment, providing favourable conditions for tumour cell growth and dissemination. Tissue factor-mediated local generation of thrombin leads to fibrin deposition, which serves as a provisional matrix for tumour angiogenesis. Furthermore, it promotes VEGF and IL-8 production, which also induces angiogenesis. Thrombin activates platelets, which release growth factors that stimulate tumour cell proliferation and survival. Besides, platelets also form a shield to protect tumour cells from immune detection and destruction, thus favouring tumour metastasis. Furthermore, thrombin induces the expression of proteins implicated in the epithelial-mesenchymal transition (such as MMPs), which ultimately favour tumour invasion and metastasis. Tissue factor can modulate endothelial cell activity, increasing adhesion molecules' expression and endothelium permeability. Also, it can induce inflammation, stimulating the release of tumour-favourable growth factors and cytokines, while contributing to immune evasion [3,14,76,93].

Abbreviations: EGFR, epithelial growth factor receptor; F3, coagulation factor 3; IL-8, interleukin-8; MMPs, metalloproteinases; PTEN, phosphatase and tensin homolog; VEGF, vascular endothelial growth factor.

Post-translational modifications also influence protein activity, including glycosylation, phosphorylation and palmitoylation [90–92]. Beyond activity, TF expression level is also under the influence of several factors. At a transcriptional level, *F3* expression is regulated by cytokines (tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin (IL)-1 β , IL-4, IL-6, IL-10, IL-13 and IL-33) and growth factors (vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor- β (TGF- β), among others), with hypoxia also having a role [76]. At a post-transcriptional level, multiple miRNAs have been reported to modulate TF synthesis. Given the roles of TF in thrombosis promotion, these miRNAs might be promising tools in predicting cancer-associated thrombosis.

3.2. MiRNAs targeting tissue factor

A total of 18 studies conducted in the last 12 years have identified TF-targeting miRNAs in physiological but mostly pathological conditions (Table 3). The first study was performed by Zhang et al. (2011) [94], in which, using a reporter gene assay, miR-19 binding to *F3*-3'-UTR was found to downregulate TF expression in a cell line of human breast cancer, an effect inverted by deleting miR-19-binding site. Since then, miR-19a/b have been consistently found to impact TF expression

across physiological conditions and multiple diseases, being implicated in the progression of colon and non-small-cell lung cancers [95–103]. Other TF-targeting miRNAs include miR-20a/b, miR-106b ~ 25 cluster (miR-25, miR-93 and miR-106b), miR-126, miR-223, miR-520 g, miR-145, miR-92a and miR-365a. Of note, this review only focuses on miRNAs whose interaction with *F3* was validated by methods that provide strong evidence, namely luciferase reporter assay, Western blot and qRT-PCR. Among these *F3*-targeting miRNAs, miR-19a, miR-93, miR-106b and miR-126 are reported to be differently expressed among cancer patients with VTE compared to their counterparts without the condition (Table 2; Fig. 5). Additionally, miR-126, as previously mentioned, was also found to be both a diagnostic and prognostic biomarker of VTE in the general population [67,68]. Furthermore, the *F3*-targeting miRNA miR-145 has been implicated in VTE in the general population [62]. Collectively, these five RNAs could be valuable biomarkers for cancer-associated VTE.

3.3. Involvement of tissue factor pathway inhibitors in cancer

Another key player in the TF coagulation pathway is TFPI. TFPI1 and TFPI2, encoded by different genes, are coagulation inhibitors with distinct roles in haemostasis [112]. Briefly, TFPI1 (or simply TFPI) is

Table 3
miRNAs targeting tissue factor (TF) in different pathophysiological conditions.

Study	Country	Pathophysiological condition	Sample/compartment	MiRNA	Technique to analyse miRNA-mRNA interaction**
Zhang et al. (2011) [94]	United States of America	Breast cancer	Breast cancer cells	miR-19	Luciferase reporter assay
Teruel et al. (2011) [95]	Spain	Systemic lupus erythematosus Antiphospholipid syndrome	Monocytes	miR-19b and miR-20a	Luciferase reporter assay for miR-20a
Chuang et al. (2012) [104]	United States of America	Leiomyoma	Leiomyoma cells	miR-93/106b (miR-106b ~ 25 cluster)	Microarray gene profiling
Yu et al. (2013) [96]	China	Colon cancer	Colon cancer cells	miR-19a	Luciferase reporter assay
Eisenreich and Rauch (2013) [97]	Germany	Endothelial cellular thrombogenicity	Human microvascular endothelial cells	miR-19a and miR-126	Luciferase reporter assay
Yu et al. (2013) [105]	China	Hematopoietic and trophoblastic differentiation of human embryonic stem cells	Human embryonic stem cells	miR-20b	Luciferase reporter assay
Li et al. (2014)a [99]	China	Unstable angina	HUVECs Lung carcinoma cells	miR-19b	Luciferase reporter assay
Li et al. (2014)b [106]	China	Atherosclerosis	Vascular endothelial cells	miR-223	Luciferase reporter assay
D'Asti et al. (2016) [107]	Canada	Primitive Neuronal Brain Tumour	Medulloblastoma cells	miR-520 g	Luciferase reporter assay
Balia et al. (2017) [100]	Italy	TF expression in PBMCs	PBMCs	miR-19a and miR-20a	qRT-PCR
Sahu et al. (2017) [62]	India	Venous thrombosis	Inferior vena cava of rats	miR-145	Luciferase reporter assay
Witkowski et al. (2018) [101]	Germany	Diabetes mellitus	Plasma	miR-19a and miR-126	Luciferase reporter assay
Chen et al. (2019) [102]	China	Non-small-cell lung cancer	Human lung cancer cells Nude mouse xenograft model	miR-19a	Luciferase reporter assay
Li et al. (2020) [108]	China	Angiogenesis	HUVECs	miR-92a-3p	Luciferase reporter assay
Tian et al. (2021) [109]	Australia	High oestradiol-related blood hypercoagulability	Plasma	miR-365a-3p	Luciferase reporter assay
Wang et al. (2021) [110]	China	Stress urinary incontinence	Extracellular vesicles released by adipose-derived mesenchymal stem cells	miR-93	Luciferase reporter assay
Zhang et al. (2021) [103]	China	Sepsis-induced disseminated intravascular coagulation	PBMCs HUVECs	miR-19a-3p	Luciferase reporter assay qRT-PCR
Collier et al. (2022) [111]	United Kingdom	Monocyte TF expression	Monocytic cells	miR-223-3p	Western Blot

Abbreviations: HUVECs, Human umbilical vein endothelial cells; PBMCs, Peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction.

** Methods that provide strong evidence.

encoded by *TFPI* (2q32.1) and constitutes the principal inhibitor of the TF coagulation pathway (Fig. 3), modulating the severity of bleeding and clotting disorders. Two primary isoforms of TFPI are produced by alternative splicing, namely TFPI α and TFPI β , both with 46 kDa [81,113,114]. While the former is a soluble protein released by endothelial cells and activated platelets, the latter is found at the endothelium surface as a glycosylphosphatidyl inositol-anchored protein [115]. In terms of function, the former inhibits the tenase complex and prothrombinase, whereas the latter blocks the extrinsic tenase complex more effectively, but it cannot inhibit prothrombinase [116]. TFPI2, in turn, is encoded by *TFPI2* (7q21.3) and is expressed by endothelial cells, fibroblasts and smooth muscle cells. In terms of its roles in thrombogenesis, the mechanisms are not entirely understood. Although with homology to TFPI1, TFPI2 appears to have a weak inhibition activity towards the tenase complex [113,117]. Instead, it seems to be mainly a plasmin inhibitor, regulating fibrinolysis and the digestion and remodelling of the endothelial cell matrix (ECM) by controlling the release of matrix metalloproteinases. Through its functional versatility, TFPI2 modulates the thrombogenic potential, as well as cancer invasion and dissemination [112,118–120]. The current evidence indicates that cancer patients with VTE have lower TFPI1 levels than their counterparts without VTE [112]. On the other hand, higher TFPI2 levels seem to promote tumour-related blood coagulability [121]. Indeed, the overall

impact of TFPI2 might differ from TFPI1, potentially favouring rather than inhibiting thrombogenesis, which aligns with the assumption that TFPI2 is a primary plasmin inhibitor [112,121]. Further investigation is needed to clarify the roles of TFPI2 in thrombosis as both an anticoagulant and anti-fibrinolytic protein. All in all, given the roles of TFPI1/2 in thrombogenesis, miRNAs targeting the expression of these haemostatic components might help predict the development of cancer-associated thrombosis.

Although with less evidence, TFPI1/2 are also thought to play a part in tumour growth and dissemination [122]. Specifically, TFPI1/2 have been demonstrated to have anti-angiogenic, anti-metastatic and pro-apoptotic effects [114]. For instance, TFPI2, for which there is more data, its downregulation is associated with aggressive tumours and a poor prognosis [114,123–127].

3.4. MiRNAs targeting tissue factor pathway inhibitors

Eight studies have identified *TFPI*-targeting miRNAs across different disorders (Table 4). Ma et al. (2011) [128] reported *TFPI2* as a target of miR-616 and pinpointed a role of the *TFPI2*-miR-616 axis in the androgen-dependent growth of prostate cancer. This same interaction was later corroborated by Xu et al. (2018) [129] in a study regarding preeclampsia. Another miRNA reported to target *TFPI2* is miR-195,

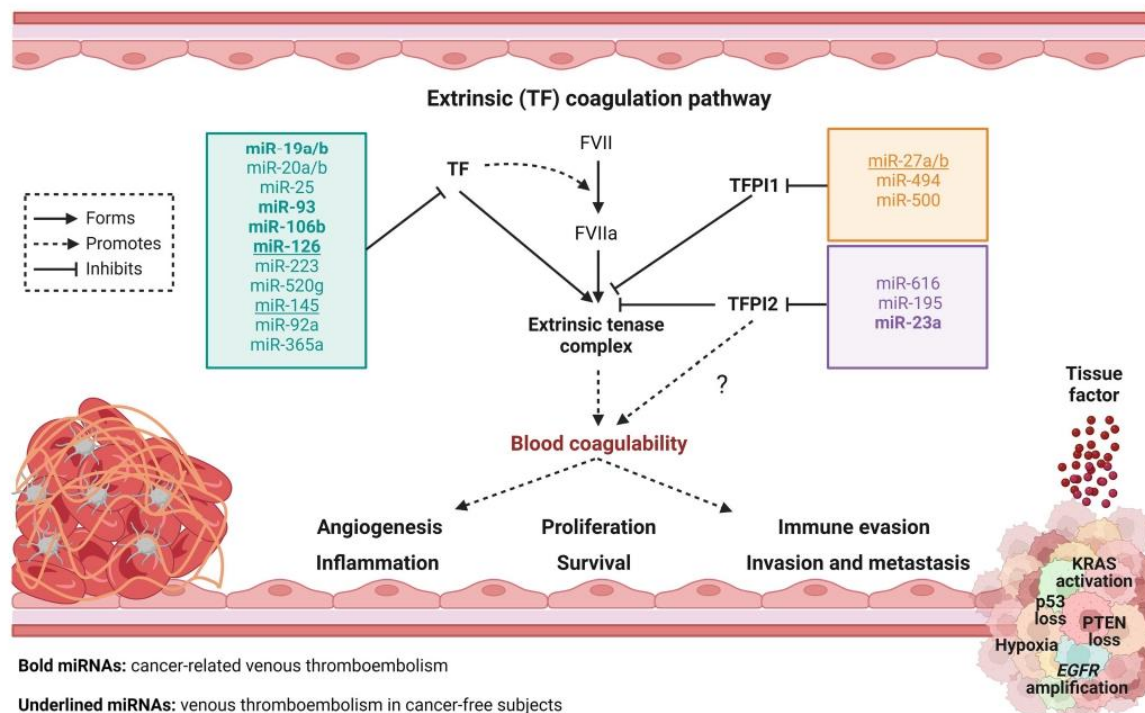


Fig. 5. MiRNAs targeting the TF coagulation pathway with reported roles in venous thromboembolism (VTE). The figure was created with [BioRender.com](https://www.biorender.com) (accessed on 28 November 2023).

Despite the limited data, miRNAs targeting the TF pathway have been found to be associated with VTE in the general population and among cancer patients. Abbreviations: FVII, coagulation factor VII; FVIIa, activated coagulation factor VII; TF, tissue factor; TFPI1, tissue factor pathway inhibitor 1; TFPI2, tissue factor pathway inhibitor 2; VTE, venous thromboembolism.

Table 4
miRNAs targeting tissue factor pathway inhibitor 1 (TFPI1) and tissue factor pathway inhibitor 2 (TFPI2) in different pathophysiological conditions.

Study	Country	Pathophysiological condition	Sample/compartment	MiRNA/Target	Technique to analyse miRNA-mRNA interaction**
Ma et al. (2011) [128]	China	Androgen-independent prostate cancer	Prostate cancer tissue and cells	miR-616/TFPI2	Microarray gene profiling
Ali et al. (2016) [133]	Norway	Breast cancer and estrogenic-regulation of TFPI	Breast cancer cells	miR-27a/b and miR-494/TFPIα	Luciferase reporter assay
Cai et al. (2017) [135]	China	Prostate cancer	Prostate cancer tissue and cells	miR-500/TFPI1	Luciferase reporter assay
Xu et al. (2018) [129]	China/United States of America	Preeclampsia	Preeclampsia placental tissues	miR-616-3p/TFPI2	qRT-PCR
Xu et al. (2018) [130]	China/United States of America	Temozolomide chemosensitivity in glioblastoma	Glioblastoma cells	miR-195/TFPI2	Luciferase reporter assay
Geng et al. (2020) [134]	China	Gastric cancer	Gastric cancer cells	miR-27a-3p/TFPI1	Luciferase reporter assay
Wang et al. (2020) [132]	China	Pancreatic cancer	Pancreatic cancer cells	miR-23a/TFPI2	Luciferase reporter assay
Zhou et al. (2022) [131]	China	Hypoxia-induced damage of trophoblast cells	Human umbilical cord mesenchymal stem cells	miR-195/TFPI2	Luciferase reporter assay

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction.

** Methods that provide strong evidence.

which was identified in two distinct studies, one concerning temozolomide chemosensitivity among glioblastoma patients, and the other on hypoxia-induced damage of trophoblast cells [130,131]. With less evidence, the *TFPI2*-miR-23a interaction has also been demonstrated in a study conducted by Wang et al. (2020) [132]. According to the results, miR-23a might promote pancreatic carcinogenesis by inhibiting *TFPI2* expression. As for *TFPI1*, Ali et al. (2016) [133] were the first to report targeting miRNAs. In the study, miR-27a/b and miR-494 were found to

be implicated in estrogenic downregulation of *TFPIα* in breast cancer cells. Later, Geng et al. (2020) [134] confirmed the interaction between *TFPI1* and miR-27a in gastric cancer cells. The authors suggested that this miRNA might have a negative impact on the clinical outcome of patients with this neoplasia. Likewise, miRNA-500 was also found to target *TFPI1* favouring prostate cancer proliferation and invasion [135]. Among these miRNAs, miR-27a/b (targeting *TFPI1*) and miR-23a (targeting *TFPI2*) have been associated with VTE events in the general

population (Table 1) and with cancer-associated VTE (Table 2; Fig. 5), respectively.

In summary, given the cumulating evidence, miRNAs targeting the TF coagulation pathway appear to be implicated both in cancer-related thrombosis and tumour progression. Further studies should focus on identifying and validating specific miRNA signatures associated with cancer-associated VTE development. In an era of liquid biopsies, these signatures could be used as non-invasive biomarkers to accurately recognise those at high risk, allowing a more targeted thromboprophylaxis, an early intervention and a more personalized treatment. Inclusively, these miRNAs could also help improve the current prevention strategies directed at cancer patients, since some develop thrombotic events even under thromboprophylaxis [28,136–140]. Additionally, as TF and TFPI1/2 are also implicated in cancer pathways, these miRNAs could also serve as prognostic biomarkers and potential therapeutic targets for cancer management.

4. Conclusion and further investigation

Identifying biomarkers and unravelling the underlying mechanisms of cancer-associated VTE pathogenesis is important to improve disease prevention and treatment. Furthermore, given the interplay between haemostatic components and tumour cells towards cancer progression and dissemination, these biomarkers might also be crucial in cancer management. Several epidemiological studies have identified miRNAs that represent potential VTE biomarkers in the general population. To a lesser extent, miRNAs have also been suggested to play a role in cancer-associated VTE. However, the restricted data available coupled with the small cohort sizes and lack of external validation in the conducted studies has contributed to the low reproducibility of findings. Hence, further investigation is needed to address these limitations. In this context, as the TF pathway is thought to have a central role in cancer-associated VTE, miRNAs targeting this pathway may constitute critical epigenetic regulators of this condition. Several miRNAs have been reported to downregulate TF expression, including miR-19a/b, miR-20a/b, miR-106b ~ 25 cluster (miR-25, miR-93 and miR-106b), miR-126, miR-223, miR-520 g, miR-145, miR-92a and miR-365a. Among them, miR-19a is the F3-targeting miRNA with more evidence. This miRNA has inclusively been associated with cancer-associated VTE. Likewise, miR-126 also deserves attention as the VTE-linked miRNA with more data in the general population, with a reported connection with the condition also among cancer patients. Regarding *TFPI1*, miR-27a/b, miR-494 and miR-500 were identified as targeting miRNAs, with miR-27a/b having a confirmed role in VTE on the broader population. As for *TFPI2*, miR-616, miR-195 and miR-23a appear to exert an effect on the expression of this procoagulant and anti-fibrinolytic protein. Among them, miR-616 is implicated in cancer-associated VTE. Given the cumulating evidence, miRNAs targeting the TF coagulation pathway can potentially be valuable non-invasive biomarkers of cancer-associated VTE to help tailor thromboprophylaxis and improve disease diagnosis and treatment. As such, researchers should put effort into this field given the clinical implications for oncological patients.

Author contributions

All authors had a significant role in the manuscript writing. Literature search and collection, VT, BVN and ISM; writing, VT, BVN and ISM; revision, VT, BVN, ISM, JA, DP and RM; Supervision and guidance on literature search, JA, DP, and RM. The authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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