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Tumour Coagulome and Venous Thromboembolism-related Biomarkers for Prognosis Assessment of Ovarian Cancer Patients

Valéria Delgado Tavares

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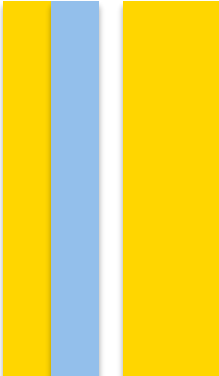
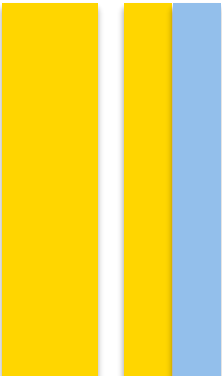
Valéria Delgado Tavares. Tumour coagulome and venous thromboembolism-related biomarkers for prognosis assessment of ovarian cancer patients



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TUMOUR COAGULOME AND VENOUS THROMBOEMBOLISM-RELATED BIOMARKERS FOR PROGNOSIS ASSESSMENT OF OVARIAN CANCER PATIENTS

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A todos que contribuíram para a realização deste trabalho e estiveram ao meu lado nesta caminhada, deixo a minha profunda gratidão.

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This thesis project was carried out in the Molecular Oncology and Viral Pathology Group, Research Centre 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).



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NOTA EXPLICATIVA / EXPLANATORY NOTE

Parte dos dados incluídos nesta tese foi apresentada à Universidade do Porto em 2023, no âmbito da dissertação de Mestrado em Biologia Celular e Molecular intitulada "*Long non-coding RNAs associated with venous thromboembolism: impact on the prognosis of ovarian cancer patients*", da aluna Inês Soares Marques. Estes dados fazem parte de publicações com autoria partilhada entre a candidata ao grau de Doutor e a Mestre Inês Soares Marques, pelo que se salvaguarda a utilização parcial ou integral dos mesmos dados no âmbito desta tese.

Part of the data included in this thesis was presented to the University of Porto in 2023 as part of the Master's dissertation in Cell and Molecular Biology entitled 'Long non-coding RNAs associated with venous thromboembolism: impact on the prognosis of ovarian cancer patients' by the student Inês Soares Marques'. These data are part of shared-authorship publications between the doctoral candidate and Master Inês Soares Marques, thus ensuring the partial or full use of the same data within this doctoral thesis.

Porto, 30 de Agosto de 2024

Valéria Delgado Tavares

(Valéria Delgado Tavares)

PUBLICAÇÕES CIENTÍFICAS / SCIENTIFIC PUBLICATIONS

Em cumprimento com o disposto no artigo 8º do Decreto-Lei nº 388/70, a autora declara que teve uma contribuição substancial na conceção e na execução do trabalho experimental e de revisão bibliográfica, bem como na análise e interpretação dos resultados e na redação dos manuscritos inframencionados. A autora declara ainda que estes manuscritos foram utilizados na redação desta tese e que a mesma inclui dados ainda não publicados que deverão ser utilizados meramente para fins académicos.

In compliance with the relevant national legislation, the author declares that she made a substantial contribution to the design and execution of the experimental work and literature review, as well as to the analysis and interpretation of the results and the writing of the aforementioned manuscripts. The author further declares that these manuscripts were used to prepare this thesis, including data that has not yet been published and is intended solely for academic purposes.

In compliance with current legislation, the following publications, which include five **literature reviews** (articles 1-5) and four **original studies** (articles 6-9), were included in this doctoral thesis:

1. Valéria Tavares, Inês Soares Marques, Inês Guerra de Melo, Joana Assis, Deolinda Pereira, Rui Medeiros. **Paradigm Shift: A Comprehensive Review of Ovarian Cancer Management in an Era of Advancements**. *International Journal of Molecular Sciences*. **2024**; 25 (3): 1845. <https://doi.org/10.3390/ijms25031845>. (Impact factor and quartile reported in 2023: **4.9** and **Q1**).
2. Joana Liz-Pimenta *, Valéria Tavares *, Beatriz Vieira Neto, Joana M.O. Santos, Catarina Brandão Guedes, António Araújo, Alok A. Khorana, Rui Medeiros. **Thrombosis and cachexia in cancer: Two partners in crime?** *Critical Reviews in Oncology/Hematology*. **2023**; 186: 103989-103989. <https://doi.org/10.1016/j.critrevonc.2023.103989>. (Impact factor and quartile reported in 2023: **5.5** and **Q1**).
3. Valéria Tavares, Beatriz Vieira Neto, Maria Isabel Vilas-Boas, Deolinda Pereira, Rui Medeiros. **Impact of hereditary thrombophilia on cancer-associated thrombosis, tumour susceptibility and progression: A review of existing evidence**. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. **2022**; 1877 (5): 188778-188778.

<https://doi.org/10.1016/j.bbcan.2022.188778>. (Impact factor and quartile reported in 2022: **11.2** and **Q1**).

4. Inês Soares Marques, Valéria Tavares, Beatriz Vieira Neto, Inês N. R. Mota, Deolinda Pereira, Rui Medeiros. **Long Non-Coding RNAs in Venous Thromboembolism: Where Do We Stand?** *International Journal of Molecular Sciences*. **2023**; 24 (15): 12103-12103. <https://doi.org/10.3390/ijms241512103>. (Impact factor and quartile reported in 2023: **4.9** and **Q1**).

5. Valéria Tavares *, Beatriz Vieira Neto *, Inês Soares Marques *, Joana Assis, Deolinda Pereira, Rui Medeiros. **Cancer-associated thrombosis: What about microRNAs targeting the tissue factor coagulation pathway?** *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. **2024**; 1879 (1): 189053. <https://doi.org/10.1016/j.bbcan.2023.189053>. (Impact factor and quartile reported in 2023: **9.7** and **Q1**).

6. Valéria Tavares, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Joana Assis, Deolinda Pereira, Rui Medeiros. **Heritable Genetic Variability in Ovarian Tumours: Exploring Venous Thromboembolism Susceptibility and Cancer Prognosis in a Hospital-Based Study**. (Submitted).

7. Valéria Tavares, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Joana Assis, Deolinda Pereira, Rui Medeiros. **Haemostatic Gene Expression in Cancer-Related Immunothrombosis: Contribution for Venous Thromboembolism and Ovarian Tumour Behaviour**. *Cancers*. **2024**; 16 (13): 2356. <https://doi.org/10.3390/cancers16132356>. (Impact factor and quartile reported in 2023: **4.5** and **Q1**).

8. Inês Soares Marques *, Valéria Tavares *, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Inês Guerra de Melo, Joana Assis, Deolinda Pereira, Rui Medeiros. **Long Non-Coding RNAs: Bridging Cancer-Associated Thrombosis and Clinical Outcome of Ovarian Cancer Patients**. *International Journal of Molecular Sciences*. **2024**; 25 (1): 140. <https://doi.org/10.3390/ijms25010140>. (Impact factor and quartile reported in 2023: **4.9** and **Q1**).

9. Valéria Tavares, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Joana Assis, Deolinda Pereira, Rui Medeiros. **Plasma microRNA environment linked to tissue factor pathway and cancer-associated thrombosis: prognostic significance in ovarian cancer**. *Biomolecules*. **2024**; 14 (8): 928.

<https://doi.org/10.3390/biom14080928>. (Impact factor and quartile reported in 2023: **4.8** and **Q1**).

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

Beyond the scientific works presented in this thesis, the author contributed to additional articles during the doctoral period, broadening the understanding of the thesis topic. Many of these papers delved into cancer-associated thrombosis in different tumour models or investigated the biomarkers discussed in this thesis within diverse pathophysiological contexts, such as cancer patients and high-performance athletes. Notably, some of these publications laid the groundwork for discussing the findings of this thesis.

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2. Hugo-Henrique Silva, Maria-Raquel G. Silva, Fátima Cerqueira, Valéria Tavares, Rui Medeiros. **Genomic profile in association with sport-type, sex, ethnicity, psychological traits and sport injuries of elite athletes: review and future perspectives.** *The Journal of Sports Medicine and Physical Fitness*. **2021**; 62 (3): 418-434. <https://doi.org/10.23736/s0022-4707.21.12020-1>.
3. Sofia Coelho Abreu *, Valéria Tavares *, Filipa Carneiro, Rui Medeiros. **Venous thromboembolism and prostate cancer: what about genetic markers?** *Pharmacogenomics*. **2021**; 22 (6): 365-373. <https://doi.org/10.2217/pgs-2020-0094>.
4. Gisseila Andrea Ferreira Garcia, Valéria Tavares, Maria José Carvalho Moreno, Delisa Soraia Monteiro Vera-Cruz Martins, Cilliavini Fonseca de Sousa Afonso Varela, Mara Yone Soares Dias Fernandes, Andréa Jaqueline Fortes Ferreira. **Novo Coronavírus SARS-CoV-2 em Cabo Verde: Análise situacional após um ano de notificação do 1º caso.** *Multidisciplinary Reviews*. **2021**; 4 (1): e2021019-e2021019. <https://doi.org/10.29327/multi.2021019>.
5. Hugo-Henrique Silva *, Valéria Tavares *, Maria-Raquel G. Silva, Beatriz Vieira Neto, Fátima Cerqueira, Rui Medeiros. **FAAH rs324420 Polymorphism Is Associated with**

- Performance in Elite Rink-Hockey Players.** *Biology*. **2022**; 11 (7): 1076-1076. <https://doi.org/10.3390/biology11071076>.
6. Beatriz Vieira Neto *, Valéria Tavares *, Joana M. O. Santos, Fátima Cerqueira, Deolinda Pereira, Rui Medeiros. **Map of thrombogenesis in viral infections and viral-driven tumours.** *Discover Oncology*. **2023**; 14 (1): 3-3. <https://doi.org/10.1007/s12672-022-00610-1>.
 7. Jani Silva *, Valéria Tavares *, Ana Afonso, Juliana Garcia, Fátima Cerqueira, Rui Medeiros. **Plasmatic MicroRNAs and Treatment Outcomes of Patients with Metastatic Castration-Resistant Prostate Cancer: A Hospital-Based Cohort Study and In Silico Analysis.** *International Journal of Molecular Sciences*. **2023**; 24 (10): 9101-9101. <https://doi.org/10.3390/ijms24109101>.
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 10. Valéria Tavares, Joana Assis, Ricardo Pinto, Margarida Freitas-Silva, Rui Medeiros. **Venous thromboembolism-related genetic determinant F11 rs4253417 is a potential prognostic factor in ischaemic stroke.** *Molecular and Cellular Probes*. **2023**; 70: 101917-101917. <https://doi.org/10.1016/j.mcp.2023.101917>.
 11. Joana M. O. Santos, Valéria Tavares, Rui M. Gil da Costa, Rui Medeiros. **MiR-150 and miR-155 expression predicts survival of cervical cancer patients: a translational approach to novel prognostic biomarkers.** *Biomarkers*. **2023**; 28 (7): 617-627. <https://doi.org/10.1080/1354750X.2023.2269320>.
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13. Inês Soares Marques *, Valéria Tavares *, Beatriz Vieira Neto, Lucas Rafael Lopes, Rodrigo Araújo Goes, João António Matheus Guimarães, Jamila Alessandra Perini, Rui Medeiros. **Genetic Variations in Susceptibility to Traumatic Muscle Injuries and Muscle Pain among Brazilian High-Performance Athletes.** *International Journal of Molecular Sciences*. **2024**; 25 (6): 3300. <https://doi.org/10.3390/ijms25063300>.
14. Joana Liz-Pimenta, Valéria Tavares, João Gramaça, João Rato, Maria Menezes, Mafalda Baleiras, Helena Guedes, Joana Reis, Catarina Guedes, Rosa Gomes, Miguel Barbosa, Marta Sousa, Alok A. Khorana, Rui Medeiros. **Primary thromboprophylaxis in cancer outpatients – real-world evidence.** *Journal of Thrombosis and Thrombolysis*. **2024**; 57: 805–814. <http://dx.doi.org/10.1007/s11239-024-02984-1>.
15. Beatriz Vieira Neto, Valéria Tavares, José Brito da Silva, Joana Liz-Pimenta, Inês Soares Marques, Lurdes Salgado, Luísa Carvalho, Deolinda Pereira, Rui Medeiros. **Haemostatic gene variations in cervical cancer-associated venous thrombosis: considerations for clinical strategies.** *Journal of Thrombosis and Thrombolysis*. **2024**; 57: 815-827. <https://doi.org/10.1007/s11239-024-02983-2>.
16. Inês Guerra de Melo, Valéria Tavares, Deolinda Pereira, Rui Medeiros. **Contribution of Endothelial Dysfunction to Cancer Susceptibility and Progression: A Comprehensive Narrative Review on the Genetic Risk Component.** *Current Issues in Molecular Biology*. **2024**; 46 (5): 4845-4873. <https://doi.org/10.3390/cimb46050292>.
17. Ana Vaz-Ferreira *, Valéria Tavares *, Inês Guerra de Melo, Patrícia Rafaela Rodrigues, Ana Afonso, Maria Joaquina Maurício, Rui Medeiros. **Connecting Gene Variation to Treatment Outcomes in Metastatic Castration-Resistant Prostate Adenocarcinoma: Insights into Second-generation Androgen Receptor Axis-Targeted Therapies.** *International Journal of Molecular Sciences*. **2024**; 25 (18): 9874. <https://doi.org/10.3390/ijms25189874>.
18. Lucas Rafael Lopes, Marcus Vinícius Galvão Amaral, Rodrigo Araújo Goes, Valéria Tavares, Francisca Dias, Rui Medeiros, Daniel Escorsim Machado, Jamila Perini. **Tenascin-C-Matrix Metalloproteinase-3 Phenotype and the Risk of Tendinopathy in High-Performance Athletes: A Case–Control Study.** *Diagnostics*. **2024**; 14 (22): 2469. <https://doi.org/10.3390/diagnostics14222469>.
19. Lucas Rafael Lopes, Jamila Perini, Valéria Tavares, Francisca Dias, Rui Medeiros. **A systematic review and meta-analysis on aerobic fitness dynamics in post-COVID-19 athletes: implications in the return-to-play performance.** (Submitted).

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LIST OF MAIN ABBREVIATIONS

A

ADCs	Antibody-drug conjugates
aHR	Adjusted hazard ratio
aPTT	Activated partial thromboplastin time
APC	Activated protein C
AUC	Area under the curve

B

BMI	Body mass index

C

CA-125	Cancer antigen 125
CAC	Cancer-associated cachexia
CAT	Cancer-associated thrombosis
CCC	Clear cell carcinoma
cDNA	Complementary DNA
CeRNAs	Competitive endogenous RNAs
CI	Confidence interval
CNV	Copy number variation
CRP	C-reactive protein

D

DD	D-dimer
DOAC	Direct oral anticoagulants
DVT	Deep venous thrombosis

E

EC	Endometrioid carcinoma
ECOG PS	Eastern Cooperative Oncology Group Performance Status
EMA	European Medicine Agency
EOC	Epithelial ovarian cancer
EPC	Endothelial progenitor cell
EPCR	Endothelial protein C receptor
ESMO	European Society for Medical Oncology

F

<i>F2</i>	<i>Coagulation factor 2</i>

List of Main Abbreviations

<i>F3</i>	<i>Coagulation factor 3</i>
<i>F5</i>	<i>Coagulation factor 5</i>
<i>F8</i>	<i>Coagulation factor 8</i>
<i>F11</i>	<i>Coagulation factor 11</i>
<i>F13A1</i>	<i>Coagulation factor 13 A chain</i>
FDA	Food and Drug Administration
FGG	Fibrinogen gamma chain
FI	Coagulation factor I (fibrinogen)
FIGO	International Federation of Gynecology and Obstetrics
FII	Coagulation factor II (prothrombin)
FIII	Coagulation factor III (tissue factor)
FIV	Coagulation factor IV (Calcium ions)
FV	Coagulation factor V
FVII	Coagulation factor VII
FVIII	Coagulation factor VIII
FIX	Coagulation factor IX
FR α	Folate receptor alfa
FX	Coagulation factor X
FXI	Coagulation factor XI
FXII	Coagulation factor XII
FXIII	Coagulation factor XIII
F _a	Activated coagulation factor
G	
GWAS	Genome-wide association study
H	
HGSC	High-grade serous carcinoma
HIPEC	Hyperthermic intraperitoneal chemotherapy
HR	Hazard ratio
HUVEC	Human umbilical vein endothelial cell
HWE	Hardy-Weinberg equilibrium
I	
Indel	Insertion and deletion
INR	International normalised ratio
IL ₋	Interleukin
K	
KS	Khorana score

L

LGSC	Low-grade serous carcinoma
LincRNA	Intergenic long non-coding RNA
LMWH	Low-molecular-weight heparin
LncRNA	Long non-coding RNA

M

MAF	Minor allele frequency
MC	Mucinous carcinoma
MiR or MiRNA	MicroRNAs
MLR	Monocyte-to-lymphocyte ratio
mRNA	Messenger RNA

N

NCCN	National Comprehensive Cancer Network
NLR	Neutrophil-to-lymphocyte ratio
NO	Nitric oxide

O

OC	Ovarian cancer
OR	Odds ratio
OS	Overall survival

P

PAI-1	Plasminogen activator inhibitor-1
PARP	Poly (ADP-ribose) polymerase
PARPi	Poly (ADP-ribose) polymerase inhibitors
PBCs	Peripheral blood components
PDNP	Podoplanin
PE	Pulmonary embolism
PFS	Progression-free survival
PLR	Platelet-to-lymphocyte ratio
PT	Prothrombin time

R

RT-PCR	Real-time Polymerase Chain Reaction

S

SD	Standard deviation
SNP	Single-nucleotide polymorphism

T

TAFI	Thrombin-activable fibrinolysis inhibitor

List of Main Abbreviations

TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TFPI1	Tissue factor pathway inhibitor 1
TFPI2	Tissue factor pathway inhibitor 2
TM/THBD	Thrombomodulin
TPO	Thrombopoietin
U	
UTR	Untranslated region
V	
vEC	Vascular endothelial cell
VEGF	Vascular endothelial growth factor
VKA	Vitamin K antagonists
VTE	Venous thromboembolism
vWF	von Willebrand factor

OUTLINE OF THE THESIS

This doctoral thesis is a collection of adapted reviews and original articles organised into eight chapters (**Figure 1**).

Chapter I is the introduction, offering a state-of-the-art research topic overview. This chapter unfolds in six segments. **Chapter IA** provides a general introduction, featuring unpublished data that sets the stage for the detailed discussions in **Chapters IB-F**. **Chapter IB** delves into the current landscape of ovarian cancer (OC) management, highlighting the need for new biomarkers and treatment strategies. **Chapter IC** gathers information on the influence of inflammation in cancer-related complications, including the most common ones – venous thromboembolism (VTE) and cachexia. Since VTE is a strongly heritable disease, **Chapter ID** explores the existing evidence linking haemostasis-related genetic factors to cancer-associated thrombosis (CAT) development and tumour susceptibility and progression. **Chapter IE** shifts focus to the cutting-edge realm of long non-coding RNAs (lncRNAs) in venous thrombogenesis, presenting the latest findings. **Chapter IF** explores the frontier of microRNAs (miRNAs), discussing their promising applications in VTE prevention, diagnosis, and prognosis assessment, focusing on those targeting the tissue factor (TF) coagulation pathway.

Chapter II establishes the main and specific aims of this thesis project based on the scientific background and the existing knowledge gaps.

Chapters III, IV, V and VI, composed of both published and unpublished original articles, detail the methodology and project findings that address the thesis aims outlined in Chapter II. **Chapter III** encompasses an unpublished original paper exploring the implications of haemostasis-related single-nucleotide polymorphisms (SNPs) in CAT development and OC patients' prognosis, regardless of thrombosis. Given the pivotal role of the coagulation system in VTE pathogenesis and particularly CAT among OC patients, **Chapter IV** presents an original paper evaluating the expression of genes encoding coagulation factors and anticoagulants of the extrinsic and common coagulation pathways in patients' peripheral blood entities, relying on the concept of cancer thrombo-inflammation. **Chapters V and VI** feature original papers assessing, respectively, the impact of VTE-related lncRNAs and miRNAs on CAT development and the clinical outcomes of OC patients.

The general discussion of all the findings is provided in **Chapter VII**, while **Chapter VIII** encompasses the main conclusions obtained through the data from Chapters III, IV, V, and VI, and pinpoints future perspectives.

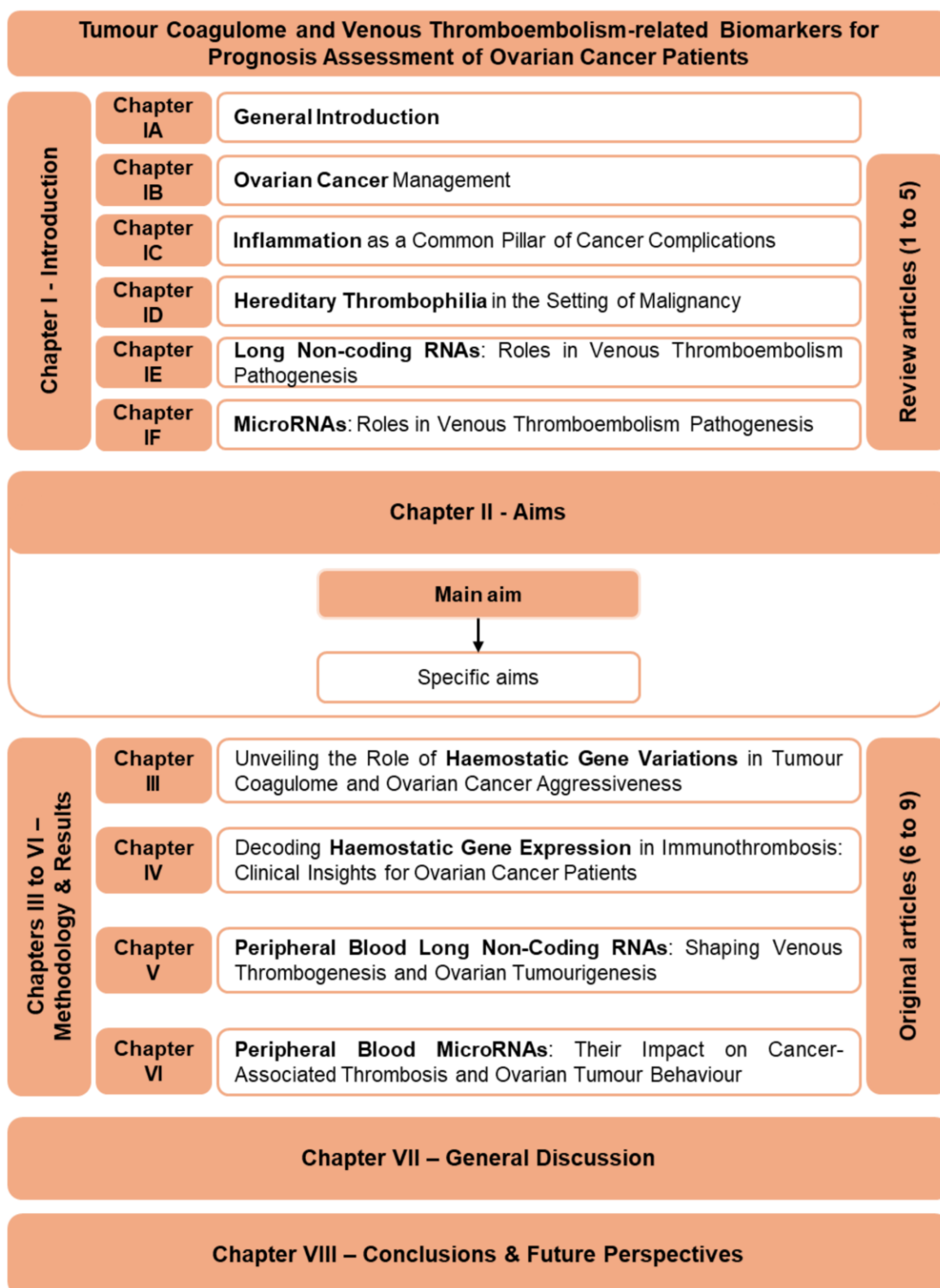


Figure 1. Outline of the doctoral thesis.

ABSTRACT

Ovarian cancer (OC) is the most lethal gynaecological tumour, with a 5-year survival rate lower than 50% in most countries. A paradigm shift in the management of the disease requires more reliable prognostic biomarkers to personalise treatment approaches, aiming for better clinical outcomes. In parallel, accumulating evidence highlights the significant role of haemostatic components in OC progression and dissemination. Indeed, OC patients often present venous thromboembolism (VTE), which is their second cause of death, the first being cancer progression. The bilateral relationship between cancer and VTE, known as Trousseau syndrome, raises the possibility that cancer-associated thrombosis (CAT) biomarkers, which act as surrogates for coagulation activation, could enhance prognostication, monitor treatment responses, and aid in the early detection of OC recurrence.

Given the existing data, this thesis aimed to identify clinically relevant prognostic biomarkers for OC by exploring the tumour coagulome and CAT pathogenesis through liquid biopsies. Thus, haemostasis-related biomarkers, including single-nucleotide polymorphisms (SNPs), expression of haemostasis-related genes in peripheral blood components (PBCs) in the setting of cancer immunothrombosis, long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) were evaluated as potential CAT predictors and prognostic tools for OC (regardless of venous thrombogenesis). Collectively, the findings indicate that OC patients exhibit a significant pro-thrombotic risk, with incidence rates between 19% and 23%. The occurrence of CAT in these patients was observed to negatively influence the time to OC progression and overall survival, particularly when dismissing those with VTE events and concurrently under anticoagulation therapy before tumour diagnosis and treatment. Notably, CAT was defined as a VTE event occurring from six months before to two years after an OC diagnosis. Aside from leucocyte count, patient characteristics did not reliably predict CAT development. The frequently studied and validated CAT risk assessment model, the Khorana score, exhibited poor predictive performance, highlighting the need for better CAT predictors. Additionally, traditional coagulation assays used in the clinical practice of our institution – prothrombin (PT), international normalised ratio (INR) and activated partial thromboplastin time (aPTT) – were found to be unsuitable for assessing the pro-thrombotic potential in OC patients. However, INR demonstrated prognostic value.

Regarding the evaluated haemostasis-related biomarkers, starting with SNPs, *protein C receptor (PROCR)* rs10747514 was found to be a predictor of CAT in our cohort of OC patients, while *serpin family E member 1 (SERPINE1)* rs2070682 was identified as both a

CAT predictor and a prognostic factor for OC (regardless of venous thrombogenesis). The SNPs *regulator of G protein signalling 7 (RGS7)* rs2502448, *coagulation factor 3 (F3)* rs1361600 and *fibrinogen gamma chain (FGG)* rs2066865 were shown to be solely prognostic biomarkers of OC. Among them, *F3* rs1361600 stands out as a predictor of both OC progression and mortality, aligning with the pivotal role that tissue factor (TF) has on malignant diseases, including OC. Concerning the expression of haemostasis-related genes in OC patients' PBCs, pre-chemotherapy expression levels of *F3* and *coagulation factor (F8)* in platelets and leukocytes were predictive of CAT development. However, only *F8* expression was associated with patients' time to disease progression and survival. In the study assessing the clinical implications of lncRNAs, pre-chemotherapy *maternally expressed 8 (MEG8)* [targeting *tissue factor pathway inhibitor 2 (TFPI2)*] levels in PBCs were a predictor of OC-related VTE. This lncRNA was also demonstrated to impact the risk of OC progression regardless of VTE. Moreover, *nuclear paraspeckle assembly transcript 1 (NEAT1)* expression in PBCs was associated with the risk of death, emerging as a potential OC prognostic biomarker. Lastly, in the evaluation of the miRNAs, pre-chemotherapy plasma levels of miR-20a-5p (targeting *TF* and *TFPI2*, the latter with less evidence) and miR-616-3p (targeting *TFPI2*) were shown to predict OC-related VTE, with miR-20a-5p being a relevant indicator of OC progression and miR-616-3p significantly impacting survival. Furthermore, miR-23a-3p (targeting *TFPI2*) influenced patients' prognosis but not CAT susceptibility. When performing subgroup analysis, all the evaluated miRNAs were shown to have a detrimental impact on OC progression and/or patients' survival. Considering all the studies, TF and TFPI2 seem to impact CAT and ovarian tumourigenesis significantly.

Collectively, this project underscores several promising prognostic biomarkers for OC that could be assessed through liquid biopsies, facilitating their clinical application. These biomarkers can serve as predictors of CAT, enabling personalised thromboprophylaxis based on individual risk profiles. They also hold potential for VTE treatment monitoring, ensuring the effectiveness of therapeutic interventions and guiding timely adjustments. In-depth studies may further uncover novel therapeutic targets for VTE management, addressing the ongoing challenge of thrombotic events in patients receiving thromboprophylaxis and the associated risk of bleeding complications. Furthermore, the independent prognostic value of some of these biomarkers highlights their potential to refine OC management strategies and improve clinical outcomes. Future research should focus on gaining more insights into the functional consequences of these biomarkers. Their clinical utility should also be validated in larger and well-designed prospective studies, using preferentially a multicentric approach. Also, their implications in different physiopathological settings should be investigated.

RESUMO

O cancro do ovário (CO) é o tumor ginecológico mais letal, com uma taxa de sobrevivência a 5 anos inferior a 50% na maioria dos países. Melhorias nas abordagens terapêuticas da doença requerem novos biomarcadores de prognóstico para personalizar o tratamento oncológico, e assim melhorar a evolução clínica das doentes. Paralelamente, estudos têm demonstrado que componentes hemostáticos têm um papel significativo na progressão e disseminação do CO. De facto, doentes com esta neoplasia são frequentemente diagnosticadas com tromboembolismo venoso (TEV). Esta doença cardiovascular é a segunda causa de morte destas doentes, sendo a primeira a progressão do cancro. A relação bilateral entre malignidade e o TEV, conhecida como a síndrome de Trousseau, sugere que biomarcadores de trombose associada a cancro (TAC), que indicam a ativação da coagulação sanguínea, podem aumentar a precisão na avaliação do prognóstico e auxiliar na avaliação das respostas ao tratamento e na deteção precoce da recorrência do CO.

Tendo em conta os dados existentes, esta tese de doutoramento teve como objetivo identificar biomarcadores de prognóstico clinicamente relevantes para o CO, através do estudo do coaguloma tumoral e a patogénese da TAC com recurso a biópsias líquidas. Assim, biomarcadores relacionados com a hemostasia, incluindo polimorfismos de nucleotídeo único [do inglês, *single-nucleotide polymorphisms* (SNPs)], expressão de genes relacionados com o sistema de coagulação em componentes do sangue periférico no contexto da imunotrombose associada a cancro, RNAs longos não codificantes (lncRNAs) e microRNAs (miRNAs), foram avaliados como potenciais preditores de TAC e biomarcadores de prognóstico para o CO (independentemente da trombose venosa). Coletivamente, os resultados indicam que doentes com CO apresentem um risco pró-trombótico substancial, com taxas de incidência entre 19% e 23%. A ocorrência de TAC influenciou negativamente o tempo de progressão do CO e a sobrevivência global das doentes, particularmente ao excluir as com eventos de TEV e simultaneamente sob terapia anticoagulante antes do diagnóstico e tratamento do tumor. De ressaltar que TAC foi definido como um evento de TEV diagnosticado seis meses antes até dois anos após um diagnóstico de CO. Com a exceção da contagem de leucócitos, as características demográficas e clinicopatológicas das doentes não denotaram capacidade preditiva para o desenvolvimento de TAC. De igual modo, o modelo de avaliação de risco de TAC mais frequentemente estudado e validado - o score de Khorana - exibiu um desempenho preditivo fraco, destacando a necessidade de melhores preditores de TAC. Além disso, os

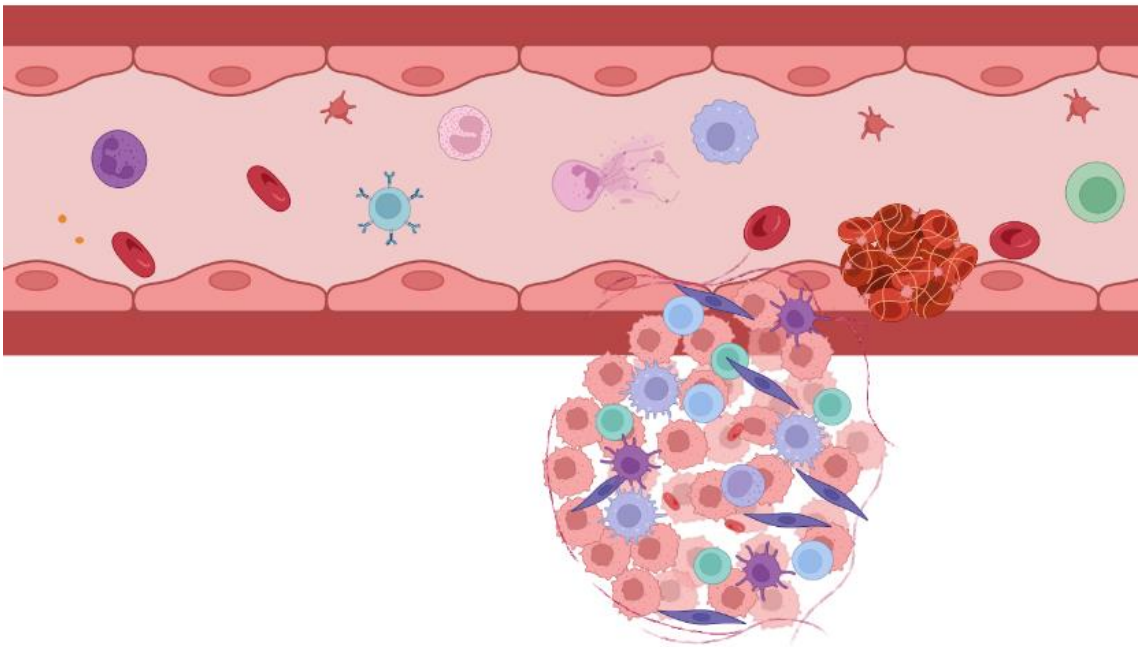
ensaios de coagulação tradicionalmente utilizados na prática clínica na nossa instituição – tempo de protrombina, razão normalizada internacional [do inglês, *international normalised ratio* (INR)] e tempo de tromboplastina parcial ativado - foram inadequados para avaliar o potencial pró-trombótico nas doentes com CO. No entanto, a INR demonstrou valor prognóstico.

No que diz respeito aos biomarcadores relacionados com a hemostasia avaliados nesta tese, a começar pelos SNPs, o *protein C receptor (PROCR)* rs10747514 foi um preditor de TAC, enquanto o *serpin family E member 1 (SERPINE1)* rs2070682 foi identificado não só como preditor de TAC, mas também como fator de prognóstico (independentemente da trombose venosa). As variantes genéticas *regulator of G protein signaling 7 (RGS7)* rs2502448, *coagulation factor 3 (F3)* rs1361600 e *fibrinogen gamma chain (FGG)* rs2066865 apresentaram apenas valor prognóstico. Entre eles, o *F3* [gene que codifica para o fator tecidual (TF)] rs1361600 destacou-se como preditor da progressão de CO e mortalidade, o que é concordante com o papel fundamental que o TF tem na tumorigénese, incluindo a tumorigénese ovárica. Quanto à expressão de genes relacionados com o sistema de coagulação nos componentes do sangue periférico das doentes com CO, os níveis de expressão pré-quimioterapia de *F3* e *coagulation factor (F8)* em plaquetas e leucócitos foram preditores do desenvolvimento de TAC. No entanto, apenas a expressão de *F8* foi associada ao tempo de progressão do CO e à sobrevivência global das doentes. No estudo que avaliou as implicações clínicas dos lncRNAs, os níveis pré-quimioterapia do *maternally expressed 8 (MEG8)* [tem como alvo a expressão do *tissue factor pathway inhibitor 2 (TFPI2)*] nos componentes do sangue periférico foram preditores de TAC. Este lncRNA também foi associado ao risco de progressão do CO independentemente do TEV. Além disso, a expressão do *nuclear paraspeckle assembly transcript 1 (NEAT1)*, nesses mesmos componentes, foi associada ao risco de morte, emergindo como um potencial biomarcador prognóstico de CO. Por fim, na avaliação dos miRNAs, os níveis plasmáticos pré-quimioterapia de miR-20a-5p (que parece modular a expressão de *F3* e *TFPI2*) e miR-616-3p (tem *TFPI2* como alvo) revelaram-se preditores de TEV relacionado ao CO, sendo o miR-20a-5p um indicador relevante da progressão do CO e o miR-616-3p um preditor de risco de morte. Além disso, o miR-23a-3p (tem *TFPI2* como alvo) influenciou o prognóstico das doentes, mas não a suscetibilidade a TAC. Em análises estratificadas, todos os miRNAs avaliados demonstraram impacto significativo na progressão do CO e/ou na sobrevivência global das doentes. Considerando todos os estudos, TF e TFPI2 aparentam ter um papel crucial na ocorrência de TAC e na progressão da tumorigénese ovárica.

Coletivamente, este projeto evidenciou vários biomarcadores de prognóstico promissores para o CO, que podem ser avaliados através de biópsias líquidas, facilitando

a sua translação para a prática clínica. Estes biomarcadores podem servir como preditores de TAC, permitindo uma trombopprofilaxia personalizada com base nos perfis de risco individuais. Os mesmos também poderão auxiliar na identificação de novos alvos terapêuticos para o TEV e na monitorização do tratamento anticoagulante de forma a maximizar a eficácia terapêutica e minimizar o risco associado de complicações hemorrágicas. Além disso, alguns destes biomarcadores apresentaram um valor prognóstico independente, o que evidencia o seu potencial para aperfeiçoar as estratégias de tratamento do CO e melhorar a qualidade de vida e sobrevivência das doentes. Todavia, para concretizar estas aplicações, serão necessários estudos adicionais. Primeiramente, estudos funcionais são fundamentais para uma melhor compreensão da consequência funcional destes biomarcadores e os mecanismos biológicos subjacentes. Além disso, a utilidade clínica dos biomarcadores deve ser validada em estudos prospetivos, com coortes maiores e, preferencialmente, de natureza multicêntrica. Igualmente, devem ser investigadas as implicações destes biomarcadores em diferentes contextos fisiopatológicos.

Chapter I



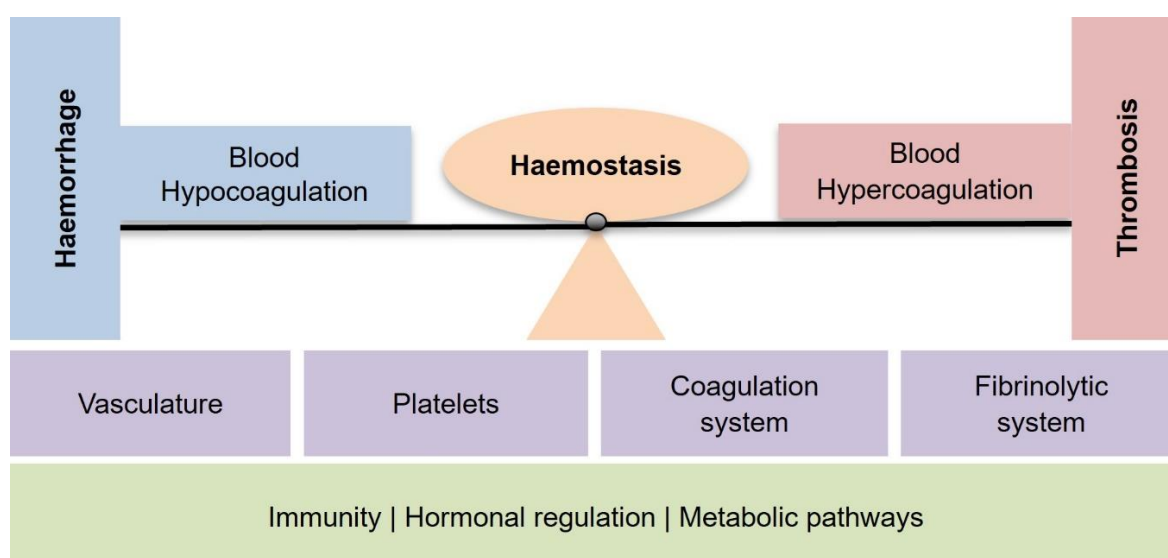
CHAPTER IA – General Introduction

Worldwide, cancer and cardiovascular diseases are the leading causes of mortality, significantly impacting healthcare costs [1-3]. Ovarian cancer (OC), in particular, is regarded as the most lethal gynaecological tumour due to late-stage diagnosis and a high rate of disease recurrence after treatment [4,5]. Based on the latest GLOBOCAN data, OC ranks as the eighth most common cancer and the eighth leading cause of death among women worldwide, with approximately 324,000 new cases and 207,000 related deaths reported in 2022. In Portugal, OC was the eleventh most diagnosed malignant disease and the eighth deadliest among women. In the same year, 682 new cases (age-standardised world (ASW) incidence of 5.8 per 100,000), 472 deaths (ASW mortality of 3.1 per 100,000) and a 5-year prevalence exceeding 1,900 cases were accounted for [6].

Despite improvements in disease management, OC patients still have a 5-year survival rate lower than 50% in most countries [7]. The disease research has observed a paradigm shift, focusing on a deeper understanding of OC, aiming for effective screening, early diagnosis and personalised treatment strategies (**see Chapter IB**). One significant challenge in improving OC management and, consequently, patients' prognosis is the lack of reliable prognostic biomarkers. This issue is critical given the high degree of heterogeneity that characterises OC, which prevents conventional prognostic factors, such as tumour histology and stage, from effectively aiding in personalised disease management [8]. Also, disease monitoring with biomarkers such as serum cancer antigen 125 (CA-125) has restricted clinical applications, not demonstrating a benefit in overall survival (OS) [9]. Thus, novel biomarkers are needed, preferably those that can be detected through minimally invasive techniques. In this context, liquid biopsies offer a promising avenue not only for early disease detection but also for prognostication and monitoring of treatment efficacy [10].

To uncover and validate prognostic biomarkers for OC, delving into the tumour microenvironment (TME) is essential. Within this complex milieu, OC cells interact with and exploit key haemostatic components, including vascular endothelial cells (vECs), platelets, and coagulation and fibrinolytic systems (**Figure 1**) [11-13]. In physiological conditions, these components work together to maintain blood circulation and the integrity of blood vessels. However, in malignancy, tumour cells hijack haemostatic elements to promote their growth, neo-angiogenesis, invasion, migration, immune evasion, and metastasis. As a result of haemostatic abnormalities induced by tumour cells (tumour coagulome), OC patients are prone to coagulopathies, including both haemorrhage and thrombotic events

[12]. Cancer-associated thrombosis (CAT), in particular, is thought to result from disrupted haemostasis in a pro-inflammatory environment, a condition referred to as immunothrombosis or thromboinflammation [14]. Notably, inflammation, a hallmark of cancer, plays crucial roles in various cancer complications, including CAT and cancer-associated cachexia (CAC) (**see Chapter IC**). Indeed, cancer patients often present with a state of blood hypercoagulation, marked by abnormalities in crucial coagulation parameters routinely monitored in clinical practice, such as activated partial thromboplastin time (aPTT), prothrombin time (PT) and international normalised ratio (INR). These tests measure the time required for blood to clot relative to an average value and assess various coagulation factors' functionality, highlighting significant coagulation process disruptions [15,16].



Chapter IA - Figure 1. Haemostasis and the pathological states of blood hypocoagulation and hypercoagulation. Blood vasculature, platelets and the coagulation and fibrinolytic systems cooperate to maintain normal haemostasis. When one of these haemostatic components is deregulated, pathological states of either blood hypocoagulation (haemorrhage) or blood hypercoagulation (thrombosis) may arise. These haemostatic disturbances are often driven by inflammation, as well as endocrine and metabolic derangements [17].

Thromboembolism can occur in arteries (arterial thromboembolism, or ATE) and veins (venous thromboembolism, or VTE). Although both arterial and venous pathological blood clots (i.e., thrombi) are composed of red cells, platelets and fibrin, arterial thrombi are typically rich in platelets ('white thrombi'), whereas venous thrombi tend to contain more red cells and fibrin ('red thrombi') [18]. Also, arterial thrombi usually occur at sites of atherosclerotic plaques with active inflammation and high shear rates [19]. Upon plaque disruption, combined with endothelial malfunction and high shear rates, there is a rapid platelet recruitment to the site followed by the formation of a 'white thrombus', a condition

known as atherothrombosis [20]. Regarding venous thrombus, it usually occurs at sites with normal vein walls, but the shear rates and blood flow are low, leading to a 'red thrombus'. Due to thrombi characteristics, platelets play a central role in arterial thrombogenesis, while the coagulation system is the key component in venous thrombogenesis. Thus, antiplatelet therapy is thought to be more effective in ATE, whereas anticoagulation therapy is the standard treatment for VTE [18].

Among cancer patients, VTE is the most common manifestation of thromboembolic events [21]. In particular, the incidence of the disease in OC patients is reported to be between 10% and 30%, making them among the cancer patients most affected by VTE [22-30]. Beyond being common, venous thrombogenesis also stands as their second leading cause of mortality, following cancer progression [17]. Venous thrombogenesis in cancer patients (i.e., CAT) is thought to be a more complex pathological entity than VTE in the general population [31]. Over the years, numerous risk assessment models (RAMs) for CAT prediction have been developed to enhance disease prevention (thromboprophylaxis). The most extensively studied one is the Khorana score (KS). This score assesses clinical and laboratory parameters to gauge the likelihood of VTE in cancer patients, thus providing crucial guidance for prophylactic strategies (**Figure 2**). Notably, this score is specifically tailored for individuals about to begin chemotherapy [32]. Unfortunately, KS has shown poor performance, potentially underestimating the risk in particular cancer types, including OC [33]. To address these gaps, various modified RAMs have been developed to enhance the score's sensitivity, especially for those classified as low-risk by the KS. The list includes the scores of ONKOTEV, PROTECHT, CONKO, Vienna-CATs, TicOnco and COMPASS-CAT, among other emerging ones [34]. Despite improvements in predictive ability, their performance can still vary across tumour types and patient populations [32,34]. These limitations underscore the need for new biomarkers that can more accurately reflect the dynamic changes in TME, providing a more precise risk assessment for CAT.

Risk factors for CAT development		Score
Primary tumour site		
Very high risk (gastric and pancreatic)		2
High risk (lung, lymphoma, gynaecological, bladder, testicular)		1
Prechemotherapy haemoglobin level < 100 g/L or use of red cell growth factors		1
Prechemotherapy platelet count $\geq 350 \times 10^9/L$		1
Prechemotherapy leukocyte count > $11 \times 10^9/L$		1
Prechemotherapy BMI $\geq 35 \text{ kg/m}^2$		1

KS		Total
KS cut-off = 2	0: Low risk	1: Intermediate risk
	≥ 2 : High risk	
KS cut-off = 3	0: Low risk	1-2: Intermediate risk
	≥ 3 : High risk	

Chapter 1A - Figure 2. Application of Khorana score (KS) in cancer-associated thrombosis (CAT) risk assessment. KS is an important clinical tool for identifying cancer patients who may need thromboprophylaxis (i.e. high-risk patients). The optimal cut-off score, whether 2 or 3, is determined based on the specific clinical context and risk stratification needs [21]. Abbreviations: BMI, body mass index.

Several biomarkers have been extensively studied for their roles in predicting and/or diagnosing CAT. Briefly, D-dimer (DD), a degradation product of cross-linked fibrin (represents fibrin turnover and coagulation activation), is the most used predictive and diagnostic biomarker of CAT in clinical practice, with high levels also linked to poor prognosis in cancer patients [35,36]. Curiously, DD levels have been found to correlate with CA-125 levels positively [37]. Its elevated pre-operative levels were also recently linked to a poor prognosis in OC patients, regardless of VTE status [38]. Beyond its predictive and diagnostic value of CAT, DD measurement can also be used to assess the risk of potential thrombotic recurrent events and mortality [39]. However, this marker is unspecific to VTE and CAT presence, as DD levels can be elevated in settings of inflammation, other cardiovascular diseases, prolonged immobilisation and even affected by patients' advanced age [35,40,41].

Additional predictive biomarkers of CAT under investigation include tissue factor (TF), alone or within microparticles (MPs), soluble P-selectin, leukocyte count, neutrophil extracellular traps (NETs), platelet count and mean platelet volume (MPV), podoplanin (PDPN), fibrinogen, thrombin-antithrombin complex (TAT), prothrombin fragment 1+2 (F1+2) and circulating tumour cells (CTCs) with coagulant properties [42-46]. As the initiator of the extrinsic (or exogenous) coagulation pathway, TF is the most extensively studied for its function in CAT pathogenesis and cancer progression. This coagulation factor is

overexpressed in many solid tumours, including OC, promoting tumourigenesis [42,47]. TF exists in two isoforms: the membrane-bound full-length TF (flTF) and the soluble alternatively spliced TF (asTF), with the latter lacking the transmembrane and cytoplasmic domains, which reduces its procoagulant activity. Beyond asTF, flTF is also found in plasma, primarily associated with MPs (TF+MPs) shed from both cancerous and noncancerous cells [48]. Unfortunately, the predictive value of plasma TF levels and/or activity for assessing CAT risk remains controversial. For instance, in OC, neither plasma TF antigen nor TF+MPs activity proved effective [49]. Others have suggested that TF+MPs could be helpful in OC diagnosis [50]. This controversy is compounded by technical issues. Namely, the immunological detection of plasma TF antigen is particularly contentious due to issues with specificity, a critical concern given the complexity of plasma, which contains numerous proteins that can produce non-specific signals. Additionally, the circulating levels of this coagulation factor are typically low [48,51]. Likewise, the MPs' isolation, purification, and characterisation from biological fluids demand sophisticated, time-consuming, and expensive techniques, with the lack of standardised protocols exacerbating issues of reproducibility and reliability [50]. A possible solution to facilitate translation to clinical practice could be studying the expression of TF gene - *coagulation factor 3*. Messenger RNA (mRNA) provides earlier indications of pathological changes compared to proteins and can be more easily and reliably quantified using techniques such as quantitative real-time polymerase chain reaction (qRT-PCR), which offers high sensitivity and specificity [52]. The dynamic nature of RNA expression profiles makes them suitable for monitoring disease progression and response to therapy in real time.

Adding to the potential CAT predictors are heritable genetic variations. In the general population, VTE is considered a highly heritable condition, with numerous genetic mutations and polymorphisms linked to increased susceptibility [53,54]. Compared to genetic mutations, genetic polymorphisms such as single-nucleotide polymorphisms (SNPs) have a more preponderant role from a population perspective due to their higher frequency and stronger association with common diseases [53]. As expected, multiple haemostasis-related SNPs have been associated with CAT development and tumour progression (regardless of thrombosis) (**see Chapter 1D**). Beyond these genetic factors, the epigenetic landscape has also been explored with the emergence of two promising CAT biomarkers: long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). The former can modulate gene expression at both the transcriptional and post-transcriptional levels, acting as key regulators in venous thrombogenesis by influencing vEC activity and modulating the levels and/or activity of haemostatic proteins [55,56]. Despite their potential, this area of research is still in its early stages, with limited data available specifically for cancer patients (**see Chapter 1E**). MiRNAs, in turn, regulate gene expression by targeting mRNAs for

degradation or translational repression [57]. In recent years, several miRNAs have been linked to VTE in the general population and among cancer patients, with some targeting the TF coagulation pathway (**see Chapter 1F**).

Given the interplay between cancer and VTE, where haemostatic components drive both tumour progression and dissemination, biomarkers related to haemostasis and VTE may provide valuable insights into the dynamic changes occurring within the OC microenvironment. Thus, these biomarkers hold significant clinical potential, not only for stratifying CAT risk but also for prognostication, monitoring treatment response, and early detection of OC recurrence [45]. By facilitating a more personalised approach to OC management, these biomarkers can enhance the precision of patient care. Furthermore, they may also lead to the identification of novel therapeutic targets for OC, opening new avenues for intervention and improving outcomes. Inclusively, low-molecular-weight heparin (LMWH), widely used in anticoagulation therapy, is believed to possess antineoplastic properties. However, robust evidence supporting its role in enhancing survival rates among cancer patients is still lacking. To establish a definitive link, more studies are necessary [58].

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CHAPTER IB – Ovarian Cancer Management

This chapter includes the following paper:

Valéria Tavares, Inês Soares Marques, Inês Guerra de Melo, Joana Assis, Deolinda Pereira, Rui Medeiros. **Paradigm Shift: A Comprehensive Review of Ovarian Cancer Management in an Era of Advancements**. *International Journal of Molecular Sciences*. **2024**; 25 (3): 1845. <https://doi.org/10.3390/ijms25031845>. (Impact factor and quartile reported in 2023: **4.9** and **Q1**).

Paradigm Shift: A Comprehensive Review of Ovarian Cancer Management in an Era of Advancements

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Abstract: Ovarian cancer (OC) is the female genital malignancy with the highest lethality. Patients present a poor prognosis mainly due to the late clinical presentation allied with the common acquisition of chemoresistance and a high rate of tumour recurrence. Effective screening, accurate diagnosis, and personalised multidisciplinary treatments are crucial for improving patients' survival and quality of life. This comprehensive narrative review aims to describe the current knowledge on the aetiology, prevention, diagnosis, and treatment of OC, highlighting the latest significant advancements and future directions. Traditionally, OC treatment involves the combination of cytoreductive surgery and platinum-based chemotherapy. Although more therapeutical approaches have been developed, the lack of established predictive biomarkers to guide disease management has led to only marginal improvements in progression-free survival (PFS) while patients face an increasing level of toxicity. Fortunately, because of a better overall understanding of ovarian tumourigenesis and advancements in the disease's (epi)genetic and molecular profiling, a paradigm shift has emerged with the identification of new disease biomarkers and the proposal of targeted therapeutic approaches to postpone disease recurrence and decrease side effects, while increasing patients' survival. Despite this progress, several challenges in disease management, including disease heterogeneity and drug resistance, still need to be overcome.

Keywords: ovarian neoplasms; biomarkers; early detection of cancer; diagnosis; antineoplastic protocols.

1. Introduction

In 2020, ovarian cancer (OC) was the eighth most diagnosed malignancy worldwide, affecting approximately 314,000 women and also ranking as the eighth most deadly cancer, with over 207,000 attributed deaths [1,2]. Like other tumours, the incidence and mortality of OC vary worldwide. While the disease is more common in European countries with high Human Development Index (HDI) levels, the lowest incidence rates are observed in African countries with low HDI. In opposition, the mortality rates tend to have a reversed inclination [2,3].

Worldwide, OC has consistently been regarded as the most lethal gynaecological tumour. Despite improvements in disease management, particularly in surgical techniques and maintenance therapy (treatment after the first-line therapeutic approach to delay disease recurrence), OC patients still have a 5-year survival rate lower than 50% in most

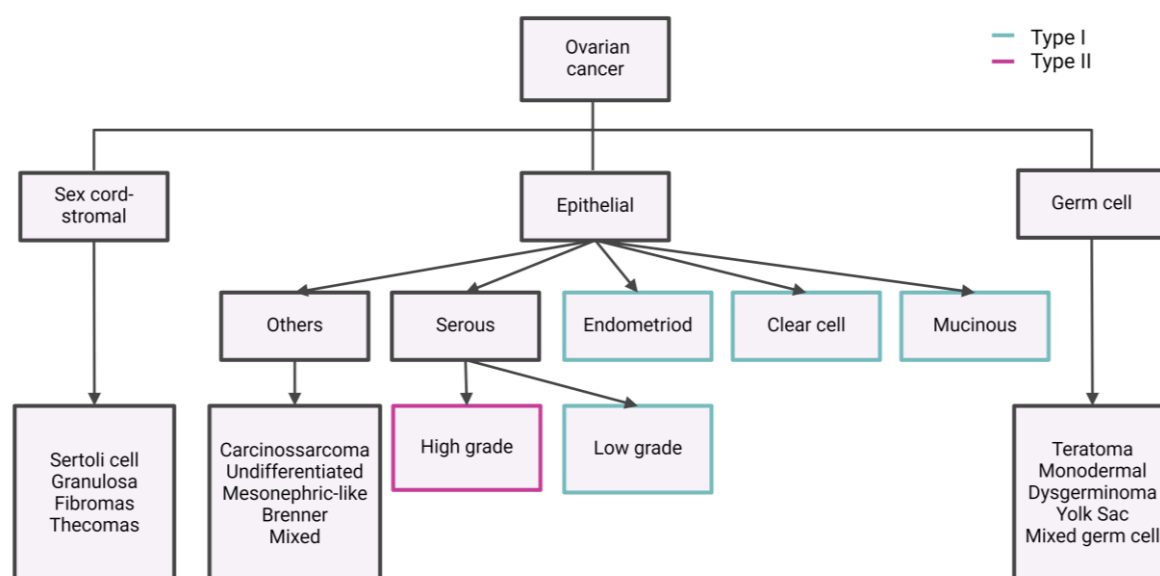
countries [4]. This is primarily driven by late disease diagnosis, owing to its non-specific symptoms and the lack of appropriate screening methods, combined with the frequent acquisition of chemoresistance leading to disease recurrence [5,6].

Although ovarian tumourigenesis is poorly comprehended, the disease is thought to arise from the ovarian surface epithelium. Also, it is closely related to tumours originating from the peritoneum and the fallopian tube, according to the serous tubal intraepithelial carcinoma (STIC) theory [7]. Indeed, the three primary tumours are typically deemed as a single tumour entity classified as “ovarian or tubal cancers” [8,9]. Ovarian tumours constitute a heterogeneous group of malignant diseases with distinct aetiology, origin, pathogenesis, differentiation, patterns of spread, and molecular profiles [10]. According to the 2020 World Health Organization (WHO) classification, OC includes epithelial (EOC; 90%), germ cell (5%), and sex cord–stromal tumours (2–5%). EOCs (i.e., ovarian carcinomas) are the most common OC type, encompassing five main subtypes that are distinguished based on molecular analysis, histologic and immune profile: high-grade serous (HGSC; 70%), endometrioid (EC; 10%), clear cell (CCC; 10%), low-grade serous (LGSC; 5%) and mucinous (MC; 3%) carcinomas (**Figure 1**) [11,12]. According to the dualistic carcinogenesis model, these subtypes can be further subdivided into type I and type II according to specific histological and molecular features [13–15]. Type I tumours (~25% of EOCs) typically exhibit slow growth and tend to be diagnosed at earlier stages (stages I/II). Furthermore, these tumours appear to be associated with endometriosis and usually present a genetic stability phenotype with a pattern of mutations in *BRAF*, *KRAS*, *PTEN*, *CTNNB1*, *ARID1A*, *PIK3CA*, and *PPP2R1A*. Type II tumours (75% of EOCs), on the other hand, generally have rapid growth, with the disease being diagnosed at advanced stages (stages III/IV). These tumours also display a high degree of genetic instability, frequently exhibiting *BRCA* and *TP53* mutations. Despite the recognised clinical value of this classification system, it does not always reflect tumour aggressiveness, as even type I tumours can be very aggressive [14–18]. Not surprisingly, this heterogeneity impacts treatment response and clinical outcomes [10].

A paradigm shift has been observed in OC research with the evolution to a better disease understanding, aiming for effective screening, early diagnosis, and personalised treatment strategies. This shift was catalysed by innovations in genomics, including the widespread use of microarrays and next-generation sequencing (NGS), which have enabled cost-effective germline and tumour genomic profiling [19]. Notably, the available technology has led to the identification of more disease subtypes related to the molecular and genetic makeup of ovarian tumours (**see Section 3**) [20]. Furthermore, progress in molecular pathology, particularly integrating artificial intelligence and machine learning

technologies, is shown to be determined [21]. Not dismissing technical and ethical challenges, existing data advocate that artificial intelligence models may aid in early and accurate OC diagnosis while providing important prognostic information to guide disease treatment [22].

Given the recent advancements in OC management, an in-depth overview of the current knowledge is critical for researchers and healthcare professionals to stay updated with the latest developments. Furthermore, it could help pinpoint research gaps and guide future investigations. Therefore, this comprehensive narrative review article aims to discuss the current body of evidence on the aetiology, prevention, diagnosis, and treatment of OC, highlighting recent progress in disease management and future directions for OC research. To perform this, a search in the PubMed database was conducted using combinations of the terms “ovarian cancer”, “ovarian tumour”, “ovarian carcinoma”, “advances”, “updates”, “overview”, “screening”, “prevention”, “diagnosis”, “prognosis”, “therapy” and “treatment” that appeared anywhere in the article. The retrieved papers were published between 2013 and 2023. Additional relevant publications were identified in the references list of the retrieved papers.



Chapter IB - Figure 1. Subtypes of ovarian cancer. Figure created with BioRender.com (accessed on 28 December 2023). Ovarian cancer is a heterogeneous disease, encompassing numerous malignant subtypes with distinct aetiology, origin, pathogenesis, differentiation, spread patterns, and molecular profiles. The most common subtype is epithelial ovarian cancer (~90%), which can be further subclassified into type I and type II according to specific histological and molecular features [10–12,14,15].

2. Disease Aetiology and Prevention

Various aetiological determinants are thought to impact ovarian tumourigenesis showing heterogeneity depending on tumour histology [3,23,24]. The most impactful ones are advanced age, genetic predisposition, and a family history of cancer. These factors are particularly related to continuous ovulation, hormonal changes, cumulative genetic damage, and chronic inflammation [3,25–27]. Ovarian tumours are rare among young women, particularly those under the age of 30. After the age of 50, especially following menopause, OC risk drastically increases, with the average diagnosis occurring between 50 and 70 years [12]. Concerning the genetic component, OC is one of the most heritable tumours, mainly linked to germline genetic mutations associated with the hereditary breast and OC syndrome (predominantly mutations in *BRCA1* and *BRCA2*) and hereditary nonpolyposis colorectal cancer syndrome (mutations in *MLH1*, *MSH2*, *MSH6*, and *PMS2*) [25,28]. Thus, a family history of breast, ovarian, and colorectal tumours, particularly at young ages, could be indicative of a high risk of OC onset [29,30]. For instance, while the risk of developing OC in the general population is <2%, women with *BRCA1* and *BRCA2* mutations have an overall lifetime risk of 20–40% and 10–20%, respectively [31].

Despite inconsistent data, reproductive factors such as early menarche, late menopause onset, long-term hormone replacement therapy, and nulliparity also constitute risk factors [32–36]. In opposition, pregnancy, breastfeeding, and the use of oral contraceptives are considered to be protective factors [37–39]. The impact of these determinants on a predisposition for OC is commonly attributed to the cumulative number of ovulatory cycles, as fewer cycles are associated with a lower OC risk [12,40,41]. Also, oestrogen exposure could be a contributing factor [42,43]. Other important risk determinants include lifestyle-related factors (e.g., diet, tobacco use, high body mass index, and obesity), a history of gynaecological conditions (e.g., endometriosis, ovarian cysts, and pelvic inflammatory disease), a personal history of endometrial, breast or colorectal cancers and ethnicity [44–47].

Identifying predisposing factors for OC development is important for tailoring prevention measures. However, there is no effective method for OC's primary prevention. Nonetheless, tubal sterilisation and salpingo-oophorectomy for women at high risk, particularly those with hereditary syndromes, are possible prophylactic options. As such, according to the National Comprehensive Cancer Network (NCCN) guidelines (version 2.2021, 2021), genetic testing should be offered to women with a family history of the disease [48–50]. Furthermore, although conflicting, some studies have found that low-dose aspirin and other anti-inflammatory medications may decrease the risk of OC [40,51–53].

The secondary prevention of OC, which refers to disease screening, has also been challenging [54,55]. Ideally, an adequate screening exam should be easy to conduct, steadily reliable, inexpensive, and induce minimal discomfort. Importantly, it must have high sensitivity and specificity. For instance, an adequate test to screen for OC should have a sensitivity and specificity superior to 75% and 99.6%, respectively, to reach a positive predictive value (PPV) of 10% [56]. Also, a suitable exam should target the subpopulation with the highest prevalence of this condition of interest to establish an adequate PPV. Lastly, it should improve the morbimortality rates in the target population [54,57]. Several potential methods for OC screening have been reviewed, including serum CA-125 measurement, a transvaginal ultrasound, colour Doppler ultrasonography, and pelvic examination. However, none of them have shown adequate performance in trials involving the general population and high-risk groups [57–60]. For instance, CA-125 (also known as mucin 16 or MUC16), which is widely used in the clinical setting for OC monitoring, exhibits limited sensitivity in early disease stages. Also, its levels can be elevated in benign conditions such as ovarian cysts and endometriosis [24,61–63]. More recently, novel molecular markers have been proposed, including HE4, CA 72-4, CA 19-9, folate receptor alpha (FR α), microRNA profiles, DNA methylation patterns, circulating tumour DNA and antibodies in liquid biopsies, particularly blood and cervical mucus and swabs [62,64–68]. The use of liquid biopsies in disease screening is attractive since they can capture the disease's heterogeneity through minimally invasive sample collection and at a low cost. However, the tumour material in these biopsies is usually scarce and does not provide information about the tumour's architecture or its primary site [68]. According to existing data, a multimodal approach combining several tests might be the most effective tool to screen OC accurately [69]. In this context, several multivariate index assays have been proposed to help detect early-stage OC, including the risk of malignancy index (RMI), OVA1, and risk of ovarian malignancy algorithm (ROMA) [70–73]. Another advancement in this field is the development of new imaging techniques, namely auto-fluorescence and magnetic relaxometry, which could help detect the disease at earlier stages, enabling timely therapeutic intervention and better outcomes [67]. Despite these improvements, screening for asymptomatic and average-risk women is still controversial, given the low prevalence of this disease and the high probability of false-positive findings, which may lead to excessive interventions [74,75]. Consequently, 60–70% of OC patients are diagnosed at advanced stages upon symptom presentation, which, as formerly mentioned, significantly impacts their prognosis [5,12,76]. Of note, the list of possible symptoms encompasses vaginal bleeding, diarrhoea, constipation, abdominal distension allied to pain, eating difficulties, urinary frequency, fatigue, nausea, anorexia, dyspepsia, and early satiety [24]. The time of

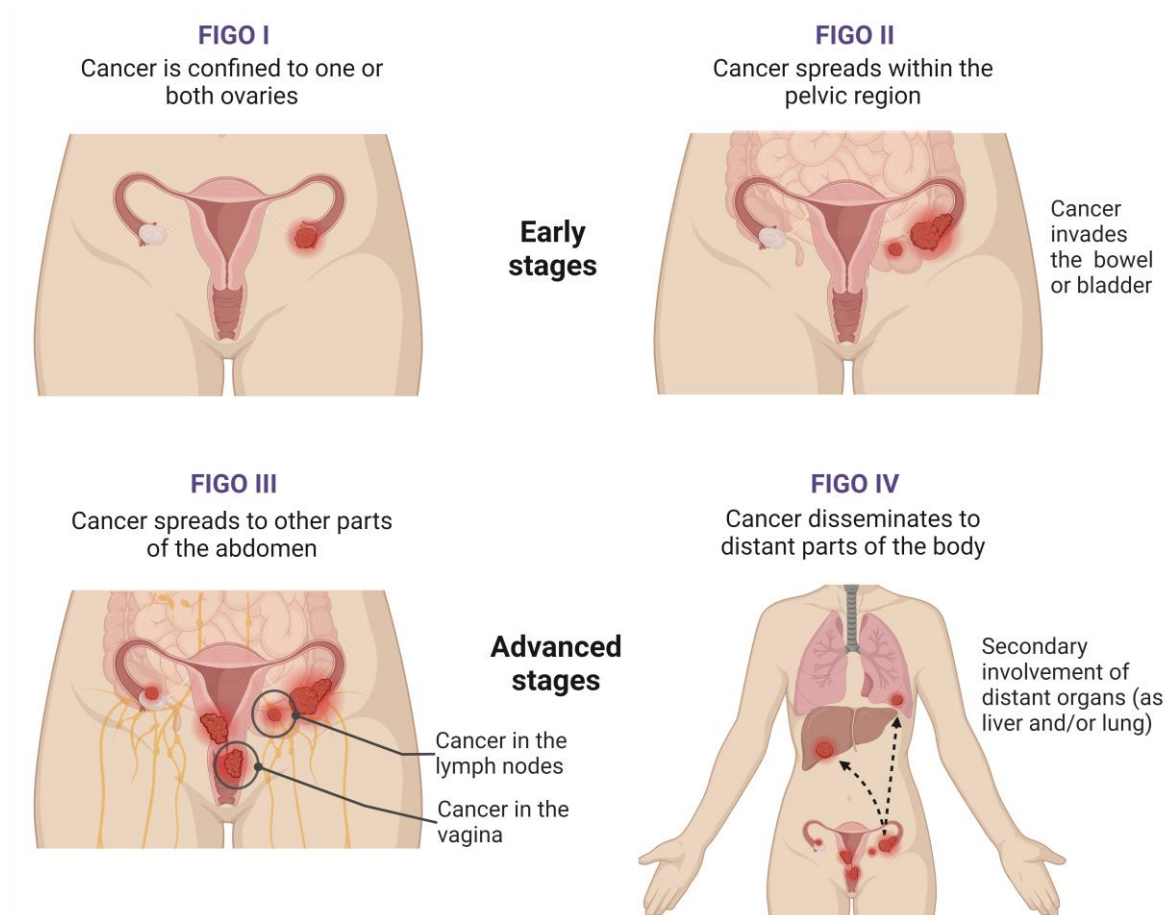
presentation of these symptoms may vary depending on the histological nature of the disease [77].

Given their implications, education on the risk factors underlying OC onset is crucial to increase patients' health awareness and self-advocacy.

3. Disease Diagnosis and Prognosis Assessment

Current strategies to diagnose OC include a medical history evaluation combined with the gynaecological exam, serum CA-125 quantification, and imaging tests (transvaginal ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), and/or positron emission tomography (PET)), while also demanding a histopathological examination from either a diagnostic biopsy or, if possible, a surgical specimen for a definitive diagnosis and staging [78–80]. For MC, the evaluation of the tumour markers CEA and CA 19-9 is also recommended according to the European Society for Medical Oncology (ESMO) 2023 guidelines for OC management [24].

At diagnosis, the International Federation of Gynecology and Obstetrics (FIGO) staging system is one of the most important tools to predict the clinical outcomes of OC patients and evaluate their therapeutical options [81]. This system, first published in 1973 and last revised in 2021, includes four stages, each with subdivisions (**Figure 2**) [75,81,82]. Ovarian carcinomas can also be subclassified based on histologic grading, with two systems being applied [60]. For non-serous tumours, according to cell architecture, the disease can be deemed as GX (grade not determined), G1 (well differentiated), G2 (moderately differentiated), and G3 (poorly differentiated). On the other hand, serous carcinomas can be categorised as low or high-grade based on their distinct cellular characteristics and behaviours [60,82].



Chapter IB - Figure 2. Stages of ovarian cancer. Figure created with BioRender.com (accessed on 28 December 2023). The FIGO staging system for ovarian cancer (OC) includes four stages that address the disease's extent and severity by evaluating the tumour burden, dissemination within the abdomen, and the secondary involvement of distant organs. 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 or primary peritoneal cancer. In stage III, OC cells spread to the peritoneum outside the pelvis, and there might be metastasis to the retroperitoneal lymph nodes. Lastly, stage IV is characterised by OC's dissemination to other body parts beyond the pelvis and abdomen, namely the liver and lungs [82]. Abbreviations: FIGO, International Federation of Gynecology and Obstetrics.

Regarding the prognosis assessment, the FIGO stage, histologic subtype, grade, baseline serum CA-125 levels, the extent of debulking surgery, and chemotherapy schemes are traditionally deemed the most relevant independent prognostic factors of OC. For instance, those with early disease stages, type I tumours and lower baseline CA-125 levels usually have higher survival [12,83–88]. However, ongoing research has recently identified several molecular biomarkers associated with OC treatment response and prognosis, including mutations, gene expression patterns, and/or epigenetic changes [89–91]. This is

particularly relevant given the high heterogeneity that characterises HGSC, with the predominant and most lethal OC subtype accounting for 70% of OC-related deaths [92]. Notably, Tothill et al. (2008) [93] were the first to propose HGSC subtypes based on the following genomic signatures: C1 (high stromal response), C2 (high immune signature), C4 (low stromal response) and C5 (mesenchymal). Next, Kurman and Shih (2010) [13] proposed the classic dualist model—type I vs. type II. Later, in 2011, data on histological structure and gene expression profile from the Cancer Genome Atlas (TCGA) Research Network led to the recognition of four HGSC subtypes: mesenchymal (with a gene expression profile that resembles mesenchymal tissues with increased cell motility and invasiveness), proliferative (displaying a molecular pattern indicative of high cell proliferation and limited inflammatory infiltration), differentiated (with a gene expression profile related to more specialised cell types) and immunoreactive (tumours with high infiltration of immune cells and with a gene expression profile characteristic of immune activation) [15,94]. Although not mutually exclusive, these subgroups correlate with prognosis. According to the “Classification of Ovarian Cancer” (CLOVAR) signature, the mesenchymal subtype is the most lethal with a related five-year OS of 18%, followed by the proliferative, differentiated, and, finally, the immunoreactive subtype, which is associated with a survival rate of 45% [95]. Importantly, these signatures also influence therapy response [96]. Since the proposal of these models, the integrative analysis of tumour (epi)genetic and molecular signatures has more or less confirmed the existence of these four HGSC subtypes with an impact on prognosis and/or treatment response (**Table 1**). This is anticipated to change OC management by facilitating personalised treatment [91].

Chapter IB - Table 1. (Epi)genetic and molecular signatures of high-grade serous ovarian carcinoma (HGSC) with implications for therapy response and patients' clinical outcomes.

Authors (Year)	Number of Cases	Methods	HGSC Clusters and/or Main Features	Main Findings	Reference
Macintyre et al. (2018)	132 patients 112 patients (Pan-Cancer Analysis of Whole Genomes) 415 patients (TCGA)	Genome sequencing	Signatures of copy number variations: Signature 1—telomere shortening and RAS/MAPK activation; Signature 2—tandem duplication; Signature 3—BRCA1/2-related HRR deficiency; Signature 4—whole genome duplication; Signature 5—subclonal catastrophic chromothriptic-like events; Signature 6—focal amplification; Signature 7—non-BRCA1/2-related HRR deficiency.	Signature 1—platinum-resistant recurrence and poor survival; Signature 2—poor survival; Signatures 3 and 7—prolonged survival; Signatures 4, 5 and 6—unclear implications.	[97]
Harris et al. (2019)	Not reported	Tumour xenografting DNA and RNA NGS DNA fingerprinting Immunohistochemistry	DNA alternations in genes involved in the ERBB2 pathway.	Deregulation in the ERBB2 pathway—favourable results by combining platinum-based chemotherapy with anti-HER2 drugs.	[98]
Li et al. (2019)	Seven patients	Tumour xenografting RNA and whole exome sequencing Immunohistochemistry	Deregulation of <i>AKT3</i> , <i>HLA-DPA1</i> , <i>PIK3R5</i> and <i>SAP25</i> expression; <i>POLR2A</i> and <i>TMEM205</i> mutations.	Features associated with the acquisition of chemoresistance to carboplatin and paclitaxel.	[99]
McDonald et al. (2019)	450 patients with chemoresistance (TCGA)	Genome-wide cluster analysis Pathway enrichment analysis	Cluster 1—growth factor signalling; Cluster 2—cell survival; Cluster 3—cellular senescence.	Best therapeutic options: Cluster 1—tyrosine kinases or angiokine inhibitors; Cluster 2—mTOR inhibitors; Cluster 3—deacetylase inhibitors.	[100]
Hao et al. (2021)	Two patients (four matched pair samples of primary and metastatic tumours)	Single-cell RNA sequencing	Cluster EC1—glycolysis/gluconeogenesis; Cluster EC2—cytokine–cytokine receptor interaction; Cluster EC3—nucleotide and amino acid metabolism; Cluster EC4—immune response Cluster EC5—DNA repair and drug metabolism	Cluster EC5 may be most aggressive and resistant to chemotherapy and PARP inhibitors.	[101]
Li et al. (2021)	66 tumour cells 568 tumour samples and 7 normal ovary samples (TCGA)	Single-cell RNA sequencing	Differently expressed genes	Low expression of <i>ANP32E</i> , <i>EGFL6</i> , <i>GPRC5A</i> , <i>PMP22</i> and <i>STAT1</i> —prolonged survival; Low expression of <i>ANP32E</i> , <i>CYB5R3</i> and <i>FBXO21</i> —prolonged PFS	[102]

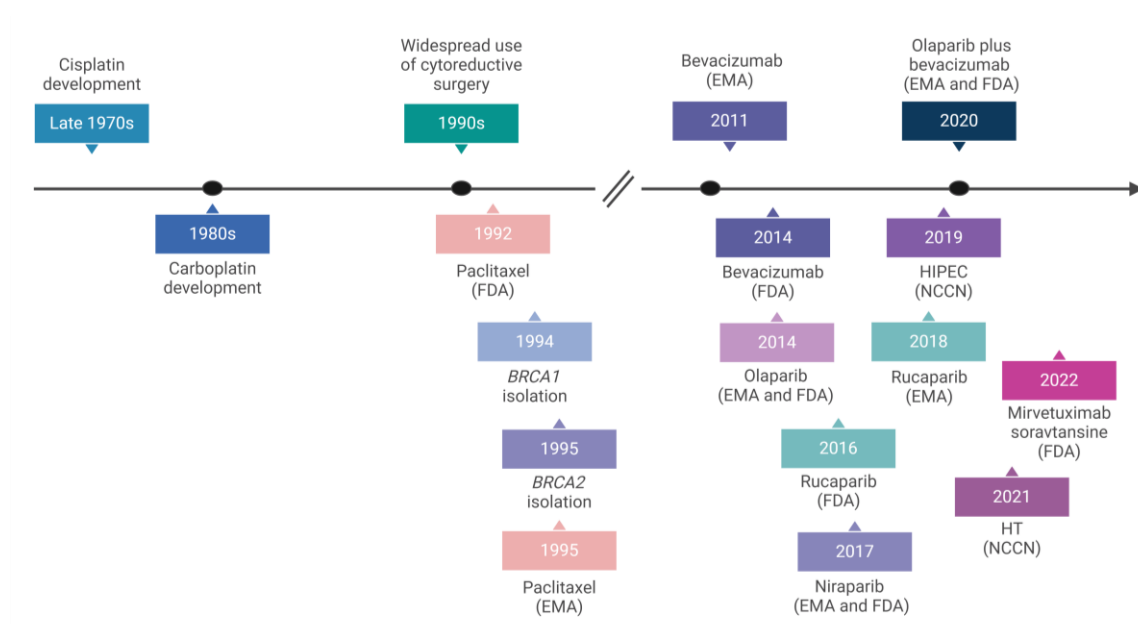
Abbreviations: HRR, homologous recombination repair; NGS, next-generation sequencing; PFS, progression-free survival; TCGA, The Cancer Genome Atlas.

4. Current Treatments and Innovations

The therapeutic management of OC mainly relies on the disease stage, with tumour histology, molecular profile, and the patient's medical background also being relevant determinants. Traditionally, the front-line approach involves cytoreductive surgery followed by intravenous chemotherapy with platinum-containing drugs (cisplatin or carboplatin) typically combined with taxane agents (paclitaxel and docetaxel) every 21 days for six cycles [79,103–106]. According to ESMO 2023 guidelines, for patients at stage I and with low-grade tumours, chemotherapy can be omitted [24]. As for those with advanced disease, the complete resection of macroscopic disease (i.e., complete debulking) is often not conceivable. As such, these patients might first be treated with neoadjuvant (induction) chemotherapy, and if there is a treatment response, an interval debulking resection can be conducted, followed by adjuvant chemotherapy [75,107]. Radiotherapy is also a possible therapeutic approach; however, due to its high toxicity and low effectiveness compared to platinum-based chemotherapy, its use is often limited to palliative care [108–110].

Although the majority of OC patients (~80%) have a complete response after front-line treatment, over 60% of the patients with <1 cm of residual disease (optimal debulking) and about 80% of those with >1 cm of residual disease (suboptimal debulking) progress to around 18 months, often due to chemoresistance [12,105,111–114]. At a phase of disease recurrence, OC treatment commonly consists of second-line chemotherapy, which depends on platinum sensitivity [114]. Based on the period between the completion of first-line platinum-based chemotherapy and disease recurrence (i.e., platinum-free interval (PFI)), OC can be classified as platinum-refractory (when it occurs during the first-line chemotherapy), resistant (within 6 months after treatment completion), partially sensitive (between 6 and 12 months) or highly sensitive (beyond 12 months) [104,114,115]. According to ESMO 2023 guidelines for recurrent OC management, patients with sensitive disease can benefit from second-line chemotherapy with a combination of platinum compounds with paclitaxel, gemcitabine or pegylated liposomal doxorubicin (PLD), followed by treatment with bevacizumab (**see Section 4.1**) or poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) (**see Section 4.2**). In the event of platinum-hypersensitivity reaction/intolerance, PLD might be combined with trabectedin [24]. As for those refractory or resistant to platinum, the best therapeutical option is monotherapy with paclitaxel, gemcitabine, PLD, or topotecan, although the overall response rate with these agents is relatively small (8 to 20%) [24,114,116]. In this setting, bevacizumab can also be added if not contraindicated [24]. It is worth mentioning that most OC patients with recurrent disease eventually develop platinum resistance [117].

The disease heterogeneity complicates OC treatment. The acquisition of chemoresistance can arise due to tumour microenvironmental, cancer cell-specific, and pharmacokinetic aberrations [116]. Additionally, chemotherapy is associated with adverse events, including but not limited to alopecia, neuropathy, neutropenia, palmar-plantar erythrodysesthesia, ototoxicity, and bone marrow depression, all of which negatively impact the patient's quality of life [118–121]. Consequently, over the past few decades, a framework change has been observed, transitioning from an era of first-line treatment mainly centred around cytoreductive surgery followed by platinum and taxane-based chemotherapy to a new phase with improved upfront interventions, such as hyperthermic intraperitoneal chemotherapy (HIPEC), to delay the disease's recurrence, reduce adverse effects and prolong patients' survival. This evolution also encompasses broadening the treatment options to include more targeted approaches, namely the use of antiangiogenic agents, DNA damage repair-based therapeutics, hormone receptor modulators, and FR α -targeting drugs (**Figure 3**). These novel therapeutical agents target signalling pathways that are central to the progression of OC and/or its mechanism of drug resistance [87].



Chapter IB - Figure 3. Pivotal gene discoveries and the approval of therapeutic agents and approaches to treat ovarian cancer. Figure created with BioRender.com (accessed on 29 January 2024). Several therapeutical agents and approaches for ovarian cancer management have emerged in recent decades due to a better understanding of the disease's pathogenesis [122,123]. Abbreviations: EMA, European Medicine Agency; FDA, Food and Drug Administration; HIPEC, hyperthermic intraperitoneal chemotherapy; HT, hormonal therapy; NCCN, National Comprehensive Cancer Network.

4.1. Antiangiogenic Agents

Tumours release proangiogenic factors, including VEGFA, which can activate the proliferation of vascular endothelial cells, fuelling tumour neoangiogenesis [124]. VEGFA and angiogenesis are crucial promoters of ovarian tumourigenesis. Both correlate directly with the disease's extent and inversely with progression-free survival (PFS) and overall survival (OS), usually regardless of other prognostic determinants [125,126].

In 2011, after the results of the GOG-0218 (NCT00262847) and ICON7 (NCT00483782) trials, bevacizumab, a recombinant humanised anti-VEGFA monoclonal antibody, was approved by the European Medicine Agency (EMA) for the first-line and maintenance treatment of advanced-stage OC in combination with platinum-taxane-based chemotherapy [125,126]. Subsequently, in 2014, the US Food and Drug Administration (FDA) granted approval for this drug to be used in the second-line therapy of platinum-resistant-recurrent OC [127]. By neutralising all active forms of VEGFA, bevacizumab suppresses angiogenesis, inhibiting tumour growth and metastatic dissemination [128]. Additionally, it is thought to enhance the delivery of chemotherapeutic agents to their designated targets by normalising the tumour's vasculature, decreasing the interstitial fluid pressure, and increasing the tumour's oxygenation [129]. This agent was the first biological drug to show a promising therapeutic response in the frontline intervention (first-line therapy) and recurrent OC (second-line therapy) [125,130]. However, its effect on PFS is limited and does not prolong OS [131,132]. Also, bevacizumab is associated with considerable toxicity, with a list of adverse events including hypertension, thrombotic events, gastrointestinal perforation, and renal and central nervous system disorders [125,133,134].

There is no unanimous agreement on the prescription of bevacizumab, given the lack of validated predictive biomarkers of response [76]. Nevertheless, those with molecular subtypes associated with poor survival, namely proliferative and mesenchymal tumours, are known to benefit most from bevacizumab-based treatment [135]. More recently, its use in combination with PARPi has proven to be beneficial, receiving approval from both the EMA and the FDA in 2020 [123,136]. Furthermore, in addition to bevacizumab, small-molecule kinase inhibitors targeting VEGFA receptors (VEGFRs) are currently under investigation (see Section 5.4).

4.2. DNA Damage Repair-Based Therapeutics

Since 2014, the landscape of OC management has been revolutionised with the approval of PARPi by the EMA and FDA for disease treatment in different settings [137,138]. These therapeutic agents inhibit the activity of PARPs, which are proteins crucial for DNA

damage repair. In malignancy, PARPs facilitate the repair of DNA damage, particularly single-strand breaks, which are induced by antineoplastic treatments [12,137]. Tumour cells with a deficient homologous recombination repair (HRR) pathway, mainly due to mutations in *BRCA1/2*, are unable to repair DNA double-strand breaks. In these cells, PARPi have a negative effect, rendering the repair of DNA damage unfeasible. Consequently, these therapeutic agents promote the apoptosis of tumour cells through a process known as synthetic lethality [12,137,139,140]. As anticipated, PARPi are particularly relevant for HGSC, given the high rate of HRR deficiencies [141].

For OC management, PARPi were initially proposed for patients with recurrent platinum-sensitive disease after the outstanding improvement in PFS observed in three randomised phase III trials—SOLO-2/ENGOT-OV21 (NCT01874353), NOVA/ENGOT-OV16 (NCT01847274) and ARIEL3 (NCT01968213) [137,142–144]. The results of these trials led to the approval of olaparib (2014), niraparib (2017), and rucaparib (2016–2018), respectively [137]. Early clinical data supported the effectiveness of these agents among those with germline or somatic *BRCA1/2* mutations. However, in the maintenance setting for those with platinum sensitivity, more recently, these drugs have shown clinical benefits even among those without these mutations [141]. Indeed, other genes implicated in the HRR pathway are known to be mutated in OC. The list includes *BARD1*, *BRIP1*, *RAD50*, *RAD51* paralogs (*RAD51C* and *RAD51D*), *MRE11* and *PALB2*. Curiously, OC patients present germline mutations in HRR-related genes more often than somatic tumour mutations (<10% of cases) [145]. In addition to recurrent disease, PARPi have been suggested to be beneficial in first-line therapy, which could affect subsequent treatment choices [137].

Despite these clinical benefits, the therapeutical impact of olaparib, niraparib, and rucaparib is constrained, translating into only a short-term survival extension as most patients inevitably develop drug resistance [146,147]. Therefore, other PARPi have emerged, including veliparib, pamiparib, fuzuloparib (formerly known as fluzoparib), and talazoparib. Veliparib is still under investigation, pamiparib and fuzuloparib were recently approved for OC treatment in China, and talazoparib was approved by the FDA in 2018 to manage HER2-negative-advanced breast cancer [148,149]. Moreover, the panorama of DNA damage repair-based therapies for OC management has evolved beyond PARPi with the development of pharmaceutical agents targeting the cell cycle checkpoint protein kinases ATR (ceralasertib), CHK1 (prexasertib) and WEE1 (adavosertib) [150]. These agents are still being investigated in clinical trials and promise to overcome PARPi-resistant ovarian tumours [150,151].

4.3. Hyperthermic Intraperitoneal Chemotherapy (HIPEC)

HIPEC involves the intraperitoneal delivery of chemotherapeutic agents after cytoreductive surgery and under hyperthermic conditions to improve patients' outcomes by more effectively removing residual disease. This is partially due to hyperthermia, which increases the penetration of chemotherapeutic drugs at the peritoneal surface while enhancing the sensitivity of the tumour to treatment. These two factors, however, notably depend on the selected drug and the achieved temperature [152,153].

While HIPEC has been adopted in the management of malignant diseases such as colorectal, gastric, and primary peritoneal carcinomatosis, for OC, its implementation has been a subject of intense debate [154]. Only in 2019 was it integrated as an optional form of treatment for the interval debulking of OC patients in the NCCN guidelines (version 1.2019, 2019) [155]. In part, this delay was due to questions on optimal patient selection, the protocol for drug delivery (open versus closed), the timing of the treatment, the choice of drug regimen, and, importantly, the risk of complications [156]. Currently, according to the NCCN guidelines, HIPEC is recommended for OC patients with peritoneal carcinomatosis (FIGO stage III) and with response or stable disease after undergoing neoadjuvant chemotherapy [155]. For these patients, the treatment has been associated with a trend towards improved PFS and OS [157]. However, for various reasons, despite the demonstrated benefits, the acceptance and implementation of HIPEC by gynaecologic oncology and surgeons have been challenging [24,158,159].

4.4. Hormone Receptor Modulators

Oestrogen is known to drive the proliferation of OC cells [160]. Oestrogen signalling is mediated by oestrogen receptor(ER)-alpha (ER α) and ER-beta (ER β), each with different isoforms, which are further amplified by G protein-coupled oestrogen receptor 1 (GPER1) [42]. *In vitro* and *in vivo* studies show that oestrogen via ER α regulates OC growth and promotes cell migration and epithelial–mesenchymal transition (EMT), influencing cell motility and survival [161–164]. These modifications proceed through the downregulation of E-cadherin: a process that ER β inhibits [165]. Indeed, ER β , the most common ER form in normal ovary tissue, is thought to be an OC suppressor [166–169]. As for GPER1, both suppressive and promotor roles have been proposed among OC patients, indicating a likely complex function [170–174]. Contrary to MC (21%) and CCC (20%), over 80% of serous EOCs (HGSC and LGSC) and EC express ER α and have demonstrated response to hormonal therapy with aromatase inhibitors (for instance, letrozole) and tamoxifen in multiple clinical studies [42,50,76,175,176]. While aromatase inhibitors block oestrogen synthesis, tamoxifen directly competes with oestrogen in order to bind to ER [177].

Progesterone, gonadotropins, androgens, and the gonadotropin-releasing hormone (GnRH) also play a role in the endocrine regulation of the ovary mediated by the hypothalamic–pituitary–ovary axis. While GnRH and progesterone seem protective against OC, gonadotropins and androgens favour its progression [111,178].

The restricted therapeutic options for the management of recurrent and platinum-resistant OC and the favourable safety profile combined with its convenient and inexpensive use make hormonal therapy an attractive option [117]. According to the ESMO-European Society of Gynaecological Oncology guidelines (ESMO-ESGO) of 2019 and the NCCN guidelines (version 2.2021) of 2021, hormonal therapy is recommended as an alternative approach to treat those with recurrent and platinum-resistant OC [50,76]. However, the clinical benefit of hormonal therapy in OC management has not been systematically evaluated in large trials (arzoxifene, an ER modulator, in NCT00003670; fulvestrant, an ER degrader, in NCT00617188, tamoxifen in NCT02728622 and NCT00041080; and mifepristone, a progesterone receptor modulator, in NCT00459290 and NCT02046421). Currently, efforts are being made to identify biomarkers that can stratify responsive OC subgroups [42].

4.5. FR α -Targeting Drugs

The folate metabolism is essential in DNA synthesis, methylation, and repair [179]. The transmembrane glycoprotein FR α transports folic acid (folate) and its derivatives into cells via endocytosis [180]. In normal tissues, its expression is restricted to the intestine, kidney, retina, lung, choroid plexus, and placenta [181]. Except for the kidney (which does not retain folate), FR α in normal tissues is only presented in polarised epithelial cells, which are inaccessible to circulating pharmaceutical agents [181,182]. On the other hand, its elevated expression is demonstrated in most carcinomas, including endometrial, breast, lung, and ovarian tumours. This selective expression and its ability to be internalised after ligand-binding makes FR α an attractive target for cancer drug delivery [179].

Most ovarian carcinomas overexpress FR α , while this receptor is absent in normal ovarian epithelium [182,183]. The synthesis of FR α is particularly common in advanced and high-grade serous EOC, which is sustained even in recurrent diseases and within metastatic niches [184]. Importantly, this receptor is reported to shed from the cell membrane into circulation [66]. In EOC patients, circulating receptor (sFR α) levels correlate with tumour FR α expression, disease burden, and treatment outcomes [66,185]. Thus, sFR α might be an attractive biomarker of early EOC. Inclusively, this marker has exhibited higher accuracy than serum CA-125 levels [66].

In the treatment setting, FR α -targeting drugs have emerged as potential therapeutic agents for OC. Antibody-drug conjugates (ADCs) are a group of agents designed to

selectively deliver chemotherapeutic agents to the site of tumours by targeting cancer-specific antigens [186]. Mirvetuximab soravtansine, one of the most extensively studied FR α -targeting ADCs, is composed of an anti-FR α antibody coupled to a potent tubulin-targeting agent named DM4. Mechanistically, the drug binds to FR α in EOC, delivering DM4 directly to the tumour cells, providing a positive balance between efficacy and toxicity. Currently, mirvetuximab soravtansine is being tested for EOC management in platinum resistance [186,187]. Based on the positive findings of the phase III trial SORAYA (NCT04296890), this drug received accelerated approval in 2022 by the FDA for the treatment of patients with FR α -positive and platinum-resistant EOC previously treated with systemic anticancer regimens [188]. Another FR α -based therapeutic strategy involves farletuzumab, a humanised monoclonal antibody to FR α . Particularly in low-folate environments, FR α provides a growth advantage to cancer cells. As expected, farletuzumab demonstrated growth-inhibitory effects on FR α -expressing OC cells in preclinical models [189,190]. Yet, clinical trials managing platinum-sensitive EOC with this drug in combination with other therapeutical approaches have shown conflicting results (NCT00318370 and NCT02289950) [191,192].

5. Emerging Therapies

The existing evidence indicates a stagnation in OC therapies, failing to extend the OS of patients significantly. As a result, there is a pressing demand for novel treatment approaches. Several therapeutical agents and schemes are being developed or are currently undergoing clinical trials, displacing encouraging preliminary results.

5.1. Immunomodulators

One of the emerging therapies for OC is cancer immunotherapy. This therapeutic method harnesses the power of the patient's immune system to eliminate the tumour [193]. Numerous immune-based interventions have been approved to treat solid and haematologic tumours, including immune checkpoint inhibitors, nonspecific immune stimulation, adoptive cell therapy, and cancer vaccines [194]. The involvement of the immune system in OC patients' outcomes is demonstrated by the observation that tumour-infiltrating lymphocytes and the lower expression of PD-L1 are associated with improved survival [195–198]. Considered an “inflamed tumour”, OC could benefit from these immune-based interventions, yet data are insufficient and inconsistent [199,200]. Thus, multiple clinical trials have explored the role of OC immunotherapy as a standalone treatment and

in combination with other therapeutical approaches, namely chemotherapy, the use of antiangiogenic agents, and PARPi [199,200]. The studies actively recruiting are described in **Table 2**. Current studies, including (epi)genetic and molecular profiling, are also focused on identifying predictive biomarkers to assess the responsiveness of OC to immune-based interventions and improve patient selection criteria [199,201]. Namely, tumour mutational burden (TMB), meaning the number of somatic mutations per unit of a tumour-interrogated genome, has surfaced as an important marker of response to immune checkpoint inhibition [202]. In 2020, the FDA granted accelerated approval to pembrolizumab (anti-PD-1 agent) for the treatment of unresectable and/or disseminated solid tumours with high TMB (≥ 10 mut/Mb) [203]. The exploration of immunotherapy and the integration of predictive biomarkers in clinical decision-making represent promising strides in the personalised management of OC.

Chapter IB - Table 2. Actively recruiting clinical trials of immunotherapy for OC management.

Clinical Trial	Trial Identifier	Immunotherapeutic Agent	Combination	N *	Phase	Setting	Reaction to Platinum	Completion Date *
Pembrolizumab and lenvatinib for the treatment of serous ovarian cancer patients	NCT05114421	pembrolizumab (anti-PD-1)	lenvatinib	30	II	First-line treatment Recurrent disease	NA NR	January 2024
Neoadjuvant dendritic cell vaccination for ovarian cancer (NEODOC)	NCT05773859	Specialised Cross-Presenting Dendritic Cells Vaccinations	Standard-of-care treatment	10	I/II	First-line treatment	NA	October 2024
Systemic immune checkpoint blockade and intraperitoneal chemo-immunotherapy in recurrent ovarian cancer	NCT03734692	rintatolimod (immune system stimulant) pembrolizumab (anti-PD-1)	carboplatin	45	I/II	Recurrent disease	Sensitive	December 2024
Durvalumab and tremelimumab in treating participants with recurrent or refractory ovarian, primary peritoneal, or fallopian tube cancer	NCT03026062	durvalumab (anti-PDL1) tremelimumab (anti-CTLA-4)	-	120	II	Refractory disease Recurrent disease	Resistant	December 2024
Pembrolizumab and carboplatin for the treatment of recurrent ovarian, fallopian tube, or primary peritoneal cancer	NCT04387227	pembrolizumab (anti-PD-1)	carboplatin	22	II	Recurrent disease	NR	April 2025
PD-1 antibody combined neoadjuvant chemotherapy for ovarian cancer	NCT04815408	tislelizumab (anti-PD-1)	paclitaxel and carboplatin	40	II	First-line treatment	NA	April 2025
A clinical study on oncolytic virus injection (R130) for the treatment of relapsed/refractory ovarian cancer	NCT05801783	Recombinant oncolytic herpes simplex virus type 1 (R130)	-	10	I	Refractory disease Recurrent disease	NR	December 2025
OSE2101 alone or in combination with pembrolizumab vs. BSC in patient with platinum-sensitive-recurrent OC (TEDOVA)	NCT04713514	OSE2101 (cancer vaccine) pembrolizumab (anti-PD-1)	-	180	II	Recurrent disease	Sensitive	December 2025
Safety and efficacy of anti-CD47, ALX148 in combination with liposomal doxorubicin and pembrolizumab in recurrent platinum-resistant ovarian cancer	NCT05467670	pembrolizumab (anti-PD-1) ALX148 (anti-CD47)	PLD	31	II	Recurrent disease	Resistant	December 2027

Data available at “clinicaltrials.gov” (accessed on 29 December 2023) until December 2023 using the terms “ovarian cancer” and “immunotherapy” as keywords. Maintenance therapy was deemed a treatment strategy employed after the first-line therapy but preceding any disease recurrence. * Estimated. Abbreviations: NA, non-applicable; NR, non-restrictive; PLD, pegylated liposomal doxorubicin.

5.2. Gene Therapies

Gene therapy is generally defined as the replacement of an abnormal gene with a functional copy of that gene aiming to correct an underlying disorder [204]. Different gene therapy strategies have been explored for OC management in preclinical studies, including the replacement of tumour suppressor genes to restore cell control (e.g., *TP53*), oncogene inhibition strategies (e.g., *EGFR*), suicide gene therapy with the delivery of genes encoding for toxins (e.g., *HSV-TK*), genetic immunopotential to reinforce immune response against tumour cells (e.g., *IL-12A/B*), antiangiogenic gene therapy (e.g., *COL18A1*), strategies to restore pharmacological sensitivity (e.g., survivin (*BIRC5*)) and cancer virotherapy (e.g., vesicular stomatitis virus). Furthermore, some of these approaches have also been investigated in clinical trials (**Table 3**). Despite continuous progress and promising results, several challenges prevent the clinical implementation of gene therapy, including low efficiency in the delivery of therapeutic genes, an unspecific expression allied to biosafety concerns, and ethical and financial issues. In addition, OC, like other malignant diseases, is a polygenic disease characterised by a higher degree of heterogeneity between individuals and even tumours in the same patient [204–206]. Thus, more clinical trials are required to explore the current preclinical strategies and the correct way to translate gene therapy to the clinical setting.

Chapter IB - Table 3. Active and completed clinical trials of gene therapy for OC management.

Approach	Therapeutical Agent	Trial Identifier (Status)	Combination	N Participants	Phase	Setting	Reaction to Platinum
Replacement of tumour suppressor genes	Ad5CMV-p53 vector (Inserting <i>TP53</i>)	NCT00003588 (completed)	-	30	I	Recurrent disease	Resistant
		NCT00003450 (completed)	-	-	I	Recurrent disease	NR
Suicide gene therapy	Ad5.SSTR/TK.RGD vector (HSV-TK + GCV)	NCT00964756 (completed)	-	11	I	Recurrent disease	NR
	p53MVA vaccine (Modified vaccinia virus Ankara expressing tumour protein p53)	NCT02275039 (completed)	gemcitabine	12	I	Recurrent disease	NR
	NYESO-1(C259)-transduced autologous T cells	NCT01567891 (completed)	-	9	I/II	Refractory disease Recurrent disease	NR
	Vigil™ tumour cell vaccine	NCT01309230 (completed)	-	145	II	First-line treatment	NA
Genetic immunopotential	Gene-modified lymphocytes with MOv-PBL	NCT00019136 (completed)	aldesleukin	13–50 *	I	Residual disease Recurrent disease	NA NR
	CDX-1401 vaccine	NCT03206047 (active)	atezolizumab and guadecitabine	75 *	I/II	Recurrent disease	Resistant
	ALVAC(2)-NY-ESO-1 (M)/TRICOM vaccine	NCT01536054 (completed)	sirolimus and sargramostim	7	I	Maintenance therapy Recurrent disease	NA NR
		NCT00803569 (completed)	sargramostim	13	I	Maintenance therapy Recurrent disease	NA NR
Cancer virotherapy	MV-CEA MV-NIS	NCT00408590 (completed)	-	37	I	Refractory disease Recurrent disease	NR

Data available at “clinicaltrials.gov” (accessed on 29 December 2023) until December 2023 using the terms “ovarian cancer” and “gene therapy” as keywords. Maintenance therapy was deemed a treatment strategy employed after the first-line therapy but preceding any disease recurrence. Completed trials with results are highlighted in bold. * Estimated. Abbreviations: MOv-PBL, MOv-gamma chimeric receptor gene; MV-CEA, Carcinoembryonic antigen-expressing measles virus; MV-NIS, oncolytic measles virus encoding thyroidal sodium iodide symporter; NA, non-applicable; NR, non-restrictive.

5.3. Drug Repurposing

Drug repurposing (also known as drug reprofiling, re-tasking, or repositioning) consists of identifying alternative uses for approved therapeutical agents that are outside the original prescription scope, even regarding non-cytotoxic drugs [207]. This strategy cuts research costs and speeds up drug usage as the repurposed drugs have already been deemed safe in preclinical models and humans. As a result, drug repurposing has achieved great success, leading to the identification of candidate drugs for a plethora of diseases [208].

Focusing on the therapeutic agents approved for non-oncological diseases, one of the repurposed drugs under investigation for OC management is vitamin D (VD) and its

analogues. VD consists of a group of steroid-like molecules, namely cholecalciferol (vitamin D3), ergocalciferol (vitamin D2), calcidiol (25-hydroxy-vitamin D) and calcitriol (with the active form also known as 1,25-dihydroxy vitamin D3 or 1,25D3), with the latter binding to the vitamin D receptor (VDR) to modulate the expression of several genes [209]. The most studied role of VD and its analogues is the maintenance of serum calcium and phosphorus homeostasis. Beyond their functions in physiological conditions, these steroid-like molecules are also reported to have antitumour effects in preclinical models. Namely, they can induce tumour cell differentiation and apoptosis while reducing the cells' proliferation and dissemination potential [209–211]. Consequently, synthetic VD analogues, which do not possess the side effect of hypercalcemia, have been developed to target malignant diseases [212]. Many epidemiological studies have linked VD deficiency to cancer risk and mortality [213–215]. The implications of VD are best characterised by breast, colorectal, and prostate cancers [212]. Regarding OC, although *in vitro* and *in vivo* studies have obtained promising results, the impact of VD and its analogues is still blurred. Current evidence suggests that VD-based therapy could potentiate the activity of chemotherapeutic agents and PARPi [216–221]. The combination of VD with immunotherapy has also been considered potentially beneficial, given its immunomodulatory effect [222]. However, clinical trials assessing the efficacy of VD-based therapy in OC are lacking.

Other repurposed drugs have been investigated in clinical trials to help manage OC. This list includes statins (hypercholesterolemia; NCT04457089 and NCT00585052), hydroxychloroquine (malaria, rheumatoid arthritis and lupus erythematosus; NCT03081702), metformin (type 2 diabetes mellitus; NCT02312661 and NCT01579812), itraconazole (fungal infections; NCT03081702), beta-blockers (hypertension; NCT01504126) and sodium valproate (bipolar disorder and epilepsy; NCT00529022) [223,224]. Of note, the off-labelled use of drugs approved for other malignant diseases in OC management is beyond the scope of this review.

Given the implications of drug repurposing, more investigation in this field is needed to better understand the underlying mechanisms.

5.4. Small-Molecule Kinase Inhibitors

Kinases are implicated in several signalling pathways that are often deregulated in cancer. These proteins regulate cell survival and growth, promoting tumour progression [225]. In OC, the kinases involved in angiogenesis (e.g., VEGFRs), cell growth (e.g., EGFR), and intracellular signalling (e.g., PI3K/AKT/mTOR pathway) are reported to be overactivated, being attractive therapeutic targets [226]. Numerous small-molecule kinase inhibitors have been evaluated in clinical trials for OC management (**Table 4**). Despite their potential, the high heterogeneity of ovarian tumours and drug resistance are significant

obstacles to the implementation of these drugs [226–229]. Nevertheless, progress in disease (epi)genetic and molecular profiling may help solve some of the current issues [91].

Chapter IB - Table 4. Clinical trials of small-molecule kinase inhibitors for OC management.

Inhibitor	Target	Trial Identifier (Status)	Combination	N Participants	Phase	Setting	Reaction to Platinum
anlotinib	VEGFRs, FGFRs, PDGFRs, c-Kit and RET kinases [230]	NCT05188781 (completed)	pembrolizumab	34	II	Refractory or recurrent disease	NR
		NCT05130515 (completed)	niraparib	6	II	Recurrent disease	Resistant
		NCT02584478 (active)	paclitaxel, PLD, topotecan and carboplatin	294	III	Recurrent or metastatic disease	NR
apatinib	VEGFR-2 [231]	NCT02867956 (completed)	etoposide	35	II	Refractory or recurrent disease	Resistant
		NCT03075462 (completed)	fuzuloparib	98	I	Recurrent	Sensitive
		NCT04348032 (active)	PLD	152	II	Recurrent disease	Resistant
		NCT04229615 (active)	fuzuloparib	690	III	Maintenance therapy	Sensitive
alpelisib	PI3K [232]	NCT04729387 (Active)	olaparib, paclitaxel and PLD	358	III	Refractory or recurrent disease	Resistant
cediranib	VEGFRs [233]	NCT00275028 (completed)	-	47	II	Recurrent disease	Resistant
		NCT00278343 (completed)	-	74	II	Refractory or recurrent disease	NR
		NCT02340611 (completed)	olaparib	4	II	Recurrent disease	NR
		NCT02889900 (completed)	olaparib	62	II	Recurrent disease	Resistant
		NCT02681237 (completed)	olaparib	34	NA	Recurrent disease	NR
		NCT03117933 (active)	olaparib and paclitaxel	139	II	Recurrent disease	Resistant
		NCT02502266 (active)	olaparib, paclitaxel, PLD and topotecan	562	II/III	Recurrent disease	Resistant
		NCT02345265 (active)	olaparib	72	II	Recurrent disease	NR
		NCT02446600 (active)	olaparib	579	III	Recurrent disease	Sensitive
		NCT01116648 (active)	olaparib	155	I/II	Recurrent disease	Sensitive
erlotinib	EGFR [234]	NCT00217529 (completed)	docetaxel and carboplatin	30 *	I/II	First-line treatment	NA
		NCT00263822 (completed)	-	835	III	Maintenance therapy	NR
		NCT00030446 (completed)	carboplatin	50	II	Recurrent disease	NR
		NCT00126542 (completed)	bevacizumab	35	II	Recurrent or metastatic disease	NR
		NCT00130520 (completed)	bevacizumab	40	II	Recurrent disease	Resistant
		NCT00059787 (completed)	carboplatin and paclitaxel	56	II	First-line treatment	NA
		NCT00520013 (completed)	bevacizumab, paclitaxel and carboplatin	60	II	Maintenance therapy	NR
palbociclib	CDK4 and CDK6 [235]	NCT01536743 (completed)	-	26	II	Recurrent disease	NR
pazopanib	VEGFRs, PDGFRs and FGFRs [236]	NCT00281632 (completed)	-	35	II	Refractory disease	Refractory
		NCT01227928 (completed)	-	145	II	Maintenance therapy	NA
		NCT01238770 (completed)	cyclophosphamide	10	I/II	Recurrent disease	Resistant
		NCT01644825 (completed)	paclitaxel	72	II	Refractory or recurrent disease	Resistant
		NCT01262014 (completed)	-	28	II	Recurrent disease	Resistant
		NCT01608009 (completed)	paclitaxel	16	I	Recurrent disease	Resistant
		NCT00866697 (completed)	-	940	III	Maintenance therapy	NA
		NCT01468909 (completed)	paclitaxel	106	II	Recurrent disease	Sensitive
		NCT01402271 (completed)	paclitaxel and carboplatin	88	I/II	Refractory or recurrent disease	Resistant
		NCT01610206 (completed)	gemcitabine	148	II	Recurrent disease	NR
		NCT02383251 (completed)	paclitaxel	118	II	Refractory or recurrent disease	Resistant

sorafenib	Raf serine/threonine kinases, VEGFRs and PDGFR- β [237]	NCT00096395 (completed)	gemcitabine	33	II	Recurrent disease	NR
		NCT00093626 (completed)	-	73	II	Recurrent disease	Resistant
		NCT00096200 (completed)	carboplatin and paclitaxel	44	II	Recurrent disease	Sensitive
		NCT00791778 (completed)	-	246	II	Maintenance therapy	NA
		NCT00390611 (completed)	paclitaxel and carboplatin	85	II	First-line treatment	NA
sunitinib	VEGFR1, VEGFR2, PDGFR- α , PDGFR- β , c-Kit, FLT3, RET and CSF1R [238]	NCT00436215 (completed)	bevacizumab	55	II	Refractory or recurrent disease	Resistant
		NCT01047891 (completed)	topotecan	174	II	Recurrent disease	Resistant
		NCT00388037 (completed)	-	31	II	Recurrent disease	NR
		NCT00768144 (completed)	-	36	II	Refractory or recurrent disease	NR
		NCT01824615 (completed)	-	30	II	Recurrent disease	Resistant
		NCT00979992 (completed)	-	35	II	Recurrent disease	NR

Data available at “clinicaltrials.gov” (accessed on 29 December 2023) until December 2023 using the terms “ovarian cancer” and “small-molecule kinase inhibitor” as keywords. Also, the term of each inhibitor was used. Maintenance therapy was deemed a treatment strategy employed after the first-line therapy but preceding any disease recurrence. Completed trials with results are highlighted in bold. * Estimated. Abbreviations: NA, non-applicable; NR, non-restrictive; OC, ovarian cancer; PLD, pegylated liposomal doxorubicin.

5.5. Coagulation-Targeting Approaches

Patients with ovarian tumours are commonly diagnosed with venous thromboembolism (VTE), with an incidence ranging from 10 to 30% [239]. This thrombotic event constitutes the second cause of death among oncological patients [240]. Importantly, even in the absence of VTE, most cancer patients present a state of blood hypercoagulation. Cumulative evidence suggests that underling this state, deregulated haemostatic components—endothelial cells, platelets, and coagulation/fibrinolysis systems—exhibit protumourigenic functions, including tumour cell growth, survival, proliferation, and invasion while also supporting cancer neoangiogenesis and metastatic dissemination [226]. Several haemostatic components have been suggested to play critical roles in OC progression and ascite formation, creating potential avenues for therapeutic intervention [241]. Namely, the overexpression of coagulation factor 3, commonly known as the tissue factor (TF), and the presence of tumour-educated platelets are some of the most studied mechanisms in this interface of VTE and OC progression [239,241,242].

Regarded as the initiator of the extrinsic coagulation pathway, TF is released into the blood circulation following vascular damage to trigger fibrin deposition at the injury site. This mechanism is vital to stop blood loss and restore haemostasis [243–245]. Cancer cells constitutively express TF and induce its synthesis in normal cells in the tumour microenvironment, which generates a prothrombotic cascade that favours tumour progression [246]. Inclusively, the overexpression of this coagulation factor in several tumour types, including OC, is linked to poor prognosis [247–250]. The protumourigenic

roles of TF encompass tumour cell proliferation, cancer stemness, angiogenesis, immune evasion, and metastasis through clotting-dependent and independent processes [243,251–253]. Recently, tisotumab vedotin (Tivdak™), a TF-specific human ADC conjugated to the tubulin-targeting agent called monomethyl auristatin E (MMAE), was approved by the FDA for the management of recurrent or metastatic cervical tumour [254–256]. According to the results of phase I/II innovaTV-201 (NCT02001623), this drug showed promising antitumour activity and a convenient safety profile in platinum-resistant OC, which supports the continued investigation of tisotumab vedotin in this population [255].

Platelets, also known as thrombocytes, are megakaryocyte-derived haemostatic key players in the bloodstream [257–259]. Apart from their role in haemostasis, platelets promote tumour growth and dissemination, and, in turn, tumour cells stimulate platelet production and activation, creating a feedback loop that fuels tumourigenesis and leads to paraneoplastic thrombocytosis (i.e., an elevated platelet count >450,000 per cubic millimetre) [260,261]. This well-recognised phenomenon is often associated with many solid tumours [257,262]. In the context of OC, the interaction of thrombocytes and tumour cells is so evident that one-third of women with newly diagnosed OC have paraneoplastic thrombocytosis. However, data on the impact of thrombocytosis on the patient's clinical outcomes is inconsistent [261,263,264]. Some studies have demonstrated that when considering other clinical factors, such as cancer burden, thrombocytosis in advanced OC-stage patients does not independently impact prognosis [261,265]. Others, nevertheless, have shown that this condition is associated with an advanced disease stage, high grade, and elevated preoperative CA-125 levels, which are all known OC prognostic factors [259,261]. When focusing on early stages, thrombocytosis seems to be a powerful prognostic factor, with affected patients exhibiting approximately an eightfold increase in the risk of recurrence and a fivefold increase in the risk of death. Moreover, this condition in these patients seems to correlate with disease burden, residual disease, and postoperative complications [265]. On the other hand, thrombocytosis was also found to be an independent prognostic factor, regardless of disease stage, tumour grade, histologic type, and the extent of surgical intervention ($p < 0.001$). Namely, affected patients presented a median OS of 2.65 years compared to the 4.65 years exhibited by their counterparts [261]. Thus, designing therapeutic agents to target platelets at the tumour microenvironment (i.e., tumour-educated platelets) can provide a promising breakthrough in OC treatment [266]. Epidemiological studies have suggested that acetylsalicylic acid (also known as aspirin), a non-steroidal anti-inflammatory drug, may have anticancer properties [267,268]. By inhibiting cyclooxygenase-2 (COX-2), aspirin exerts antiplatelet and anti-inflammatory effects. Although the cumulating data on aspirin's impact on OC patients' survival is

conflicting, preclinical data show that aspirin exerts anti-tumoural effects when combined with bevacizumab [269,270]. Thus, the phase II trial EORTC-1508 (NCT02659384) is currently evaluating the efficacy and safety of combining atezolizumab (monoclonal antibody targeting PD-L1), bevacizumab and aspirin to treat recurrent platinum-resistant OC. Moreover, an ongoing phase I trial (NCT05080946) aims to evaluate the effectiveness of aspirin with neoadjuvant chemotherapy for decreasing markers of immune suppression (M2 tumour-associated macrophages and immunosuppressive T-regulatory cells) within the tumour. More studies on aspirin's effect and the development of other antiplatelet agents for OC treatment should be evaluated.

6. Conclusions

Despite significant strides in disease management, OC remains the most lethal female reproductive cancer. Not dismissing the potential publication and selection bias, which is characteristic of narrative reviews, this comprehensive overview provides an in-depth analysis of the recent evidence regarding OC management, identifying gaps in the literature and suggesting future directions for disease research. Briefly, ongoing research focuses on dissecting OC pathogenesis to refine screening techniques and seek innovative and more targeted treatments to manage this malignant disease effectively, decrease side effects, and enhance OC patient outcomes. Due to a better understanding of OC's (epi)genetic and molecular profiling, several therapeutical approaches have been recently approved, with others in development. Notably, (epi)genetic and molecular changes in ovarian tumours have been found to correlate with drug efficacy and resistance, particularly in HGSC heterogeneity. Thus, integrating molecular insights might have significant implications for clinical decision-making. Future investigation endeavours should be directed at OC's heterogeneity and drug resistance challenges. Likewise, there should be an emphasis on identifying more accurate diagnostic tools, prognostic indicators, and predictive biomarkers of response for current and emerging therapies. Furthermore, more data should be collected on the impact of combining diverse treatment modalities, as it holds promise in elevating treatment effectiveness and conquering drug resistance, enhancing patient outcomes by leveraging the synergistic actions of multiple therapies.

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CHAPTER IC – Inflammation as a Common Pillar of Cancer Complications

This chapter includes the following paper:

Joana Liz-Pimenta *, Valéria Tavares *, Beatriz Vieira Neto, Joana M.O. Santos, Catarina Brandão Guedes, António Araújo, Alok A. Khorana, Rui Medeiros. **Thrombosis and cachexia in cancer: Two partners in crime?** *Critical Reviews in Oncology/Hematology*. **2023**; 186: 103989-103989. <https://doi.org/10.1016/j.critrevonc.2023.103989>. (Impact factor and quartile reported in 2023: **5.5** and **Q1**).

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Thrombosis and Cachexia in Cancer: Two Partners In Crime?

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Abstract: Among cancer patients, thrombosis and cachexia are major causes of morbidity and mortality. Although the two may occur together, little is known about their possible relationship. Thus, a literature review was conducted by screening the databases PubMed, Scopus, SciELO, Medline and Web of Science. To summarise, cancer-associated thrombosis (CAT) and cancer-associated cachexia (CAC) seem to share several patient-, tumour- and treatment-related risk factors. Inflammation alongside metabolic and endocrine derangement is the potential missing link between CAT, CAC and cancer. Many key players, including specific pro-inflammatory cytokines, immune cells and hormones, appear to be implicated in both thrombosis and cachexia, representing attractive predictive markers and potential therapeutic targets. Altogether, the current evidence suggests a link between CAT and CAC, however, epidemiological studies are required to explore this potential relationship. Given the high incidence and negative impact of both diseases, further studies are needed for the better management of cancer patients.

Keywords: Neoplasms; Venous thromboembolism; Cachexia; Inflammation; Hormones; Metabolism; Patient monitoring.

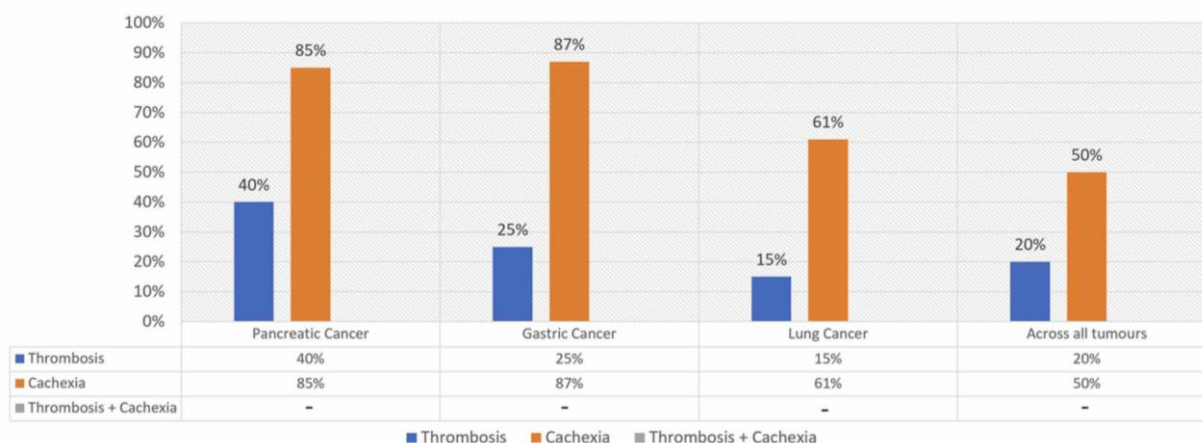
1. Introduction

Cancer is a prominent cause of mortality and a significant obstacle to improving life expectancy. Worldwide, the tumour types with higher mortality are in decreasing order: lung, colorectal, liver, gastric, breast, oesophageal and pancreatic cancers. In 2020, the number of patients that died due to cancer was almost 10.0 million, while in 2040 it is foreseen to be 28.4 million, an increase of 47% in 20 years [1].

Unfortunately, besides the disease itself, cancer patients also face life-threatening complications, namely cancer-associated thrombosis (CAT) and cancer-associated cachexia (CAC) [2,3]. Both events have a deleterious impact, negatively affecting the patient's quality of life and prognosis, and imposing a substantial cost burden on healthcare systems [2-4]. Individual genetic background and tumour-specific factors, as well as variations in anti-neoplastic treatments, might explain the inter-individual variation in terms of prevalence, severity and progression of these complications [5-7]. One shared feature is a systemic pro-inflammatory state, which plays a central role in the pathogenesis of both conditions [8-10].

The high frequency of both thrombosis and cachexia among oncological patients and the roles of pro-inflammatory cascades in their pathogenesis raise the question of whether these conditions could be related. Although they may occur together, the accurate

percentage of patients affected by both diseases is unknown (**Figure 1**) [11-15]. Furthermore, the biological mechanisms underlying such potential link are poorly explored, with no consistent evidence-based molecular pathways proposed yet. Also, the physiopathology behind these disorders is still a subject of study as neither of them is fully understood [8,16].



Chapter IC - Figure 1. Prevalence of Thrombosis and Cachexia in Cancer. Thrombosis and cachexia are major complications among cancer patients. Although they may occur together, the percentage of patients affected by both events is unknown [13-15,17-22].

Given the knowledge gaps, this review article aims to provide mechanistic insight into the putative association between CAT and CAC. To do so, during 2022 a literature review of scientific articles published in the last 60 years (between 1962 and 2022) was performed in the following research databases: PubMed, Scopus, SciELO, Medline and Web of Science. Using these databases were searched terms and interpretations of “Cancer” (malignancy, malignant neoplasms, neoplasia, neoplasm, tumour, malignancy) and either “Thrombosis” (blood clot, thrombus, thromboembolism) or “Cachexia” (wasting, malnutrition). After initial data collection, similarities between CAT and CAC were explored.

2. Thrombosis in the setting of malignancy

The first understanding of CAT, mainly venous thromboembolism (VTE), began in the 19th century with Rudolf Virchow (1821 – 1902) and Armand Trousseau (1801–1867). The former identified in 1856 the three factors underlying VTE pathogenesis, together known as Virchow's triad, namely blood hypercoagulability, venous stasis and endothelial injury [16]. The latter, in the 1860s, was the first to identify thromboembolism (*Phlegmasia alba dolens*)

as a manifestation of cancer, in some cases even preceding its diagnosis, known nowadays as Trousseau's syndrome. He noticed this condition in himself and died in the same year due to gastric cancer [23,24]. Recent data show that over 5% of patients are diagnosed with cancer within one year after an unprovoked VTE and over 60% of occult cancers (mainly colorectal and prostate cancers) are diagnosed in this context [25-29]. Current guidelines recommend limited cancer screening (only the most prevalent tumours considering patient age and sex) after an idiopathic VTE, and there is no validated predictive marker available to decide on whether to perform a more thorough investigation [30].

Generally, CAT is defined as a thromboembolic event that occurs within six months up to two years after malignancy diagnosis [31]. Importantly, the risk of developing VTE is higher in the first six months after cancer diagnosis and in the presence of metastases, being the second cause of mortality among oncological patients after cancer progression [32,33]. Among these patients, VTE is the most common manifestation of thromboembolism, which also encompasses arterial events [4]. With several displays, VTE includes deep-vein thrombosis (DVT) and pulmonary embolism (PE), the most prevalent ones, but also superficial vein thrombosis, catheter-related thrombosis and thrombosis in unusual sites [34,35]. The diagnosis is made by direct observation of the thrombi, either by duplex ultrasonography or computed tomography (CT) for DVT or PE detection, respectively [36,37]. The disease is classified according to aetiology (venous or arterial), site and its presentation: symptomatic or incidental. The latter are events that are asymptomatic and diagnosed only because the patient underwent an imaging exam for other clinical reason. Inclusively, clinically unsuspected PE is frequently detected in cancer patients undergoing CT scans for staging, treatment response evaluation and surveillance [38]. Of all VTE cases, about 20% occur in the context of malignancy, and over 20% of cancer patients will have symptomatic VTE events (50% in post-mortem studies) versus 2% in the general population [34]. Moreover, the prevalence of CAT has been increasing due to the increased prevalence of cancer, better accuracy of imaging exams (increasing the incidence of incidental CAT) and the new cancer treatments with pro-thrombotic effects [4]. The disease risk, which is more than six times greater than in the noncancer population, is mainly attributed to factors related to tumour biology and anti-neoplastic treatment leading to haemostatic disorders [33,39].

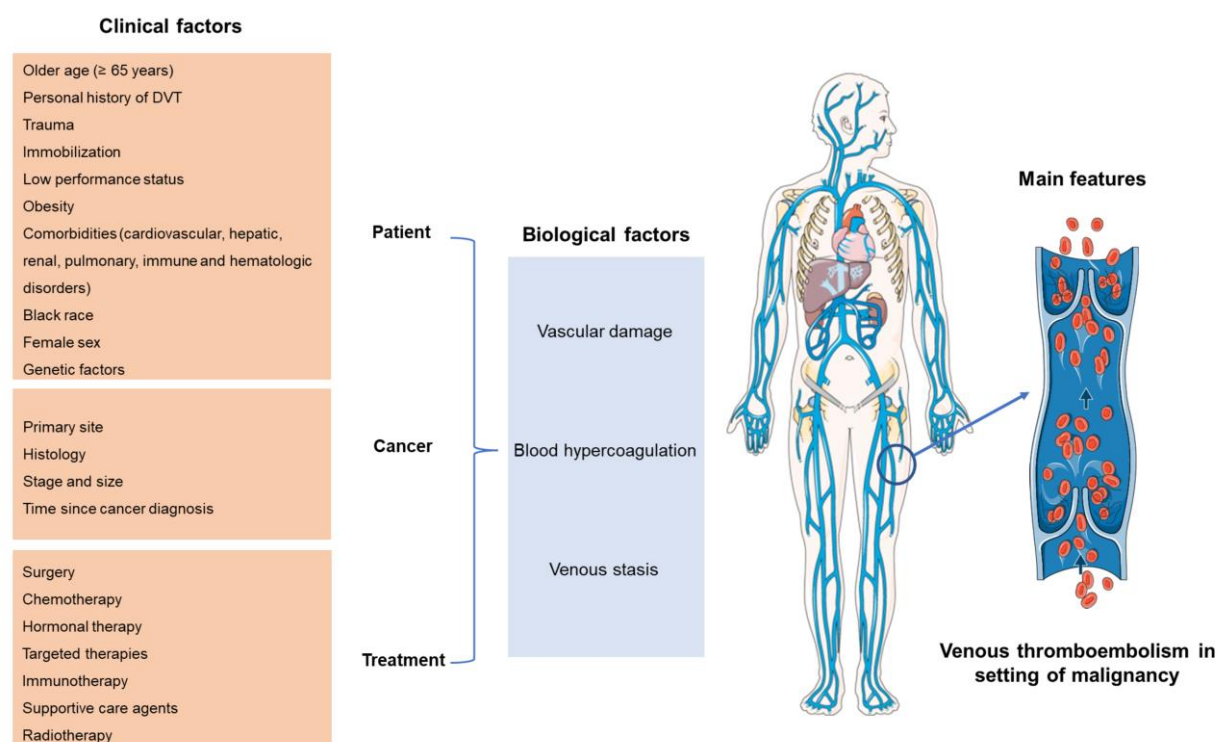
Despite the several studies performed since the 19th century, the biological mechanisms of CAT are still not fully understood, and research in this field is still ongoing, mainly given its frequency and detrimental effect on cancer patient survival [16,33]. To be noted, the incidence of arterial thromboembolic events is also increased among cancer patients compared to the general population. However, given the scarce data concerning

arterial events in this population subgroup, the focus of the present literature review is cancer-related VTE [40].

2.1. Pathophysiology

The development of VTE is the result of an intricate interplay between genetic and acquired factors. Among cancer patients, these factors can be categorised as related to the patient, cancer and oncologic treatments as summarised in **Figure 2** [16,41].

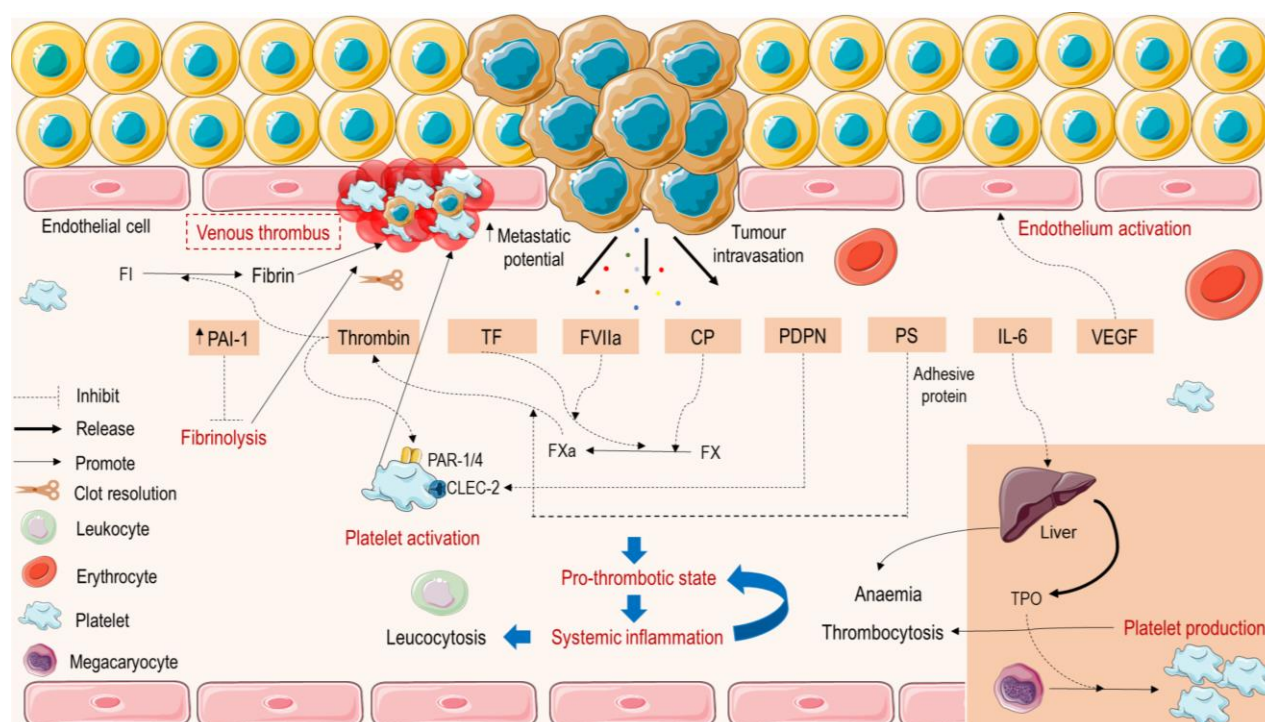
Regarding pro-thrombotic factors related to the patient, the major ones are advanced age (≥ 65 years old), previous history of VTE, obesity (body mass index (BMI) higher than 35Kg/m^2), low-performance status, physical trauma, immobilization, genetic factors (particularly haemostatic gene polymorphisms) and the existence of comorbidities (cardiovascular, hepatic, renal, pulmonary, immune and hematologic disorders). Less is known about demographic factors, however, Black ethnicity and female sex seem to constitute risk factors [16,41,42]. Despite the irrefutable effect of patient-related factors, as thrombogenesis is more frequent in cancer patients than in the general population and is a distinct condition, risk factors related to tumour and anti-neoplastic treatments may play a more preponderant role [42].



Chapter IC - Figure 2. Aetiological factors of venous thromboembolism in oncological patients. Patient, tumour biology and anti-neoplastic treatment-related factors may induce vascular damage, blood hypercoagulation and venous stasis (Virchow's triad), which are the main drivers of

venous thrombogenesis among oncological patients [16,41]. The figure includes pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

Among the risk factors related to cancer biology, a major determinant is the primary tumour site. Gastric and pancreatic cancers are the solid tumours associated with the highest VTE risk, followed by brain, lung, gynaecologic and urologic tumours (except prostate cancer) [43]. In pancreatic cancer, for instance, tissue factor (TF) seems to be the initiator of the pro-thrombotic cascade, in addition to favour tumourigenesis [44]. In glioblastoma, the high tendency has been linked with the upregulation of podoplanin (PDPN), which also correlates with a poor prognosis (**Figure 3**) [45]. Other proposed pathways include thrombocytosis in ovarian tumours and leucocytosis, neutrophilia and neutrophil extracellular traps (NETs) in lung tumours (**Figure 3**) [46]. The histologic subtype plays a role as well, with adenocarcinoma patients having a higher risk for thrombogenesis [47,48]. It is postulated that circulating carcinoma mucins might activate platelets through interaction with platelet P-selectin and leukocyte L-selectin with the following formation of microthrombi [49]. In addition, different oncogenic driver mutations seem to be associated with distinct VTE risks. For instance, in lung cancer, patients with *anaplastic lymphoma kinase* (ALK) mutations are apparently more prone [50].



Chapter IC - Figure 3. A simplistic view of cancer-associated venous thrombogenesis and the underlying mechanisms. Tumour cells can trigger thrombogenesis by releasing a set of pro-coagulant proteins (TF, thrombin, FVII, CP, PDPN, among others), fibrinolytic mediators (e.g., PAI-

1), pro-inflammatory cytokines (e.g., IL-6), proangiogenic (e.g., VEGF) and adhesive or pro-aggregating factors (PS). The generated pro-thrombotic state has an underlying pro-inflammatory cascade, which favours and augments thrombogenesis. Conversely, the generated thrombo-inflammation promotes tumour neoangiogenesis and metastasis [10,16,45,51-54]. Abbreviations: PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; FVIIa, activated coagulation factor VII; FI coagulation factor I; FX, coagulation factor X; FXa, activated coagulation factor X; CP, cancer procoagulant; PDPN, podoplanin; PS, phosphatidylserine; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; TPO, thrombopoietin; PAR, proteinase-activated receptor; CLEC-2, C-type lectin-like receptor-2. The figures include pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

Factors related to cancer treatments are relevant by their direct and indirect effects. On the one hand, the cytotoxicity of anti-neoplastic treatments results in the local release of pro-coagulants and pro-inflammatory cytokines into the blood flow. On the other hand, these treatments can induce hepatotoxicity, which can disrupt the haemostatic balance by deregulating the hepatic expression of both pro-coagulants (coagulation factors, except for factors III (TF or tissue thromboplastin), IV (calcium), VIII and von Willebrand factor (vWF)), and anti-coagulants (antithrombin, protein C and protein S). Similarly, induced nephrotoxicity may impair glomerular permeability, leading to blood hypercoagulation and increased thrombotic tendency [51,55,56]. The cancer systemic treatments with the highest pro-thrombotic potential are chemotherapy and hormone treatment. The former is associated with a six-fold increase in disease risk [57]. The agents associated with a higher risk are gemcitabine and platinum anticancer drugs (apart from oxaliplatin, with the lowest incidence (around 2%) compared with other platinum drugs [58]). Taking this into account, it was created a prognostic risk score (the PROTECHT score: PROphylaxis of ThromboEmbolism during ChemoTherapy) [57]. Regarding hormone treatment, particularly tamoxifen has a linked CAT risk that is up to seven-fold superior (one of the highest increases in VTE risk) [59]. Other systemic treatments that are pro-thrombotic are anti-angiogenic and molecular/targeted therapies, particularly directed to vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), followed by the combination of B-type Raf kinase (BRAF) and mitogen-activated protein kinase (MEK) inhibitors [60,61]. Recent studies also suggest that cyclin-dependent kinase (CDK) inhibitor 4/6 increases VTE risk, with a higher impact for palbociclib (incidence of 11%, with ribociclib presenting 8% and abemaciclib 5%) [62,63]. Regarding immunotherapy, it is associated with a significant yet underappreciated VTE risk. The treatment might initiate a thrombo-inflammatory response that augments the already elevated risk of CAT. In this process,

interleukin-8 (IL-8), vascular cell adhesion molecule 1 (VCAM-1) and myeloid-derived suppressor cells (MDSCs) seem to be key players given their increased levels during thrombogenesis under therapy with immune checkpoint inhibitors [64]. Furthermore, some supportive treatments seem to also increase VTE risk, namely the use of megestrol (a synthetic form of progesterone used to increase appetite), corticosteroids (synthetic adrenal hormones), growth colony-stimulating factors and blood transfusions [61,65]. Beyond the treatment itself, cancer patients are also predisposed to thrombotic events due to the use of venous central catheters, causing what is known as central venous catheter-related thrombosis. This condition can lead to an increased risk of infections, long-term central venous stenosis and catheter dysfunction [66]. Regarding local therapies, both radiotherapy and surgery are pro-thrombotic, mostly likely due to damage to the vascular endothelium [67,68]. Upon injury, endothelial cells release several components into the bloodstream, including the vWF that mediates platelet attachment to the subendothelium, enabling haemostasis and thrombosis [69-72]. Fibrinogen overexpression, TF release and fibrinolysis deregulation are also important processes. Furthermore, surgery can also lead to venous stasis due to prolonged immobilization, inducing thrombogenesis [41,68].

Associated with the clinical risk factors, several biological mechanisms for CAT have been described, and they integrated the arms of Virchow's triad, involving changes in the blood composition, blood flow and blood vessel integrity (**Figure 2**) [16,41]. In terms of blood composition, in the presence of malignant disease, the expression levels of several haemostatic proteins are deregulated, which impairs the haemostatic balance, shifting it towards thrombogenesis. Haemostatic elements and tumour cells have a close and complex interaction, which contributes to the blood hypercoagulability state commonly found among oncological patients [51]. Tumour cells are known to produce and release a range of components, alone or within microvesicles, that trigger a thrombo-inflammatory cascade, namely pro-clotting proteins (e.g., cancer coagulant, coagulation factors VII (FVII) and VIII (FVIII), phosphatidylserine (PS), PDPN and TF), inflammatory cytokines (e.g., interleukin-6 (IL-6), which induces megakaryopoiesis through activation of thrombopoietin (TPO) synthesis), heparinases (that degrade endogenous heparin), fibrinolytic regulators (e.g., plasminogen activator inhibitor-1 (PAI-1)) and pro-angiogenic factors (e.g., VEGF) (**Figure 3**). In addition, they also induce endothelial cells, leucocytes and platelets to release pro-thrombotic components [16,45,51-54]. Consequently, anaemia, leucocytosis and thrombocytosis are common features among oncological patients (**Figure 3**) [9,39,51]. The endocrine system also impacts haemostatic balance. Beyond sexual hormones, others are linked with thrombogenesis [73]. In a sub-study of the CASSINI trial, thyroid-stimulating hormone (TSH) and growth hormone (GH) were predictors for VTE and mortality [74]. Thyroid hormones have an effect by modulating the levels of vitamin K levels, which is a

cofactor for some haemostatic proteins, namely FII, FVII, FIX and FX, protein S and protein C [73,75,76]. Also, the thyroxine (T4) hormone seems to reduce the activity of FII, while triiodothyronine (T3) decreases the activity of FII, FVII, FIX and FX [75].

Regarding changes in the blood flow, thrombogenesis can be triggered by venous compression by the tumour lesions, increased turbulency caused by circulating cancer cells and prolonged patient immobility. Inclusively, venous compression was found to be the major pro-thrombotic factor in the context of high-grade non-Hodgkin lymphomas and pancreatic adenocarcinoma [16,77]. As for the integrity of blood vessels, direct tumour invasion, oncological treatments and the prolonged use of venous catheters and external devices can cause injuries to vascular endothelium triggering thrombogenesis [16].

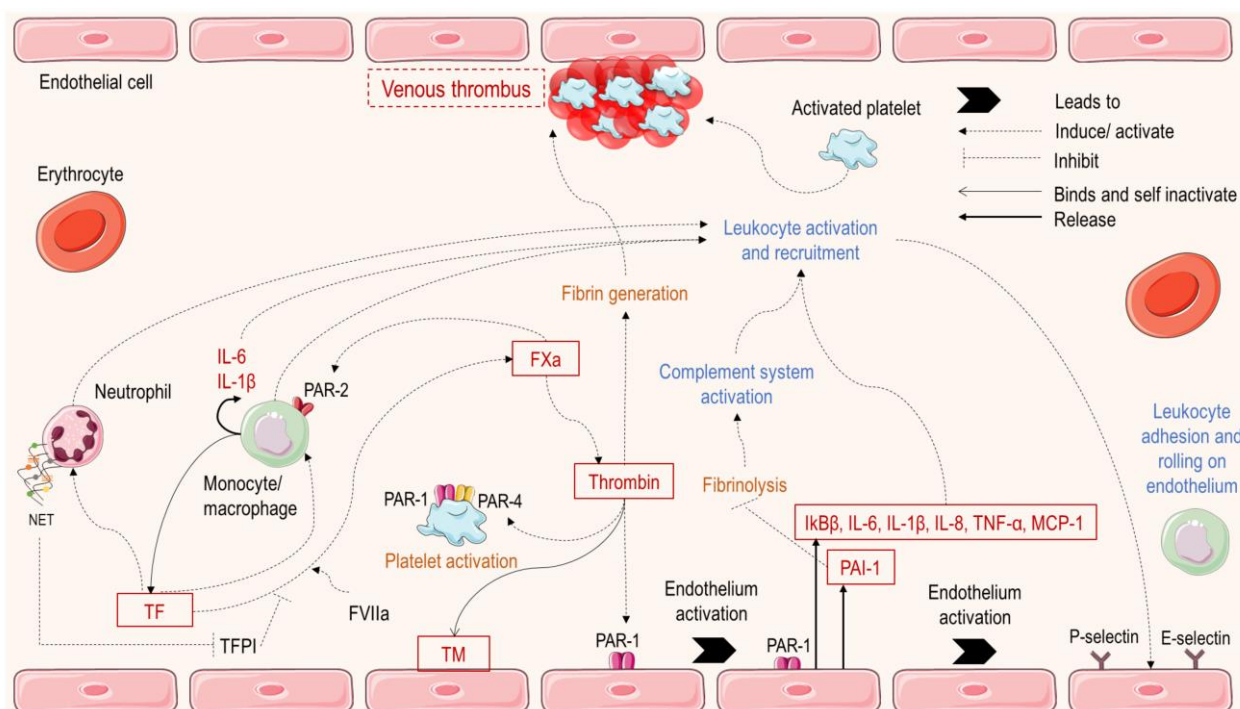
Altogether, these events are reflected in the higher levels of D-Dimers, C-reactive protein (CRP), P-selectin, platelet factor 4 (PF4), FVIII, IL-6 and TF [4,6,51]. Beyond thrombogenesis, changes in the haemostatic system also impact tumour behaviour by enhancing angiogenesis and metastatic potential, ultimately contributing to cancer progression, which may explain the correlation between VTE presence and tumour aggressiveness [16,78]. In this two-way association intercedes several haemostatic key players, including for instance platelets. Indeed, thrombocytopenia and antiplatelet drugs are thought to be associated with a reduction of metastases (**Figure 3**) [4,79,80].

2.2.Role of inflammation

The haemostatic and immune systems are interconnected in numerous ways to optimise the host response to both vascular injury and pathogen invasion. In fact, coagulation and inflammation are known to amplify each other (**Figure 4**) [10].

Some of the key players thought to provide a bridge between haemostasis and the immune system include TF, thrombin (activated FII; FIIa), FX, anticoagulation complex protein C-thrombomodulin (PC-TM), fibrinolytic regulators, toll-like receptors (TLRs) and the complement system [10]. The pro-clotting protein TF is a transmembrane glycoprotein expressed by perivascular and epithelial cells in an organ-specific manner, with high levels being found in the brain, heart, lung, placenta and uterus [81]. This protein constitutes the primary activator of the coagulation cascade (specifically in the TF pathway) but also functions as a trigger for inflammation and fibrosis. Upon infection, its expression is up-regulated in perivascular cells, while a transient-inducible TF generation is described for circulating monocytes. Once exposed, TF can trigger several pro-inflammatory events, including leukocyte recruitment and activation, with reports of subsequent formation of NETs. Furthermore, TF mediates the generation of activated FX (FXa) and thrombin, each with individual roles in the immune activity. These pro-inflammatory events, in turn, up-

regulate TF generation contributing to the augmentation of the pro-thrombotic potential (Figure 4) [10,81].



Chapter IC - Figure 4. A simplistic view of the amplifying effects haemostasis and immune response have on one another. The haemostatic and immune systems are interconnected in numerous ways, amplifying each other [10]. Abbreviations: TF, tissue factor; NET, neutrophil extracellular trap; PAR, proteinase-activated receptor; IL, interleukin; FVIIa, activated coagulation factor VII; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; FXa, activated coagulation factor X; IκBβ, I-kappa-B-beta; TNF-α, tumour necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1. The figures include pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

Thrombin is a downstream product of coagulation pathways responsible for fibrin formation. This serine protease can cleave different proteinase-activated receptors (PARs), a group of four cell surface receptors (PAR-1, PAR-2, PAR-3 and PAR-4) that belong to the family of G protein-coupled receptors and are responsible for cellular activation [82]. Due to their expression throughout the body, particularly by platelets, leukocytes, endothelial and epithelial cells, neurons and astrocytes, these receptors are involved in a wide range of pathophysiological mechanisms, including thrombosis, inflammation and carcinogenesis. On the haemostatic site, thrombin can activate platelets by cleaving PAR-1 and PAR-4, as well as activate endothelial cells through PAR-1 leading to their expression of PAI-1 and I-kappa-B-beta (IκBβ), the former an inhibitor of fibrinolysis and the latter an inhibitor of

nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway involved in platelet activation and inflammatory events [82,83]. On the inflammation site, by activating endothelial cells, thrombin induces also the expression of the pro-inflammatory cytokines IL-6, interleukin-1 beta (IL-1 β), IL-8, tumour necrosis factor-alpha (TNF- α) and monocyte chemoattractant protein-1 (MCP-1), as well as trigger the endothelial exposure of adhesion molecules E- and P-selectin leading to leukocyte recruitment, adhesion and rolling on the vascular endothelium (**Figure 4**) [10]. To amplify this thrombo-inflammation cascade, adherent monocytes are vulnerable to PAR-mediated activation (PAR-2), which increases even more the expression of IL-6 and IL-8. Likewise, FXa can cleave PAR-2 inducing pro-inflammatory events, for instance, macrophage polarization (**Figure 4**) [10,84,85].

Natural anticoagulants also impose a regulatory impact on the immune system. Thrombomodulin (TM), protein S, protein C and endothelial cell protein C receptor (EPCR) act together in the protein C anticoagulant pathway to maintain haemostasis, limit pro-inflammatory responses and protect endothelial cells from apoptosis [86,87]. Starting with TM, this endothelial transmembrane glycoprotein constitutes the initiator of the protein C anticoagulant pathway. When bound to thrombin, TM prevents the pro-clotting and pro-inflammatory effects of this clotting factor (**Figure 4**), while also generating activated protein C (APC) through the thrombin-TM-EPCR complex. Once produced, APC is released from EPCR and binds to protein S on the surface of activated endothelial cells and platelets, exerting anti-coagulant and pro-fibrinolytic effects, including FV inactivation and PAI-1 neutralization [87]. Likewise, when still bound to EPCR, although unable to exert the mentioned roles, APC provides anti-inflammatory and cytoprotective signals by, for instance, suppressing the NF- κ B pathway, decreasing the expression of pro-inflammatory cytokines and promoting the generation of inhibitors of apoptosis [10].

Despite the role in fibrin degradation, which prevents thrombogenesis, the fibrinolytic system may paradoxically promote thrombus formation by favouring pro-inflammatory processes [10,88]. Upon vascular damage, fibrinolysis is immediately activated with thrombin inducing the expression of tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), which cleaves plasminogen to produce the key fibrinolytic enzyme, plasmin [89]. Beyond fibrinolysis, plasmin is implicated in vascular remodelling and inflammation, the latter through the activation of the complement system (**Figure 4**). Furthermore, interactions between uPA and its receptor uPAR can favour cytokine production. Conversely, inflammation may induce PAI-1 expression, thus increasing the risk of thrombosis [10].

Although the exact mechanisms are not entirely understood, innate immunity is also thought to be involved in thrombogenesis as TLRs and the complement system engage with pathways that favour coagulation. Specifically, TLRs and the complement system are

thought to mediate TF expression in several different cells and promote vWF secretion and platelet activation. Platelets are also thought to promote thrombo-inflammation by, for instance, stimulating the formation of NETs, which in turn are implicated in several mechanisms that favour clot formation (degradation of TF pathway inhibitor (TFPI) allowing TF activity; **Figure 4**), resistance to fibrinolysis and impairment of the protein C anticoagulation pathway [10,90].

2.3. Treatment and prophylaxis

Thrombosis has a detrimental impact on the survival and quality of life of cancer patients. Possible VTE sequels include pulmonary hypertension (with risk of right heart strain and failure), post-thrombotic syndrome (sequel of leg's DVT) and delays in cancer treatments schedule [91]. Also, compared to the general population, oncological patients diagnosed with VTE are at higher risk of recurrent thrombotic events, bleeding under anticoagulation therapy and a higher risk of dying due to this cardiovascular disease, which highlights the need for new and improved VTE management [39].

For CAT treatment, the recommended anticoagulation options are low-molecular-weight heparins (LMWH) and direct oral anticoagulants (DOAC), with the treatment duration varying between three to six months or more, as secondary prevention if risk factors persist. The decision on anti-thrombotic treatment is made by taking into consideration the primary tumour, haemorrhagic risk, presence of comorbidities, performance status, possible drug-drug interactions and patient's preference [92,93]. Importantly, heparins are suggested to have an anti-inflammatory effect potentially impacting tumour progression by interfering with molecular pathways that contribute to cell proliferation, angiogenesis and metastasis. As such, anticoagulation may have an anti-neoplastic effect [94]. However, still, no sufficiently powered studies support the use of LMWH to improve survival in these patients. More clinical trials are required, particularly involving homogenous groups of patients in terms of the type and stage of cancer [94-97].

Thromboprophylaxis (primary prevention) must be considered in ambulatory, hospitalization and in post-surgery settings [92,93]. However, compared with treatment, thromboprophylaxis for ambulatory patients is more controversial with less evidence of survival benefits [4]. In the clinical setting, the Khorana score is the most studied CAT risk assessment tool. This validated score uses laboratory parameters, including cancer site, leukocyte, platelet and haemoglobin counts and BMI to categorise patients according to VTE risk (high, intermediate or low risk) [98,99]. Unfortunately, the Khorana score has a low sensitivity [4,100-103]. Other risk assessment models have been developed. For instance, for VTE prediction specifically in multiple myeloma patients, the most studied scores are

IMPEDE-VTE, SAVED and more recently PRISM, all with limitations [104-106]. As result, VTE risk stratification and thromboprophylaxis remain a subject of research.

Summing up, CAT is a complex disorder that results from alterations in blood flow, blood vessel integrity and blood composition [16]. Given its high incidence and detrimental impact, a better knowledge of pathogenesis is essential to improve prophylaxis and treatment. Along the way, this research may impact cancer investigation itself by allowing a better understanding of the pathways involved. On the other hand, it may positively affect overall survival and patient quality of life by decreasing the second cause of death in cancer [33].

3. Cachexia in the setting of malignancy

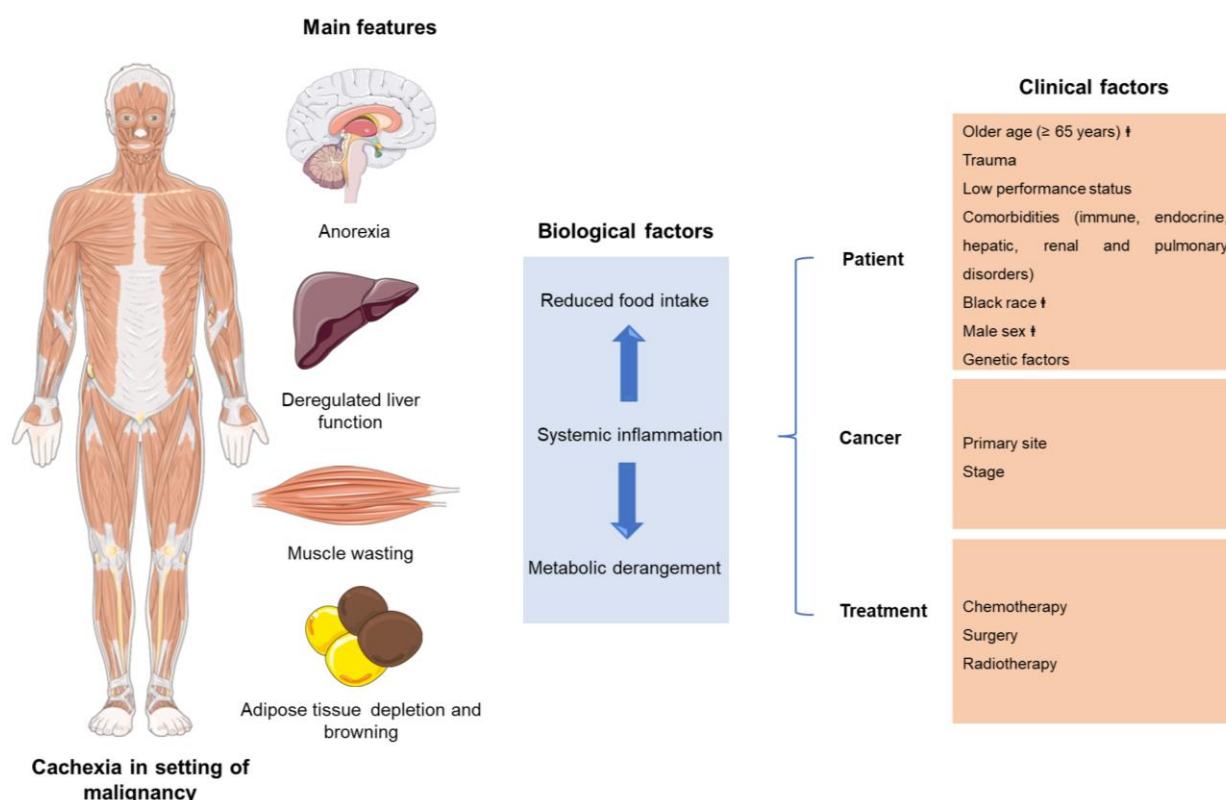
Cachexia is a term that originated from the Greek words *Kakos* and *hexus*, meaning “bad condition”. The disease, which was first recognised by Hippocrates more than 2000 years ago, is seen in patients with chronic illnesses, including, Crohn's disease, acquired immunodeficiency syndrome (AIDS) and cancer [8,107]. Characterised as a chronic syndrome of complex aetiology, cachexia is a continuing wasting of skeletal muscle mass, with or without loss of adipose tissue mass, that leads to physical disability, and that can only be partially reversed by nutritional support and physical therapy [8,108]. Sarcopenia, starvation, or malabsorption are resembling conditions often mistaken for cachexia. They all can share features such as weakness and systemic inflammation, yet they are different entities with distinctive pathophysiology. Nonetheless, a person with cachexia can also be affected by these other conditions [8,109].

Although with no consensus, the diagnosis of CAC is recommended to be made considering either a weight loss higher than 5% in six months, or a weight loss higher than 2% if the patient's BMI is less than 20, in the absence of starvation, or a weight loss superior to 2% and an appendicular skeletal muscle index (ASMI; the sum of the lower and upper extremities lean muscle mass adjusted for height) agreeable with sarcopenia ($<5.45\text{kg/m}^2$ in female patients and $<7.26\text{kg/m}^2$ in male patients) [8,108]. The standard tool to determine ASMI is dual-energy X-ray absorptiometry (DXA), a clinical imaging method that in addition to lean mass, evaluates fat mass and bone mineral density using low radiation [110,111]. A direct measure of muscle mass is recommended since CAC can simultaneously occur with conditions that mask weight loss, such as fluid retention, obesity and large tumours [8,108]. In the setting of malignancy, cachexia affects more than half of the patients, particularly those with advanced disease stages (up to 80%), being responsible for over 20% of cancer-associated deaths [112,113]. Besides the impact on overall survival, CAC

consequences include impaired quality of life due to reduced physical, emotional and social well-being, as well as reduction of both tolerance and response to anti-neoplastic therapy, with reports of increased chemotoxicity and predisposition to postoperative complications, which are associated with shortened disease-free survival [8,108,114-116]. To be noted, CAC patients may also present wasting of cardiac muscle leading to organ dysfunction, which contributes to asthenia and can be potentially deadly [8,117-119].

3.1. Pathophysiology

Like CAT, clinical risk factors of CAC can be categorised as related to patient, tumour biology and oncological treatments (**Figure 5**). The former may include advanced age, black ethnicity and male gender, however, the existing evidence concerning the effect of these factors is somewhat scarce, requiring further investigation [120-123]. To be noted, muscle wasting among individuals at an advanced age (≥ 65 years old) is particularly attributed to sarcopenia. Nevertheless, in this age group, people are also at a higher risk of developing cachexia, mostly likely due to the substantial prevalence of comorbidities that favour muscle wasting [120]. Genetic markers (particularly genetic polymorphisms implicated in inflammatory responses and metabolic pathways), low-performance status, physical trauma, infection, systemic inflammatory response syndrome (SIRS) and endocrine abnormalities such as insulin resistance, thyroid dysfunction, adrenal insufficiency and hypogonadism are also important risk factors [107,124-127]. For instance, CAC patients do not present lowering levels of thyroid hormone triiodothyronine (T3) as observed in individuals with non-malignant weight loss and non-CAC patients, suggesting a role of these hormones in the disease pathogenesis [128]. In addition, kidney failure, chronic obstructive pulmonary disease (COPD) and inflammatory bowel disease are other risk determinants, particularly in cancer patients [129-131].



Chapter IC - Figure 5. Aetiological factors of cachexia in oncological patients. Patient, tumour biology and anti-neoplastic treatment-related factors are known to induce a systemic inflammatory state, which has a central role in muscle wasting. † Scarce evidence. The figures include pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

As for cancer-related factors, CAC incidence differs mainly according to the tumour type and stage. Patients with pancreatic carcinoma are the most affected by CAC, likely due to the decline in insulin generation and its anabolic capacity [132]. The disease affects over 80% of patients facing pancreatic and gastric cancers; in non-Hodgkin lymphomas, colon, lung and prostate cancers, CAC frequency is nearly 50%, while in breast neoplasia, advanced head and neck tumour, sarcoma, and in some subtypes of leukaemia, almost 40% of patients develop this syndrome [8,133]. In general, the disease frequency seems to be higher when the tumour compromises ingestion, digestion and nutrient absorption [127].

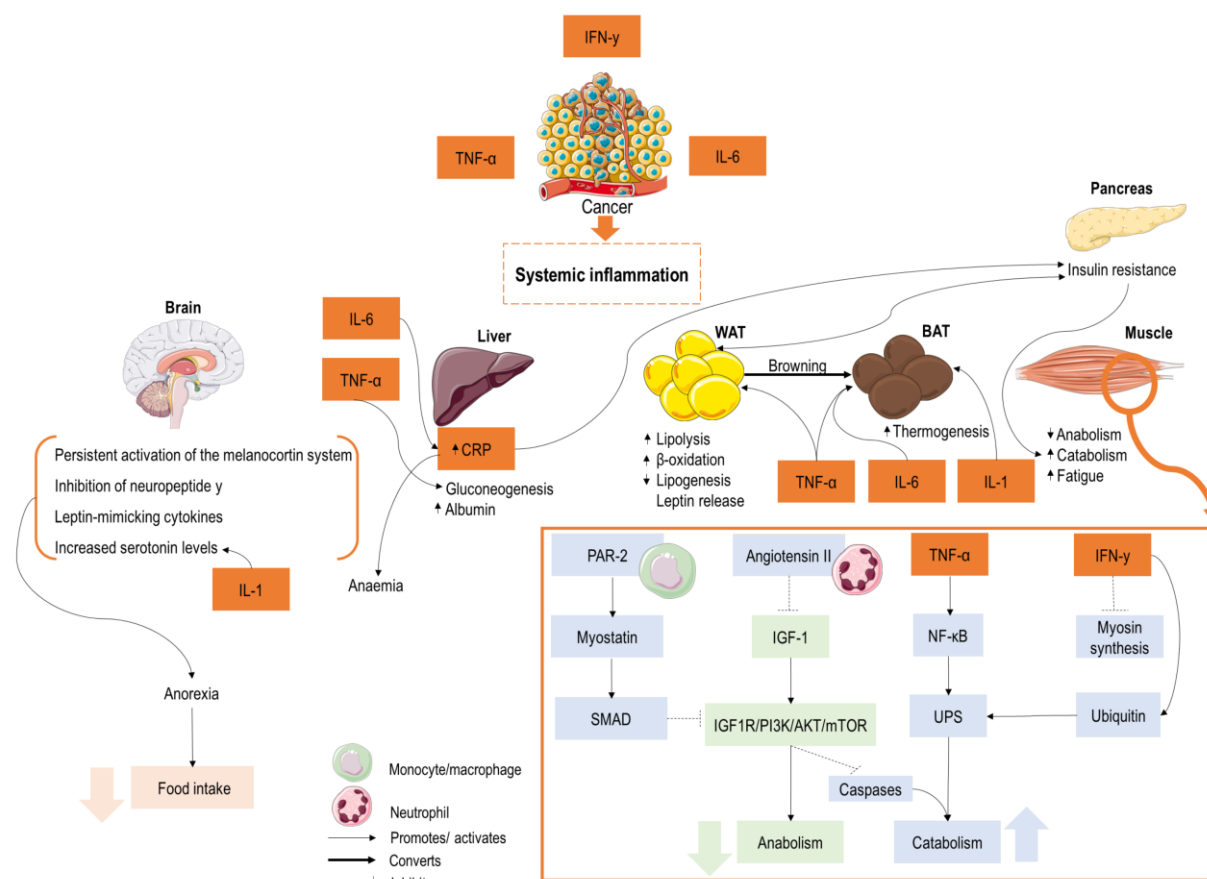
Cancer treatments also contribute to CAC development and progression. For instance, chemotherapy may induce the release of pro-cachectic molecules that are related to fatigue and inflammation [112]. The pro-cachectic chemotherapeutic agents most recognised are platinum salts, particularly cisplatin, as they activate pro-cachectic cytokines, myostatin, the NF- κ B signalling pathway and high emetogenicity [113]. Moreover, surgery and radiotherapy can also contribute to CAC due to the induction of a high catabolic state,

mucositis or alteration in the gastrointestinal tract [108,134]. Given the central role of inflammation in CAC, it would be reasonable to consider cancer immunotherapy as a potential risk factor due to its associated spectrum of inflammatory toxicities, which can affect any body organ [135]. However, to the best of our knowledge, this association is poorly explored, and the existing evidence concerns only general skeletal muscle weakness induced by this treatment [136]. Likewise, besides megestrol acetate, little is known about the impact of cancer hormone therapy on CAC [137-139].

In terms of the biological mechanisms, although only partially understood, CAC is thought to be the combination of reduced food intake and abnormal metabolism. In the center is a pro-inflammatory state, which not only drives a neuroendocrine response that leads to anorexia (the underlying cause of reduced food intake) but also induces a metabolic derangement responsible for the degeneration of muscle mass and adipose tissue (**Figure 5**). The pro-inflammation state is stimulated by tumour-derived factors and pro-inflammatory cytokines from both host and tumour cells, alongside a diminished release of anti-inflammatory mediators [8]. Relevant pro-inflammatory cytokines implicated in CAC development include TNF- α (also known as cachectin), IL-1, IL-6, IL-8 and interferon- γ (IFN- γ), all playing key roles in several biological pathways and different organs [140].

3.2. Role of inflammation

Like in thrombosis, inflammation plays a critical role in CAC pathogenesis, inducing both anorexia and metabolic derangement. Inflammation-induced anorexia occurs through the persistent activation of the melanocortin system, providing an anorexigenic (i.e., appetite suppressor) signal, and through the inhibition of neuropeptide Y in the hypothalamus, preventing its orexigenic (i.e., appetite stimulant) effect [141]. Pro-inflammatory cytokines can also induce anorexia by acting in the hypothalamus mimicking leptin, an adipose-derived hormone that regulates energy homeostasis and allows body weight control [142]. Despite controversial results, IL-1 is also thought to exert an anorexigenic effect by increasing serotonin levels, which causes early satiety [8,143]. Together, cytokines and neuropeptides may impair the hypothalamus's response to energy imbalance and peripheral metabolic signals (**Figure 6**) [141].



Chapter IC - Figure 6. A simplistic view of cancer-associated cachexia and the underlying mechanisms. Cancer cells release continuously pro-inflammatory cytokines into the bloodstream, which can cause metabolic derangement in several organs and, consequently, lead to cachexia. Abbreviations: IL, interleukin; TNF- α , tumour necrosis factor-alpha; CRP, C-reactive protein; IFN- γ , interferon- γ ; WAT, white adipose tissue; BAT, brown adipose tissue; PAR-2, proteinase-activated receptor 2; SMAD, small mothers against decapentaplegic; IGF1R, the insulin-like growth factor-1 receptor; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; NF- κ B, nuclear factor kappa B pathway; UPS, ubiquitin-proteasome pathway. The figures include pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).

The inflammation-promoted metabolic derangement is a key mechanism underlying muscle mass degeneration, in addition to lipolysis and adipose tissue browning (**Figure 6**) [8]. Muscle homeostasis is regulated by a balance between protein synthesis and breakdown. Pro-inflammatory cytokines can disrupt this balance causing muscle atrophy by either reducing the synthesis of muscle proteins (hypoanabolism) or promoting their breakdown (hypercatabolism) [144]. There are four major signalling pathways involved in muscle atrophy, namely the insulin-like growth factor-1 receptor (IGF1R)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB/AKT)/mammalian

target of rapamycin (mTOR) signalling, which is an anabolic pathway (**Figure 6**), as well as the autophagy/lysosomal, calcium/calpain and ubiquitin-proteasome (UPS) pathways, which mediate protein catabolism. All these pathways are under the influence of pro-inflammatory mediators. For instance, TNF- α , through the stimulation of the NF- κ B pathway, can trigger the ubiquitination of muscle proteins followed by their breakdown through UPS [8,145]. This process leads to muscle degeneration and favours hepatic gluconeogenesis, which fuels tumour progression [145]. The cytokine IFN- γ is thought to be also implicated by synergizing with TNF- α , individually activating the expression of ubiquitin and inhibiting the synthesis of myosin, a major muscle structural protein [8,146]. Also involved in muscle wasting is IL-6, which stimulates the liver production of acute phase proteins, including CRP, which in turn induces a prolonged inflammatory state leading to both anorexia and metabolic derangement (**Figure 6**) [8,147]. Elevated IL-8 circulating levels have been associated with CAC, even though it does not have direct participation in cachexia induction, at least according to the current evidence [8,148].

Beyond pro-inflammatory cytokines, other CAC key players are worth mentioning, including but not limited to, neutrophils, angiotensin II, myostatin and PARs (**Figure 6**). Neutrophils produce a set of proteases, including Cathepsin B, which can generate angiotensin II. Among other roles, angiotensin II can lower the levels of insulin-like growth factor-1 (IGF-1) in skeletal muscle [149]. Since IGF-1 stimulates the IGF1R-PI3K-AKT-mTOR signalling pathway, which in turn decreases muscle protein degeneration and favours protein synthesis, by decreasing IGF-1 levels, angiotensin II contributes to muscle wasting [150]. Inclusively, TNF- α -induced aggregation of neutrophils and macrophages in skeletal muscle reflects a significant critical CAC status [145]. Regarding myostatin, it is a growth factor that activates SMAD 2/3 proteins (SMAD; small mothers against decapentaplegic). These proteins are known to inhibit AKT, thus blocking the IGF1R-PI3K-AKT-mTOR pathway. Indeed, several studies confirm the involvement of this signalling pathway in muscle wasting. Conversely, this pathway is the target of some anticancer drugs, which constitutes a challenge in managing metabolic side effects [151]. As for PARs, they participate in the pro-inflammatory cascade underlying CAC pathogenesis. Namely, PAR-2 can induce the polarization of pro-inflammatory M1 macrophages, which has been reported in insulin resistance and adipose tissue inflammation, both linked with cachexia [85]. Furthermore, PAR-2 activation may directly promote the myostatin and/or growth and differentiation factor 15 (GDF15) signalling also involved in CAC [84].

3.3. Treatment

At cancer diagnosis, assessing nutritional status should include an objective assessment of body weight and its changes during the preceding months, body composition

(particularly muscle mass), food intake with a focus on energy and proteins, performance status and information regarding the presence and degree of systemic inflammation [108,152,153]. Healthcare professionals should be aware of CAC to screen it and signalise the patients at risk regularly (each three to six months and at beginning of a new anti-neoplastic strategy). Due to the clinical implications, CAC management should be initiated as early as possible, and in a multidisciplinary manner given its multifactorial aetiology [153,154].

After CAC diagnosis, treatment is mandatory. The disease demands a holistic approach with pharmacology and non-pharmacological treatments. In addition to control of secondary causes, such as nausea, therapy should be targeted towards reduced nutritional intake, physical inactivity and inflammation-derived metabolic alterations with the combination of appetite stimulation, proper nutritional support, exercise and use of anti-inflammatory agents [116,155]. Regarding non-pharmacologic measures, they start with information and education of cancer patients and their caregivers [154]. Psychological, nutritional, physical rehabilitation, and social and palliative care support are recommended. The importance of physical training with adapted aerobic, anaerobic, and flexibility muscle exercises, three times per week, is gaining more relevance as more is known about CAC [153]. Directed at nutritional support, the oral route should be privileged, with percutaneous endoscopic gastrostomy, enteral tube and parenteral nutrition being evaluated case-by-case. Nutrition should consist of at least an uptake of a minimum of 25-30 kcal/kg body weight (BW)/day and 1.2 g protein/kg BW/day with fat in 50% of the non-protein calories, divided into a high number of small meals with recourse to supplements if necessary for reaching the daily goals. Patients under chemo- or radiotherapy should be offered n-3 fatty acids (also with anti-inflammatory properties) to maintain body weight and quality of life [116,152].

Unfortunately, the disease cannot be reversed by nutritional support and there is no single unique effective pharmacological drug [3]. Under investigation are several appetite stimulators, metabolism and cytokine modulators [156]. Corticosteroids have consistent benefits in increasing appetite, but only for a short period of up to 2-3 weeks and additionally present several side effects. Progestins like megestrol acetate, despite the solid results, have an impact solely on appetite and body weight (potentially through reduction of IL-6 and other pro-inflammatory cytokines), not affecting muscle mass, quality of life or physical function [3,65,153,157,158]. Another appetite stimulator is anamorelin. The benefit of this drug, a selective agonist for the ghrelin receptor, has been evaluated in several clinical trials for CAC treatment [156,159]. Overall, anamorelin treatment seems to increase appetite and body weight and improve quality of life. However, the drug was not associated with an improvement in muscle function or overall survival of the patients [156]. Enobosarm and

espidolol are two metabolism modulators that are also under investigation. The former is a selective modulator of the androgen receptor with anabolic effects in both bone and muscle [156,160]. Based on a randomised controlled trial, this drug is associated with an improvement in lean body mass, however with no effect in restoring muscle function [161]. Espindolol is a β_1/β_2 adrenoceptor antagonist which presents anti-catabolic and pro-anabolic effects in addition to stimulating appetite [162]. A phase II trial showed that this drug could improve lean body mass, body weight and strength, with, however, no differences observed in fat mass and no benefit in terms of overall survival [163]. Also, the efficacy of cytokine modulators in restoring muscle function has been tested. The list includes, for instance, thalidomide and etanercept, which target TNF- α [156]. For other drugs, such as medical cannabis, androgens, olanzapine, nonsteroidal anti-inflammatory drugs (NSAIDs), metoclopramide or domperidone, the data are either heterogeneous or disappointing, not being recommended for CAC management [153,164]. Nevertheless, to help restore muscle function, combining these drugs with physical exercise and other therapeutic approaches could be an option [156]. Furthermore, many phase I trials of other drugs are being conducted (<https://clinicaltrials.gov/>).

Summing up, CAC is a complex and life-threatening syndrome resulting from reduced food intake and abnormal metabolism, underlined by a pro-inflammatory state. Prevention and treatment demand a multidisciplinary approach, but to this day there is no single effective treatment [153,154].

4. Thrombosis and cachexia in cancer context: is there a relation?

Thrombosis and cachexia are common complications in cancer patients, being leading causes of cancer-related deaths, after cancer progression. The two events may act together, like two partners in crime, worsening the patient's prognosis, however, there is no scientific research exploring a possible relationship between them [165]. To identify possible shared pathophysiological mechanisms, clinical and biological factors were reviewed.

Both CAT and CAC tend to increase in prevalence, and unfortunately, their pathogenesis is still not fully understood [5,13]. Both events are paraneoplastic syndromes that can precede cancer diagnosis and are also at higher risk at diagnosis and progressions and in more advanced stages, raising awareness for deterioration in prognosis [24,155]. Furthermore, they might cause delays or dose reductions in cancer treatments, representing a major burden on the health system [9,22]. In terms of aetiology, CAT and CAC shared risk determinants that are related to the patient, tumour and treatments.

Regarding patient-related factors, although further investigation is mandatory, the list seems to include mainly advanced age, Black race, physical trauma and low-performance status [98,121,125,166,167]. Likewise, comorbidities such as hepatic, kidney, immune and pulmonary diseases may also favour the development of both conditions [129,130,168-170]. For instance, liver dysfunction, a possible consequence of malignant diseases and systemic anti-neoplastic treatments, can lead to changes in the expression levels of acute-phase (for instance, CRP and FI) and coagulation proteins (including the pro- and anti-coagulant factors) [171]. As such, this condition may promote pro-cachectic and pro-thrombotic cascades, with a pro-inflammatory signal underlying both. To be noted, hepatic and cardiovascular damage can also be a consequence of CAT and CAC [8,166,171]. In opposition, although further investigation is necessary for clarification purposes, patient sex seems to pose a distinct impact on these conditions. While female cancer patients seem to be at a higher risk of thrombosis, their counterparts might be more prone to CAC [16,122,172]. Regarding thrombosis, sex hormones (either endogenous or exogenous) are thought to influence the expression levels of coagulation and inflammation factors, which might explain the inversion in the thrombotic risk pattern according to age (until 50 years old (childbearing phase), women are more susceptible than men, with the opposite being observed among older patients) [173,174]. As for cachexia, the effect of sex hormones is most likely due to their impact on metabolism and muscle fibre composition, both impacting muscle homeostasis [122]. Importantly, the influence of these hormones in CAC could be also age-dependent, highlighting the need for further studies to explore the underlying mechanisms. On top, cancer may induce a hypogonadism state that can influence the levels of these hormones. Furthermore, sex differences include not only sexual dimorphism (differences in genes and hormones) but also gender differences (distinct behaviour and societal attitudes), which also required further investigation [175].

In terms of tumour-related factors, CAT and CAC are more prevalent among patients with pancreatic, esophagogastric and lung cancers, tumour types frequently diagnosed at advanced stages and associated with bad prognosis [18,150,176,177]. Of note, patients facing these tumours, particularly at the end of life, suffer from an increased number of adverse events, which can partially explain the high incidence of CAT and CAC [115]. Nonetheless, these tumours are usually associated with a systemic and chronic pro-inflammatory state, which is a pillar stone in thrombosis and cachexia development [9,178,179]. In addition, other shared tumour-related factors might exist since tumour-related determinants of CAC are poorly comprehended.

Regarding the impact of treatments, it is important to analyse separately the effect of cancer treatments on CAT and CAC onset, treatments for CAT that may interfere with CAC and vice versa. Concerning cancer treatments, chemotherapy, particularly a cisplatin-based

regimen, may provoke both events since it leads to an increase in the circulating levels of pro-coagulant proteins and/or a decrease in the synthesis of anti-coagulant ones, and an enhanced release of pro-cachectic mediators. Likewise, surgery and radiotherapy, although not to the same extent, are also associated with a high risk for CAT and CAC [16,118,180,181]. The former through vascular damage and generation of a pro-inflammatory state, and the latter by causing alterations in the gastrointestinal tract in addition to vascular damage. Of note, besides anti-neoplastic treatments, treatments for CAC may also promote CAT, namely Megestrol Acetate and corticotherapy by altering hormonal balance [61,65]. External devices for improving nutritional support, like central venous access for parenteral nutrition and percutaneous endoscopic gastrostomy, may also increase the risk of thrombogenesis as well, due to possible vascular damage [2,182]. As for the impact of CAT treatments on CAC, little is known. The only possible relation described was between anticoagulation therapy with LMWH and its anti-inflammatory effect and endothelial protection, which theoretically may prevent CAC [183].

From a biological perspective, based on the described mechanisms underlying these events, inflammation in a setting of metabolic and endocrine derangement seems to be the potential link between CAT and CAC [33]. Regarding inflammation, among several cytokines and immune cellular mediators, TNF- α , IL-6 and monocytes/macrophages appear to be the major shared key players. The pro-inflammatory cytokine TNF- α is an important regulator of body mass weight implicated in obesity and cachexia [184]. The former is a well-known VTE risk factor, putatively due to the chronic inflammation state underlying this condition and its related metabolic disorders (metabolic syndrome, type 2 diabetes mellitus and insulin resistance) [184,185]. Despite the dichotomy, obesity and cachexia share some mechanisms, including systemic inflammation, skeletal muscle atrophy and metabolic disorders [184]. Furthermore, cachexia can affect individuals all across the BMI spectrum, meaning that obese subjects can also have cachexia [186]. Under physiological conditions, TNF- α works as an adipostat informing the central nervous system about the size of the fat mass, allowing the control of the body weight. Although controversial, it is postulated that in cachexia, TNF- α is overexpressed due to pathological conditions (e.g., cancer and infections, with TNF- α being released by tumour cells and macrophages), while in obesity, adipocytes release this cytokine, which then fails to work as an adipostat, consequently not allowing control of energy expenditure and food intake [187]. Conversely, TNF- α is also involved in venous thrombogenesis. In line with the two-way association between coagulation and inflammation previously described (**Figure 4**), TNF- α overexpression can be either a cause or consequence of VTE and to blur even more the lines, it has been reported that this pro-inflammatory adipokine has both pro- and anti-thrombotic effects [188]. As for IL-6, tumour cells can release this cytokine in the

bloodstream, triggering pro-cachectic cascades, as well as inducing platelet production and contributing to the augmentation of pro-thrombotic potential through inflammation. Concordantly, CAC patients can present a high platelet count and increased thrombin generation [121,189]. Inclusively, platelet count was recently found to be strongly and inversely associated with a 1-year survival of CAC patients, meaning that thrombogenesis may favour and/or co-occur with CAC development and progression [190]. Furthermore, platelet activation, in addition to other thrombo-inflammatory processes (for instance, monocyte expression of TF and endothelium exposure of adhesion molecules), is influenced by the NF- κ B signalling pathway, which, as previously stated, has also a pivotal role in muscle atrophy [8,83,145].

Apart from cytokines, monocytes/macrophages seem to be the main immune cell mediator presented in the setting of thrombosis and cachexia. They are known to express and release the pro-coagulant protein TF, mediate blood clot resolution, as well as participate in muscle wasting, insulin resistance and adipose tissue inflammation [84,85]. Furthermore, their related proteins, for instance, MCP-1 (also known as CCL2) and PAR-2, are also commonly reported to be associated with coagulopathies and muscle atrophy. The former regulates the migration and infiltration of monocytes/macrophages, while the latter can induce macrophage polarization [85,191-193]. Other pro-inflammatory components reported in both diseases, but with less evidence, are IL-1, IL-8, CRP, selectins and neutrophils [8,10,194,195].

Metabolism derangement in conjunction with endocrine alterations, although more discussed in the setting of cachexia, has also a role in thrombogenesis, with metabolic syndrome, type 2 diabetes mellitus and insulin resistance being often reported among VTE patients. These disorders can be either a cause or consequence of CAT and CAC, most likely because of pro- and anti-inflammatory and pro-thrombotic adipokines [184,185,196]. Furthermore, deregulated levels of thyroid hormone T3 and sex hormones seem to also be implicated in the pathogenesis of both CAT and CAC, as previously stated [75,128,173]. Another important hormone is leptin, which in addition to anorexigenic actions, it is reported to bind and activate platelets, as well as stimulate pro-coagulant and anti-fibrinolytic proteins. However, whether these effects might explain the VTE development among obese patients is controversial, and, to the best of our knowledge, the association is not explored among CAC patients, who present lower circulating levels of leptin compared to obese patients, as the hormone is proportional to fat mass [142,197-199]. To be noted, regardless of the adipose tissue release of leptin, the anorexigenic signal in the CAC context may be essentially provided by pro-inflammatory cytokines that mimic leptin in the hypothalamus [142]. Importantly, malnutrition as a consequence of anorexia may also impact the levels of important nutrients and ions implicated in haemostasis, including calcium (FIV), vitamin k

and zinc, and may lead to anaemia that, in its turn, is related to thrombosis (one of integrated component of the Khorana Score), as shown in **Figure 3** [8,98,115,200].

In summarizing, the existing evidence supports that CAT and CAC may indeed be related as they share pivotal mechanisms (chronic inflammation, metabolic derangement and hormone abnormalities). However, whether one condition can promote or prevent the other, or even influence each other severity remains to be clarified. This is relevant given the demand for improvements in the prevention, diagnosis and management of these conditions alongside our knowledge gaps concerning CAT and CAC. Awareness of health professionals is important and regular assessments for CAT and CAC should be made [154,201]. Investigation of risk and prognosis assessment tools is mandatory [3,166]. The management of these diseases should be made in a multidisciplinary context, with a multitude of healthcare professionals and medical specialities, and properly informing the patients and their caregivers [92].

5. Conclusion and future perspectives

Thrombosis and cachexia are very common complications among cancer patients, imposing a detrimental impact on both length and quality of life. Until now, the number of oncological patients affected by both conditions is unknown, and a potential relationship between them has not been explored. Nonetheless, according to existing evidence, CAT and CAC appear to share several risk determinants. Inflammation alongside metabolic and endocrine derangement seems to be the putative missing link between these conditions, with several key players influencing, in one way or the other, thrombotic and cachectic cascades. Inclusively, these key players may serve as potential predictive markers and therapeutic targets for both conditions. However, despite these noticeable similarities with a possible relationship being present, it is still unclear whether a positive or negative association exists between the incidence of CAT and CAC. If negative, factors that determine if a patient is more likely to have one condition rather than the other need to be investigated to improve the screening methods, allowing better cancer patient management and better use of health resources. On the other hand, if the association is positive, in addition to combined screening methods, the benefit of treatment approaches considering both conditions, and with a focus on anti-inflammatory and anti-coagulation drugs, should be evaluated. Inclusively, ameliorating CAC may reduce the risk of CAT since cachexia is associated with reduced mobility, which is one of the many risk factors linked to thrombosis. Given these clinical implications, epidemiological studies exploring the incidence of both

conditions among oncological patients are crucial to determine if indeed there is a connection between CAT and CAC.

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CRedit authorship contribution statement

Literature search and collection, JLP and VT; writing, JLP, VT and BVN; revision, JLP, VT, BVN, JMOS, CBG, AA, AAK and RM; supervision, RM. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

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CHAPTER ID – Hereditary Thrombophilia in the Setting of Malignancy

This chapter includes the following paper:

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Impact of hereditary thrombophilia on cancer-associated thrombosis, tumour susceptibility and progression: a review of existing evidence

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Abstract: Venous thromboembolism (VTE) is a cardiovascular disorder frequently diagnosed among cancer patients. Aside from being common, VTE severely deteriorates the prognosis of these patients as they face a higher risk of morbidity and mortality, which makes clinical tools able to identify the patients more prompt to thrombogenesis very attractive. Over the years, several genetic polymorphisms have been linked with VTE susceptibility in the general population. However, their clinical usefulness as predictive biomarkers for cancer-related VTE is yet unclear. Furthermore, as a two-way association between cancer and VTE is well-recognised, with haemostatic components fuelling tumour progression, haemostatic gene polymorphisms constitute potential cancer predictive and/or prognostic biomarkers as well. Thus, in this article, we review the existing evidence on the role of these polymorphisms on cancer-related VTE and their impact on cancer onset and progression. Despite the promising findings, the existing studies had inconsistent results most likely due to their limited statistical power and population heterogeneity. Future studies are therefore required to clarify the role of these polymorphisms in the setting of malignancy.

Keywords: venous thromboembolism; neoplasms; genetic polymorphism; incidence; prognosis

1. Introduction

Chronic diseases are heterogeneous conditions with persevering effects on patient quality of life [1]. Among them, cardiovascular disorders and cancer, both diseases with complex pathophysiology, are the most frequent cause of death, with the current evidence pinpointing a steady increase in their prevalence worldwide, which drastically affect healthcare costs [1-3].

Venous thromboembolism (VTE), encompassing deep venous thrombosis (DVT) and pulmonary embolism (PE), is one of the leading cardiovascular disease-related death in high-income countries [4]. Although common in the general population, VTE is particularly frequent among cancer patients, accompanying or even preceding malignancy [5]. Namely, the disease affects approximately 20% of these patients, with cancer being associated with a ninefold increased risk of VTE development (OR=8.9, 95% CI, 7.2–10.9) [6]. Even in the absence of cancer-related VTE (generally defined as events occurring six months before to two years after cancer diagnosis), most cancer patients present systemic blood hypercoagulability at some point of clinical intervention. Unfortunately, the incidence of this condition has been gradually increasing over the last years, most likely due to novel anti-cancer therapies (e.g., antiangiogenic therapy, which associates with an increased

thrombotic risk), improved patient survival and high-resolution imaging for VTE diagnosis [6-8]. Besides the high incidence, VTE is also associated with substantial morbidity and mortality in these patients, being, in fact, their second most common cause of death, after cancer itself [9]. Furthermore, treating VTE in a setting of malignancy is challenging as a balance between efficacy and safety of antithrombotic therapy is hard to achieve, which results in a higher rate of VTE recurrence or bleeding complications in cancer patients compared to the general population [9, 10].

Apart from VTE itself, the detrimental effect of the disease on the prognosis of cancer patients is most likely also attributed to disrupted haemostatic mechanisms, which lead to VTE development but also promote tumour growth and dissemination as multiple interactions between tumour cells and haemostatic components have been reported. In fact, cancer-related VTE seems to be a pathologic entity that reflects tumour aggressiveness [11]. Together, the data suggest that virtually all cancer patients at high risk should receive thromboprophylaxis, given the disease's negative impact on patient outcome. In fact, according to European Society for Medical Oncology (ESMO) and American Society of Clinical Oncology (ASCO) guidelines, routine thromboprophylaxis is not recommended in ambulatory patients, particularly the ones under adjuvant chemotherapy and/or hormonal therapy due to the increased risk of bleeding and possible drug-drug interactions, however, it might be an option for those at high risk [10, 12]. This highlights the importance of identifying reliable predictive biomarkers for cancer-related VTE for a more personalised thromboprophylaxis.

In terms of VTE aetiology, the disease development results from a complex interplay between acquired and genetic determinants that together disturb the haemostatic balance towards thrombosis [13]. The acquired risk factors are well-recognised and among cancer patients they can be categorised as related to the patient (e.g., advanced age and immobilization), cancer behaviour (e.g., disease primary site, histology and size) and oncological treatments (e.g., surgery and chemotherapy) [14]. Nevertheless, over 50% of the variation in VTE susceptibility is thought to be explained by genetic determinants (i.e., hereditary thrombophilia) [15]. In the general population, several VTE-related genetic factors have been identified, including mutations, particularly in genes implicated in anticoagulation (*SERPINC1*, *PROC* and *PROS1*), but mostly genetic polymorphisms [11]. Within the Caucasian population, the most common polymorphisms are Factor V Leiden (FVL) (*F5* rs6025) and prothrombin G20210A (*F2* rs1799963), which are respectively associated with reduced clearance of activated FV by activated protein C (a condition known as APC resistance) and increased expression levels of prothrombin, both favouring thrombogenesis [16-18]. Individually, about 5% of the Caucasian population has the rs6025 risk allele (T allele; R506Q allele), which is associated with a two-to-five-fold higher VTE

risk [19, 20]. As for rs1799963 risk allele (A allele), it is less common and correlates with a 2.80-fold increase in disease risk [17, 18]. Other genetic polymorphisms, including *ABO* rs8176719 (frameshift SNP), *FGG* rs2066865 (downstream gene SNP) and *F11* rs2036914 (intronic SNP), among others reported by genome-wide association studies (GWAS), were discussed in detail elsewhere [11].

Although VTE genetic factors were initially identified in the general population, there is an increasing interest in studying their predictive value concerning cancer-related thromboembolism. Besides, given the roles of haemostatic components in cancer growth and progression, these genetic determinants may also be potential cancer predictive and prognostic biomarkers [21]. In this context, this review article aims to analyse the existing evidence regarding the value of VTE genetic markers as predictors of cancer-related VTE and as biomarkers of cancer susceptibility and progression, even in the absence of VTE, considering both solid tumours and haematological malignancies. For the article's purpose, only genetic polymorphisms were taken into consideration given their relevance at a population level. In addition to well-established VTE markers in the general population, genetic determinants associated directly or indirectly with haemostatic activity with potential predictive value of VTE were also discussed.

To review the existing evidence, articles on the role of haemostatic gene polymorphisms in cancer-related VTE, tumour susceptibility and progression published until 31st December 2021 were identified by screening the PubMed database for the co-occurrence of the terms “polymorphism”, “SNP”, “genetic”, “determinant”, “variant”, “cancer”, “tumour”, “tumour”, “neoplasia”, “venous thrombosis”, “venous thromboembolism”, “deep vein thrombosis”, “pulmonary embolism”, “thrombosis”, “thromboembolic”, “coagulation” and “clotting” in the title. In addition, pertinent publications were identified by cross-referencing from the bibliographic references of the initial articles retrieved. GWAS as well as studies that tested only polygenic risk scores were not taken into consideration. Studies with only abstracts as well as reports that did not provide a measure of statistical significance were also dismissed.

2. Genetic polymorphisms and susceptibility for cancer-related VTE

Cancer is an independent predictor of VTE. During carcinogenesis, the transcriptional program enables tumour cells to express and release a set of inflammatory cytokines, proangiogenic and procoagulant factors, and to further stimulate stromal cells to express these prothrombotic components, which contribute to thrombogenesis [22, 23]. The result, cancer-related VTE, is a common and detrimental complication among oncological patients,

and given the poor prognosis associated with this condition and its treatment challenges, a search for tools able to stratify disease risk for better thromboprophylaxis implementation has been made [11].

Indeed, some clinical prediction models have been proposed. Among them is the Khorana Score, the first and most studied VTE risk assessment tool in cancer patients [24, 25]. The score makes use of laboratory parameters that are routinely measured in the clinical setting, including platelet, leukocyte and haemoglobin counts, cancer site and body mass index as VTE predictors [25]. However, while the score has high specificity, the sensitivity is poor, which makes its implementation in clinical practice difficult [26]. To solve the poor discrimination performance, several modifications of the Khorana model, by adding and/or replacing predictors, have been reported over the years including, for instance, the PROphylaxis of ThromboEmbolism during CHemoTherapy (PROTECHT) score. Although promising, these modifications still lack validation, which highlights the unmet need for better prediction models [24].

Conversely, several genetic polymorphisms associated with VTE susceptibility, mostly single-nucleotide polymorphisms (SNPs), have been identified and validated in the general population. Most of them are located in genes involved in platelet activity, coagulation, fibrinolysis and endothelium integrity, impacting either gene expression (in a cis or trans fashion) or protein function [11]. In opposition to the general population, the role of these genetic determinants in VTE among cancer patients is not as clear, partially because genetic studies on VTE susceptibility tend to exclude these individuals [27].

To the best of our knowledge, a total of at least 44 studies were carried on to identify genetic polymorphisms associated with cancer-related VTE, most of them by attempting to validate the well-established VTE-related variants found in the general population. Among these studies, 25 reported significant associations, which are summarised in **Table 1**. As for the reports with no significant results, their specific data are outside the scope of this discussion [22, 28-45].

The first study presenting significant associations was performed in 2002 by Pihusch *et al.* [46]. The authors assessed the role of *F5* rs6025 (missense SNP), *F2* rs1799963 (3 prime UTR variant) and *MTHFR* rs1801133 (missense SNP) in gastrointestinal carcinoma-related VTE in a German Caucasian population. In the study, only *F5* rs6025 was found to be a potential predictive biomarker, increasing the disease risk more than fourfold (CT genotype; $P=0.026$; **Table 1**). Considering this SNP, additional 23 studies have tested it in the setting of malignancy, with 13 reporting a significant association. Although the studies were performed in different populations from across the globe, most of those with significant results are related to the Turkey population, where this genetic variant is particularly common. Till now, the role of *F5* rs6025 in cancer-related VTE, however, remains unclear,

with some authors suggesting that it might not play a major role [40]. Regarding *F2* rs1799963, none of the 17 performed studies was able to demonstrate a role of this genetic variant in cancer-related VTE. To be noted, Falvella *et al.* [47] found a significant association between this SNP and VTE among colorectal cancer with metastatic disease [CT genotype; aOR=8.74; 95% CI, 1.32-57.78; $P=0.025$]. However, the authors considered patients who were diagnosed with VTE events in their lifetime and not only in the setting of malignancy. Nevertheless, this variant is known to induce only a moderate blood hypercoagulation, being most likely less relevant in cancer-related VTE compared to FVL. As for *MTHFR* rs1801133, an SNP associated with hyperhomocysteinemia and thus higher VTE risk, one of eight conducted studies reported a significant association ($P=0.03$; **Table 1**) [48]. The study was performed in the Egypt population and with patients with nonmetastatic cancer.

Other VTE genetic markers that have been analysed include *F13A1* rs5985 (missense SNP), *SERPINE1* rs1799889 (*PAI-1* 4G/5G polymorphism; indel), *MTHFR* rs1801131 (missense SNP), *ACE* D/I (indel), *CYP4V2* rs13146272 (stop-gain SNP), *GP6* rs1613662 (missense SNP), *F5* rs4524 (missense SNP), *ITGB3* rs5918 (missense SNP), *F11* rs2036914 (intronic SNP), *FGG* rs2066865 (intergenic SNP), *FGB* rs1800790 (upstream gene SNP) and *ABO* rs8176719 (frameshift SNP), with significant results being reported for *MTHFR* rs1801131 ($P=0.040$), *GP6* rs1613662 ($P=0.040$), *F5* rs4524 (HR=4.4; 95% CI, 1.4-13.9), *F11* rs2036914 ($P=0.009$) and *FGG* rs2066865 (HR=2.0; 95% CI, 1.1-3.6).

Haemostatic gene polymorphisms with no previous association with the disease in the general population have also been investigated in cancer patients. The list includes variants related to *CPB2* (*TAFI*), *PROZ*, *F7*, *ITGB3*, *SERPINC1*, *F3*, *FR2* (*PAR1*) and *PLAT*. Among them, *ITGB3* rs3809865 (3 prime UTR SNP) revealed to be a potential predictor of colorectal cancer-related VTE ($P=0.0005$; **Table 1**). The polymorphism regulates *ITGB3* expression and the mRNA interaction with miRNAs, impacting levels of integrin $\beta 3$, which is involved in platelet and endothelial cell activity [49].

Given the complexity of VTE pathogenesis, the investigation was also conducted with genetic variants located within or close to genes with primary roles in angiogenesis (*VEGFA*), extracellular matrix remodelling (*MMP9*), inflammation (*NLRC5*, *TNF*, *TNFRSF17* and *IL6*), metabolism (*CYP2D6*), DNA synthesis and/ or repair (*DHFR*, *CINP*, *CDKN1A*, *CHEK1* and *XRCC5*) and regulation of signalling pathways (*ESR1*, *ESR2*, *NFKB1*, *DRD2*, *ALDH1A1* and *GAN*) [50]. In terms of significant results, a marginal association was found for *VEGFA* rs3025039 (3 prime UTR SNP; $P=0.045$; **Table 1**) [51]. This SNP seems to regulate *VEGF* expression and protein circulating level, although the association direction is controversial [52, 53]. Also thought to impact *VEGFA* expression is *VEGFA* rs1570360 (upstream gene SNP), which was found to be associated with VTE in cancer patients under chemotherapy and bevacizumab-based treatment ($P=0.003$ and

$P=0.004$, respectively; **Table 1**) [54]. These findings corroborate that VEGF may have a role in VTE onset, most likely by modifying the endothelial haemostatic properties [55]. Furthermore, significant associations were also found for *MMP9* rs3918242 (intergenic SNP; $P=0.0009$), *IL6* rs1800795 (intronic SNP; $P=0.0023$), *TNF* rs1799724 (regulatory region SNP; $P=0.003$), *CDKN1A* rs3829963 (intronic SNP; $P=0.015$) and *ESR1* rs9340799 (intronic SNP; aHR=2.17; 95%CI, 1.28-3.67) (**Table 1**) [56-59].

Finally, a special highlight should be given to a study performed by Johnson *et al.* [60] among multiple myeloma patients treated with thalidomide. The authors analysed 3400 polymorphisms with potential involvement in VTE pathogenesis, response to myeloma therapy and its side effects. As a result, a total of 18 SNPs with potential predictive value for multiple myeloma-related VTE were reported ($P<0.05$; **Table 1**). Namely, *ABCB4* rs2302387 (synonymous SNP; $P=0.041$), *CASP3* rs1049216 (3 prime UTR SNP; $P=0.006$), *CHEK1* rs506504 (missense SNP; $P=0.044$), *CINP* rs7011 (missense SNP; $P=0.036$), *DCLRE1B* rs12022378 (missense SNP; 0.023), *ERCC6* rs4253211 (missense SNP; $P=0.035$), *HMMR* rs299295 (missense SNP; $P=0.041$), *IL12A* rs582537 (intron SNP; $P=0.005$), *LEP* rs10249476 (intergenic SNP; $P=0.026$), *LIG1* rs20579 (5 prime UTR SNP; $P=0.017$), *MCAT* rs13815 (missense SNP; $P=0.010$), *NAT2* rs2410558 (intergenic SNP; $P=0.024$), *NFKB1* rs3774968 (intronic SNP; $P=0.017$), *PARP1* rs1805414 (synonymous SNP; $P=0.033$), *PPARD* rs2267669 (intronic SNP; $P=0.007$), *SERPINE1* rs2070682 (intronic SNP; $P=0.029$), *TNFRSF17* rs12922317 (intronic SNP; $P=0.010$) and *XRCC5* rs2440 (3 prime UTR SNP; $P=0.047$). The polymorphisms-related genes are implicated mostly in metabolism, immune response/inflammation, apoptosis, gene expression and/or DNA damage repair and cell motility [50]. From this set, the polymorphism with a more direct role in VTE pathogenesis is *SERPINE1* rs2070682, given the involvement of PAI-1 in fibrinolysis [61].

In general, while analysing the role of genetic polymorphisms in cancer-related VTE, the studies presented conflicting results, which could be explained by several factors. Namely, differences in population ancestry and size given the variations in polymorphisms' prevalence according to ethnicity, particularly considering uncommon variants such as FVL, as well as differences in study design (cohort vs. case-control study for instance), VTE manifestations (DVT, PE or both) and tumour biology (primary site, histology, size and stage) as different tumours seem to be associated with distinct VTE risk and thrombopathogenetic mechanisms [62]. Besides, although it is irrefutable that the genetic architecture plays a relevant role in VTE development, among cancer patients the additional factors related to tumour biology and oncological treatment may have a more preponderant impact [22]. Inclusively, as VTE is a highly complex disease, particularly in the setting of malignancy, a single genetic polymorphism is unlikely to significantly contribute to its onset.

The aim should be to identify and include multiple genetic polymorphisms in risk assessment models combined with relevant clinical factors.

It should be emphasised that most of the performed studies had a relatively small size, without also considering potential confounding factors (factors related to patient, cancer biology and treatments with roles in thrombogenesis). Inclusively, most of these studies were carried out on heterogeneous populations without a discrimination of tumour primary site, which has a major influence on thrombogenesis. Therefore, future studies validating previous findings are required. Furthermore, the impact of more common genetic variants should be investigated.

Although not the focus of this discussion, similar to inherent gene variation, genetic mutations in tumour cells, involving particularly *TP53*, *KRAS*, *EGFR*, *PTEN* and *IDH1* genes, are also shown to impact haemostasis by inducing the tumour expression of tissue factor (TF) and VEGF, and the release of pro-inflammatory cytokines and extracellular vesicles with prothrombotic effects. Whether inherent genetic architecture or tumour (epi)genetics play a more preponderant role in the development of a blood hypercoagulation state and at which disease stage remains, however, to be clarified [23].

Chapter ID - Table 1. Epidemiological studies on the role of genetic polymorphisms on cancer-related VTE

First author (year)	Country/ Ethnic background	Population characteristics	Study design	Studied polymorphisms	Main findings
Pihusch <i>et al.</i> (2002) [46]	German/ Caucasian	28 patients with gastrointestinal cancer-related VTE 147 gastrointestinal cancer patients without VTE	Case-control study	<i>F5</i> rs6025, <i>F2</i> rs1799963 and <i>MTHFR</i> rs1801133	<i>F5</i> rs6025 is a potential predictor of gastrointestinal cancer-related VTE [CT genotype; OR=4.4; 95% CI, 1.3–14.9; <i>P</i> =0.026]
Eroğlu <i>et al.</i> (2005) [63]	Turkey/ Unclear	30 patients with cancer-related VTE 68 cancer patients without VTE	Case-control study	<i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>F5</i> rs6025 is a potential predictor of cancer-related VTE [T allele; A=30.0%; B=5.8%; <i>P</i> =0.002]
Curigliano <i>et al.</i> (2006) [64]	Italy/ Unclear	25 patients with breast cancer-related DVT 50 breast cancer patients without DVT	Case-control study	<i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>F5</i> rs6025 is a potential predictor of breast cancer-related DVT in patients with advanced disease stages and under chemotherapy [T allele; aOR=6.1; 95% CI, 1.1–34.3; <i>P</i> =0.040]
Eroğlu <i>et al.</i> (2006) [65]	Turkey/ Unclear	43 patients with cancer-related VTE 81 cancer patients without VTE 100 VTE patients without cancer 100 healthy individuals	Case-control study	<i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>F5</i> rs6025 is a potential predictor of cancer-related VTE [T allele; OR=8.1; 95% CI, 2.4–27.1] ^β
Eroğlu <i>et al.</i> (2008) [51]	Turkey/ Unclear	36 patients with cancer-related VTE 60 cancer patients without VTE 120 healthy individuals	Case-control study	<i>VEGFA</i> rs3025039	A marginal association was found between <i>VEGFA</i> rs3025039 and cancer-related VTE [CT genotype; OR=2.3; 95%CI, 0.9–5.7; <i>P</i> =0.045] ^β
Johnson <i>et al.</i> (2008) [60]	United Kingdom/ European origin	180 patients with multiple myeloma-related VTE 364 multiple myeloma patients without VTE	Nested case-control study	3400 SNPs were selected and analysed based on their potential involvement on the VTE pathogenesis, treatment response and side effects of myeloma therapy	<i>ABCB4</i> rs2302387, <i>CASP3</i> rs1049216, <i>CHEK1</i> rs506504, <i>CINP</i> rs7011, <i>DCLRE1B</i> rs12022378, <i>ERCC6</i> rs4253211, <i>HMMR</i> rs299295, <i>IL12A</i> rs582537, <i>LEP</i> rs10249476, <i>LIG1</i> rs20579, <i>MT</i> rs13815, <i>NAT2</i> rs2410558, <i>NFKB1</i> rs3774968, <i>PARP1</i> rs1805414, <i>PPARD</i> rs2267669, <i>SERPINE1</i> rs2070682, <i>TNFRSF17</i> rs12922317 and <i>XRCC5</i> rs2440 are potential predictors of myeloma-related VTE under thalidomide [<i>P</i> <0.05]

Chapter ID - Table 1. Epidemiological studies on the role of genetic polymorphisms on cancer-related VTE (cont.).

First author (year)	Country/ Ethnic background	Population characteristics	Study design	Studied polymorphisms	Main findings
Eroğlu <i>et al.</i> (2008) [66]	Turkey/ Unclear	63 patients with cancer-related VTE 124 cancer patients without VTE	Case-control study	<i>MTHFR</i> rs1801133, <i>DHFR</i> rs70991108, <i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>F5</i> rs6025 is a potential predictor of cancer-related VTE [T allele; A =31.7%; B =1.6%; <i>P</i> <0.0001]
Eroğlu <i>et al.</i> (2009) [67]	Turkey/ Unclear	55 patients with cancer-related VTE 115 cancer patients without VTE	Case-control study	<i>PROZ</i> rs17882561, <i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>F5</i> rs6025 is a potential predictor of cancer-related VTE [T allele; A =27.3%; B =3.5%; <i>P</i> <0.0001]
Onitilo <i>et al.</i> (2009) [59]	United States/ Caucasian	16 patients with breast cancer-related VTE 204 breast cancer patients without VTE	Cohort study	<i>ESR1</i> rs2234693 and rs9340799, <i>ESR2</i> rs4986938 and <i>CYP2D6</i> rs1065852	<i>ESR1</i> rs9340799 is a potential predictor of breast cancer-related VTE under tamoxifen [G allele; aHR=2.17; 95%CI, 1.28-3.67]
Garber <i>et al.</i> (2010) [68]	USA/ Mixed	124 patients with breast cancer-related VTE 248 breast cancer patients without VTE	Case-control study	<i>F5</i> rs6025	<i>F5</i> rs6025 is a potential predictor of breast cancer-related VTE under tamoxifen [CT genotype; OR= 4.66; 95%CI, 2.14-10.14; <i>P</i> <0.001]
Almasi <i>et al.</i> (2011) [58]	Czech Republic/ Caucasian	21 patients with multiple myeloma-related VTE 90 multiple myeloma patients without VTE	Retrospective cohort study	<i>CINP</i> rs7011, <i>NLRC5</i> rs289747, <i>ALDH1A1</i> rs610529 and rs168351, <i>CDKN1A</i> rs3829963, <i>GAN</i> rs2608555 and <i>VEGFA</i> rs699947	<i>CDKN1A</i> rs3829963 is a potential predictor of multiple myeloma-related VTE under thalidomide [AC genotype; OR=3.64; 95%CI, 1.28-10.36; <i>P</i> =0.015]
Arslan <i>et al.</i> (2011) [69]	Turkey/ Unclear	33 patients with lung cancer-related DVT 33 lung cancer patients without DVT	Cohort study	<i>F5</i> rs6025, <i>F2</i> rs1799963, <i>MTHFR</i> rs1801133 and rs1801131, <i>ACE</i> D/I, <i>SERPINE1</i> rs1799889 and <i>ITGB3</i> rs5918	<i>MTHFR</i> rs1801131 is a potential predictor of lung cancer-related DVT [T allele; A =76%; B =52%; <i>P</i> =0.040]
Eroğlu <i>et al.</i> (2011) [70]	Turkey/ Unclear	16 patients with breast cancer-related VTE 50 breast cancer patients without VTE	Case-control study	<i>F5</i> rs6025, <i>F2</i> rs1799963 and <i>MTHFR</i> rs1801133	<i>F5</i> rs6025 is a potential predictor of breast cancer-related VTE under tamoxifen [T allele; A =43.75%; B =6%; <i>P</i> <0.001]
Glurich <i>et al.</i> (2013) [71]	USA/ Caucasian	19 patients with breast cancer-related VTE 74 breast cancer patients without VTE	Case-control study	<i>DRD2</i> rs2234689, <i>ESR1</i> rs9340799, <i>CYP4V2</i> rs13146272, <i>SERPINC1</i> rs2227589 and <i>GP6</i> rs1613662	<i>GP6</i> rs1613662 is a potential predictor of breast cancer-related VTE following tamoxifen [G/C/T alleles; aOR=0.19; <i>P</i> =0.04]

Chapter ID - Table 1. Epidemiological studies on the role of genetic polymorphisms on cancer-related VTE (cont.).

First author (year)	Country/ Ethnic background	Population characteristics	Study design	Studied polymorphisms	Main findings
Malaponte <i>et al.</i> (2013) [56]	Italy/ Unclear	130 patients with cancer-related DVT 190 cancer patients without DVT 215 healthy individuals	Case-control study	<i>IL6</i> rs1800795 and <i>MMP9</i> rs3918242	<i>IL6</i> rs1800795 [GG genotype; OR=2.07; 95% CI, 1.30–3.30; $P=0.0023$] ^α and <i>MMP9</i> rs3918242 [CC genotype; OR=2.60; 95% CI, 1.48–4.57; $P=0.0009$] ^α are potential predictors of cancer-related DVT
Roselli <i>et al.</i> (2013) [57]	Italy/ Caucasian	15 patients with gastrointestinal cancer-related VTE 142 gastrointestinal cancer patients without VTE 157 healthy individuals	Case-control study	<i>TNF</i> rs1800630, rs1799724, rs1800750, rs1800629 and rs361525	<i>TNF</i> rs1799724 [T allele; OR=6.51; 95% CI, 1.96–21.6; $P=0.003$] ^β is a potential predictor of gastrointestinal cancer-related VTE during chemotherapy
Bianconi <i>et al.</i> (2015) [49]	Austria/ Unclear	13 patients with colorectal cancer-related VTE 99 colorectal cancer patients without VTE	Prospective cohort study	<i>ITGB3</i> rs3809865, rs5918 and rs4642	<i>ITGB3</i> rs3809865 is potential predictor of colorectal cancer-related VTE [AA genotype; HR=7.87; 95% CI, 2.31–40.00; $P=0.0005$]
Pabinger <i>et al.</i> (2015) [72]	Austria/ Unclear	79 patients with cancer-related VTE 903 cancer patients without VTE	Prospective cohort study	<i>F5</i> rs6025	<i>F5</i> rs6025 is a potential predictor of cancer-related VTE [T allele; aHR=2.0; 95% CI, 1.0–4.0; $P=0.037$]
Wahba <i>et al.</i> (2015) [73]	Egypt / Unclear	40 patients with nonmetastatic cancer-related VTE 40 nonmetastatic cancer patients without VTE	Case-control study	<i>F5</i> rs6025, <i>F2</i> rs1799963 and <i>MTHFR</i> rs1801133	<i>F5</i> rs6025 [T allele; A =30%; B =10%; $P=0.03$] and <i>MTHFR</i> rs1801133 [T allele; A =45%; B =25%; $P=0.03$] are potential predictors of nonmetastatic cancer-related VTE
Eroğlu <i>et al.</i> (2016) [74]	Turkey/ Unclear	46 patients with cancer-related VTE 196 cancer patients without VTE	Case-control study	<i>F3</i> rs1361600 and rs3917643, <i>F5</i> rs6025, <i>F2</i> rs1799963 and <i>MTHFR</i> rs1801133	<i>F5</i> rs6025 is a potential predictor of cancer-related VTE [T allele; A =41.3%; B =4.1%; $P<0.05$]
Ferroni <i>et al.</i> (2016) [54]	Italy/ Caucasian	26 patients with cancer-related VTE 271 cancer patients without VTE 140 healthy individuals	Prospective cohort study	<i>VEGFA</i> rs699947, rs35569394, rs1005230, rs35864111, rs833061, rs13207351, rs1570360 and rs2010963	<i>VEGFA</i> rs1570360 is a potential predictor of cancer-related VTE associated with chemotherapy [A allele; aHR= 0.21; 95% CI, 0.07–0.58; $P=0.003$] ^β and bevacizumab-based treatment [A allele; HR=0.09; 95% CI, 0.01–0.86; $P=0.004$] ^β

Chapter ID - Table 1. Epidemiological studies on the role of genetic polymorphisms on cancer-related VTE (cont.).

First author (year)	Country/ Ethnic background	Population characteristics	Study design	Studied polymorphisms	Main findings
Gran <i>et al.</i> (2016) [6]	Norway/ Unclear	117 patients with cancer-related VTE 1691 patients without VTE (170 with a history of cancer)	Case-control study	<i>F5</i> rs6025 and rs4524	<i>F5</i> rs6025 [CT genotype; HR=1.9; 95% CI, 1.1-3.3] and rs4524 [TT genotype; HR=4.4; 95% CI, 1.4-13.9] are potential predictors of cancer-related VTE
Heraudeau <i>et al.</i> (2018) [75]	France/ Unclear	182 patients with cancer-related VTE 182 cancer patients without VTE	Case-control study	<i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>F5</i> rs6025 is a potential predictor of cancer-related VTE [T allele; aOR= 7.04; 95%CI, 2.01-24.63; <i>P</i> =0.002]
Jarvis <i>et al.</i> (2019) [76]	Denmark, Estonia, Finland, Iceland, Lithuania, Norway, and Sweden/ Caucasian	89 patients with acute lymphoblastic leukemia-related VTE 1163 acute lymphoblastic leukemia patients without VTE	Prospective cohort study	<i>F5</i> rs6025, <i>F11</i> rs2036914, <i>FGG</i> rs2066865 and <i>ABO</i> rs8176719	<i>F11</i> rs2036914 is a potential predictor of acute lymphoblastic leukemia-related VTE [C allele; aHR= 1.52; 95% CI, 1.11-2.07; <i>P</i> =0.009] A marginal association was also found for <i>FGG</i> rs2066865 [C allele; aHR= 1.37; 95% CI, 0.99-1.91; <i>P</i> =0.058]
Paulsen <i>et al.</i> (2020) [27]	Norway/ Unclear	626 patients with cancer-related VTE 3734 cancer patients without VTE	Case-cohort study	<i>FGG</i> rs2066865	<i>FGG</i> rs2066865 is a potential predictor of cancer-related VTE [AA genotype; HR=2.0; 95% CI, 1.1-3.6]

Highlighted SNPs represent the genetic variants that are not validated as VTE-related genetic variants in cancer-free populations. For studies that did not provide an odds ratio (OR), the frequency of the polymorphism allele or genotype in patients with cancer-related VTE (**A**) and cancer patients without VTE (**B**) were indicated. **α** results were obtained by comparing healthy individuals (without cancer and DVT) with cancer patients with DVT; **β** considering cancer patients without VTE as the reference group; Abbreviations: TE thromboembolism; VTE venous thromboembolism; DVT deep vein thrombosis; OR odds ratio; HR hazard ratio; aHR adjusted hazard ratio; CI confidence interval

3. Haemostatic gene polymorphisms and cancer susceptibility

According to recent data, 5% of individuals with idiopathic VTE present an underlying occult malignancy, which is detected within one year after VTE diagnosis [77]. Conversely, cancer patients often exhibited at diagnosis a state of blood hypercoagulability, presenting also a higher frequency of some prothrombotic genetic variants compared to the general population [46, 78]. Taken together, the evidence suggests a close relationship between haemostasis deregulation and tumour growth, indicating a putative role of haemostatic gene polymorphisms in cancer initiation. To assess whether these genetic variants may also be potential cancer predictors, at least 14 studies were carried on, regardless of patient VTE history. Among those, eight reported significant associations, which are described in **Table 2**. For the studies with no significant results, their findings are beyond the focus of this review [79-84].

Although not the primary aim of the study, Pihusch *et al.* [46] demonstrated that *F2* rs1799963 might be a risk factor for gastrointestinal carcinoma (AG genotype; $P=0.028$; **Table 2**). As previously mentioned, this SNP is known to modulate prothrombin levels. Upon prothrombin activation, there is the generation of thrombin, which in turn induces fibrin formation and platelet activation to maintain haemostasis. Beyond its haemostatic functions, thrombin, particularly through interactions with receptor PAR-1, is also thought to influence critical mechanisms in tumour initiation, including for instance cell survival, proliferation and adhesion [85-87]. Thus, it is plausible that *F2* rs1799963 by favouring thrombin synthase might contribute to cancer onset [46].

In the setting of oral squamous cell carcinoma (OSCC), Vairaktaris *et al.* [88], Vairaktaris *et al.* [89] and Vylliotis *et al.* [90] together identified as potential predictors *F13A1* rs5985, *SERPINE1* rs1799889 and *ACE* I/D. The *F13A1* rs5985 polymorphism was found to be associated with the disease at early stages, with no association with metastasis (AA genotype; OR=3.73; 95% CI, 1.23-11.33; **Table 2**) [90]. This variant seems to modulate FXIII enzyme activity altering fibrin clot properties, with the risk allele (A) being associated with smaller pores and thinner fibres in the fibrin network, which may impair fibrinolysis, supporting initial tumour cell proliferation and stroma formation [88]. As for the polymorphisms *SERPINE1* rs1799889 and *ACE* I/D, both seem to modulate the expression levels of the respective encoded proteins (PAI-1 and ACE). While PAI-1 is thought to promote epithelial-mesenchymal transition, favouring tumour cell proliferation and motility, ACE does not have a clear role in cancer initiation, with studies reporting conflicting results [91, 92]. Both *SERPINE1* rs1799889 4G/4G and *ACE* I/I genotypes were associated with an increased OSCC risk in two studies, being more preponderant in early cancer stages [89, 90].

Considering the breast cancer model, Eroğlu *et al.* [93] and Tinholt *et al.* [78] showed significant associations for *F7* rs510317 (upstream gene SNP), *F5* rs12120605 (intronic SNP), rs6427202 (intronic SNP), rs9332542 (intronic SNP) and rs6427199 (intronic SNP), *F10* rs3093261 (upstream gene SNP) and *PROCR* rs2069948 (non-coding transcript exon SNP). The *F7* rs510317 A allele was associated with an increased risk for breast cancer ($P=0.004$; **Table 2**) [93]. Concordantly, this allele correlates with increased circulating levels of FVII, an activating enzyme of the extrinsic coagulation pathway [94, 95]. Under hypoxic conditions, cancer cells constitutively express FVII, which combined with TF (TF/FVIIa complex) increases coagulation activity and may favour tumour neoangiogenesis, cell migration and invasion through coagulation-independent mechanisms [96]. Regarding the *F5* SNPs, they were tendentially associated with triple-negative tumours, indicating a possible influence on breast cancer aetiology [78]. However, other than inducing thrombin generation, the role of FV in carcinogenesis is unknown. Nevertheless, in another study, *F5* expression was found to be upregulated in breast cancer tissue compared to normal tissue, being particularly increased in aggressive tumours. As for the SNPs functionality, rs6427202 and rs9332542 seem to regulate FV tumour and plasma expression levels, while for rs12120605 and rs6427199 no clear function is known [87, 97]. The *F10* rs3093261 polymorphism seems to influence circulating levels of FX, which has a central role in both intrinsic and extrinsic coagulation pathways. Beyond thrombin generation, FX is thought to promote synthase of nitrogen oxide, inflammatory cytokines, adhesion molecules and growth factors, all contributing to tumourigenesis [98]. To be noted, tumour cells produce a proclotting protease known as cancer procoagulant that activates FX [99]. The rs3093261 T allele may constitute a risk factor for breast cancer ($OR=1.33$; $P=0.011$; **Table 2**). As for *PROCR* rs2069948, this SNP seems to modulate *PROCR* mRNA, potentially influencing the expression level of EPCR [100]. This protein is an endothelial receptor for activated protein C, being the effector of its anticoagulant roles. In combination with PAR receptors, EPCR seems to also facilitate tumour cell survival, migration and angiogenesis and influence tumour-stroma interactions [101]. In breast cancer, rs2069948 was associated with oestrogen and progesterone receptor positivity ($P=0.030$; **Table 2**) [78].

As for colorectal cancer, Vossen *et al.* [102] and Baghdad *et al.* [103] have identified FVL, *F2* rs1799963 and *F13A1* rs5985 as potential predictive biomarkers. Regarding FVL, Vossen *et al.* [102] were the only ones pinpointing a role of this variant in carcinogenesis, possibly due to the large cohort of patients (1801 colorectal cancer patients and 1853 healthy individuals). Interestingly, while the TT genotype was associated with an increased disease risk ($OR=5.77$; 95% CI, 1.69-19.72; **Table 2**), the CT genotype demonstrated a protective effect ($OR=0.68$; 95% CI, 0.52-0.90; **Table 2**). In terms of *F2* rs1799963, contrary to findings of Pihusch *et al.* [46] concerning gastrointestinal cancer, Vossen *et al.* [102]

found a protective effect of AG genotype in the setting of colorectal cancer (OR=0.69; 95% CI, 0.49-0.96; **Table 2**). Baghdad *et al.* [103], in turn, showed an increased disease risk associated with the A allele (OR=21.24; 95% CI, 10.45–43.16, **Table 2**). As for *F13A1* rs5985, Vossen *et al.* [102] reported a protective effect of CA genotype (OR=0.85; 95% CI, 0.74-0.97; **Table 2**), opposing Vairaktaris *et al.* [88] findings in the setting of OSCC. Whether the effect of these polymorphisms is cancer type-dependent needs, therefore, to be explored.

In sum, given the scarce and somewhat controversial evidence, future studies with larger cohorts are required to validate these results, particularly in prospective cohorts of patients, as well as to clarify the role of these genetic polymorphisms on cancer susceptibility. To be noted, until now guidelines only recommend limited cancer screening in patients with unprovoked VTE, and there is no validated predictive marker used to decide on whether to perform or not cancer screening among these subjects [104].

Chapter ID - Table 2. Epidemiological studies on the role of haemostatic gene polymorphisms on cancer susceptibility

First author (year)	Country/Ethnic background	Population characteristics	Study design	Studied polymorphisms *	Main findings
Pihusch <i>et al.</i> (2002) [46]	German/ Caucasian	175 gastrointestinal cancer patients 128 healthy individuals	Case-control study	<i>F5</i> rs6025, <i>F2</i> rs1799963 and <i>MTHFR</i> rs1801133	<i>F2</i> rs1799963 is a potential predictor of gastrointestinal cancer [AG genotype; A =5.7%; B =0.8%; <i>P</i> =0.028]
Vairaktaris <i>et al.</i> (2007) [88]	Greece and German/ European origin	130 oral squamous cell carcinoma patients 135 healthy individuals	Case-control study	<i>F12</i> rs1801020 and <i>F13A1</i> rs5985	<i>F13A1</i> rs5985 is a potential predictor of oral squamous cell carcinoma [AA genotype; OR=2.893; 95% CI, 1.056–7.890; CA genotype; OR=1.868; 95% CI, 1.126–3.101]
Vairaktaris <i>et al.</i> (2009) [89]	Greece and German/ European origin	162 oral squamous cell carcinoma patients 168 healthy individuals	Case-control study	<i>ITGA2</i> rs1126643, <i>SERPINE1</i> rs1799889, <i>ACE</i> I/D and <i>CPB2</i> rs2296642	<i>SERPINE1</i> rs1799889 [4G/4G genotype; OR=5.00; 95% CI, 1.32-18.92] and <i>ACE</i> I/D [I/I genotype; OR=9.16; 95% CI, 1.14-73.50] are potential predictors of oral squamous cell carcinoma
Eroğlu <i>et al.</i> (2011) [93]	Turkey/ Unclear	92 breast cancer patients 80 healthy individuals	Nested case–control study	<i>F7</i> rs36208070, rs510335 and rs510317	<i>F7</i> rs510317 is a potential predictor of breast cancer [A allele; A =16%; B =4%; <i>P</i> =0.004]
Vossen <i>et al.</i> (2011) [102]	German/ Caucasian	1801 colorectal cancer patients 1853 healthy individuals	Case-control study	<i>F5</i> rs6025, <i>F2</i> rs1799963, <i>SERPINE1</i> rs1799889, <i>MTHFR</i> rs1801133, <i>FGG</i> rs2066865 and <i>F13A1</i> rs5985	<i>F5</i> rs6025 [TT genotype; OR=5.77; 95% CI, 1.69-19.72; CT genotype; OR=0.68; 95% CI, 0.52-0.90], <i>F2</i> rs1799963 [AG genotype; OR=0.69; 95% CI, 0.49-0.96] and <i>F13A1</i> rs5985 [CA genotype; OR=0.85; 95% CI, 0.74-0.97] are potential predictors of colorectal cancer
Vylliotis <i>et al.</i> (2013) [90]	Greece and German/ Caucasian	160 oral squamous cell carcinoma patients 168 healthy individuals	Case-control study	<i>MTHFR</i> rs1801133, <i>F5</i> rs6025, <i>F2</i> rs1799963, <i>F12</i> rs1801020, <i>F13A1</i> rs5985, <i>SERPINE1</i> rs1799889, <i>ACE</i> I/D, <i>AGT</i> rs699 and <i>CPB2</i> rs1926447	<i>SERPINE1</i> rs1799889 [4G/4G genotype; OR=3.09; 95% CI, 1.30-7.36; <i>P</i> =0.001], <i>ACE</i> I/D [I/I genotype; OR=5.92; 95% CI, 2.00-17.48; <i>P</i> <0.001] and <i>F13A1</i> rs5985 [AA genotype; OR=3.73; 95% CI, 1.23-11.33; <i>P</i> =0.01; only early stage] are potential predictors of oral squamous cell carcinoma

Chapter ID - Table 2. Epidemiological studies on the role of haemostatic gene polymorphisms on cancer susceptibility (cont.)

First author (year)	Country/Ethnic background	Population characteristics	Study design	Studied polymorphisms *	Main findings
Tinholt <i>et al.</i> (2014) [78]	Norway/ Scandinavian	366 breast cancer patients 307 healthy individuals	Case-control study	37 SNPs in <i>F2</i> , <i>F3</i> , <i>F5</i> , <i>F7</i> , <i>F10</i> , <i>TFPI</i> and <i>PROCR</i> genes	<i>F5</i> rs12120605 [T allele; OR=1.47; <i>P</i> =0.026], rs6427202 [C allele; OR=1.28; <i>P</i> =0.028], rs9332542 [A allele; OR=0.76; <i>P</i> =0.028] and rs6427199 [A allele; OR=0.79; <i>P</i> =0.037], <i>F10</i> rs3093261 [T allele; OR=1.33; <i>P</i> =0.011] and <i>PROCR</i> rs2069948 [C allele; OR=1.27; <i>P</i> =0.030] are potential predictors of breast cancer
Baghad <i>et al.</i> (2017) [103]	Morocco/ Unclear	76 colorectal cancer patients 182 healthy individuals	Case-control study	<i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>F2</i> rs1799963 is a potential predictor of colorectal cancer [AA genotype; OR=21.24; 95% CI, 10.45–43.16; <i>P</i> <0.0001]

Highlighted SNPs represent the genetic variants that are not validated as VTE-related genetic variants in cancer-free populations.

For studies that did not provide an odds ratio (OR), the frequency of the polymorphism allele or genotype in patients with cancer-related VTE (**A**) and cancer patients without VTE (**B**) were indicated.

* Polymorphisms associated with VTE susceptibility in cancer-free populations or polymorphisms within or close to genes directly implicated in haemostasis.

Abbreviations: OR odds ratio; CI confidence interval

4. Haemostatic gene polymorphisms and cancer progression

Beyond cancer initiation and with more compelling evidence, haemostatic components are thought to promote cancer progression towards metastasis. However, the role of coagulation-related polymorphisms on cancer progression and/ or patient outcome is poorly explored. Until now, only six studies were performed to provide insights into this topic, with five reporting significant associations, which are summarised in **Table 3**. Data from the study with non-significant results were excluded from this analysis [83].

To the best of our knowledge, the first study was conducted by De Haas *et al.* [35]. The authors showed that *SERPINE1* rs1799889 4G/4G genotype is associated with an increased risk for early disease relapse and reduced survival among patients with testicular cancer under platinum-based chemotherapy ($P=0.004$ and $P=0.010$, respectively; **Table 3**), which corroborates previous findings linking PAI-1 to cancer aggressiveness. Namely, independent of uPA, PAI-1 is thought to enhance tumour neoangiogenesis and block apoptosis in endothelial and tumour cells, promoting tumour progression [105].

In breast cancer, Tinholt *et al.* [106] reported different SNP haplotypes of *TFPI* associated with tumour size, lymph node spread and distinct tumour molecular subtypes ($P<0.05$; **Table 3**). Concordantly, current evidence suggests that TFPI has antimetastatic properties and its increased expression levels correlate with better prognosis in breast cancer patients. This anticoagulant protein is known to regulate the activity of TF, which is the initiator of the extrinsic coagulation pathway and constitutes the most widely studied coagulation factor in the setting of malignancy [42, 106]. Induced by oncogenes, tumour expression of TF is linked to cell survival, proliferation and invasion, angiogenesis and metastasis, being overall associated with poor prognosis in different tumour models. The mechanisms through which TF favour cancer progression can either be coagulation-related through thrombin generation or coagulation-independent through FVIIa and activation of PARs [11, 106]. Therefore, by inhibiting TF activity, TFPI might deaccelerate tumour dissemination.

Not targeting any specific tumour model, Wahba *et al.* [73] found an association between the *F5* rs6025 T allele (risk allele for VTE development) and reduced overall survival among patients with nonmetastatic malignant disease (aHR=2.42; $P=0.01$; **Table 3**). Interestingly, in another study, increased FV expression in aggressive tumours was associated with favourable outcome in breast cancer patients [97]. As previously mentioned, no direct role of FV in carcinogenesis is known, being this association most likely attributed to the generation of thrombin and its repercussions in tumour biology.

In colorectal cancer, Bazzarelli *et al.* [42] reported an association of *TFPI* rs8176592 (intronic SNP) GG genotype with improved disease-free survival (aHR=0.28; $P=0.008$;

Table 3). Concordantly, this genotype was previously associated with higher TFPI circulating levels, which explains its protective role in terms of tumour recurrence given TFPI antimetastatic properties [42, 107]. To be noted, this polymorphism is in almost complete linkage disequilibrium (LD) with the *TFPI* rs8176541 variant (pairwise LD $r^2 > 0.95$ in European descents), which Tinholt *et al.* [106] found to be correlated with triple-negative status in breast cancer patients.

Lastly, our group have recently found significant associations of *ZFPM2* rs4734879 (intronic SNP), *SLC19A2* rs2038024 (upstream gene SNP) and *CNTN6* rs6764623 (upstream gene SNP) with overall survival and disease-free survival in patients with epithelial ovarian cancer ($P < 0.05$; **Table 3**) [108]. All these variants were previously identified as VTE-related by GWAS in the general population and they have yet to be validated as predictors of cancer-related thrombogenesis [11, 87]. Briefly, among several potential roles, we hypothesised that *ZFPM2* rs4734879 may influence tumour neoangiogenesis through *VEGF* expression, *SLC19A2* rs2038024 might impact glucose metabolism by modulating the uptake of thiamine and *CNTN6* rs6764623 may have a role in the Notch pathway contributing to cancer aggressive behaviour. Remarkably, these effects seem to be only relevant prior to metastasis as the significant associations were only observed at early disease stages (FIGO I/II stages). We hypothesised that these genetic variants might contribute to tumour dissemination, but once it happens other mechanisms might impose a more preponderant impact on cancer progression. Inclusively, at advanced stages, the tumour epi(genetic) signature, rather than inherent genetic variation, might be the engine of tumour cell ability to hijack haemostatic components to benefit their survival. Interestingly, no study has yet detected a significant association between *F2* rs1799963 and cancer progression, metastasis and/or patient outcome, which suggests that this variant might also be relevant only at an early stage of carcinogenesis [46].

In sum, few studies have explored the role of haemostatic gene polymorphisms in cancer progression, and they were focused on only a few tumour types (testicular, breast, colorectal and gynaecological cancers). Although further investigation is mandatory, the current evidence pinpoints an effect of the genetic polymorphisms in cancer behaviour, which can be explained by their involvement in several pivotal mechanisms towards tumour dissemination.

For clarification and validation purposes, additional studies with larger cohort sizes and considering additional potential confounding factors are required. Furthermore, investigation of the role of these genetic determinants in different cancer stages should be also conducted.

Chapter ID - Table 3. Epidemiological studies on the role of haemostatic gene polymorphisms on cancer progression and/ or patient outcome

First author (year)	Country/Ethnic background	Population characteristics	Study design	Studied polymorphisms	Main findings
de Haas <i>et al.</i> (2010) [35]	The Netherlands/ Mixed	324 patients with testicular cancer under platinum-based chemotherapy	Cohort study	<i>SERPINE1</i> rs1799889	<i>SERPINE1</i> rs1799889 was associated with reduced survival [4G/4G genotype; aHR=2.69, 95% CI, 1.26-5.73; $P=0.010$] and increased risk for early relapse [4G/4G genotype; OR=3.35; 95% CI, 1.48-7.59; $P=0.004$]
Tinholt <i>et al.</i> (2015) [106] **	Norway/ Caucasian	152 treatment-naive breast cancer patients	Cohort study	6 SNPs of <i>F3</i> and 14 SNPs of <i>TFPI</i>	Different <i>TFPI</i> SNP haplotypes were associated with tumour size [OR=3.14; $P=0.004$], triple negativity [OR=2.40; $P=0.004$], lymph node spread [OR=3.34; $P=0.006$], basal-like [OR=2.30; $P=0.011$], luminal B [OR=3.50; $P=0.005$] and normal-like [OR=22.17; $P=0.0002$] subtypes
Wahba <i>et al.</i> (2015) [73]	Egypt / Unclear	80 patients with nonmetastatic cancer	Case-control study	<i>F5</i> rs6025, <i>F2</i> rs1799963 and <i>MTHFR</i> rs1801133	<i>F5</i> rs6025 was associated with reduced overall survival [T allele; aHR=2.42; $P=0.01$]
Bazzarelli <i>et al.</i> (2016) [42]	Canada/ Unclear	127 patients with colorectal cancer	Cohort study	<i>TFPI</i> rs8176592, <i>F5</i> rs6025 and <i>F2</i> rs1799963	<i>TFPI</i> rs8176592 was associated with improved disease-free survival [GG genotype; aHR=0.28; $P=0.008$]
Tavares <i>et al.</i> (2021) [108]	Portugal/ Caucasian	336 patients with epithelial ovarian cancer	Cohort study	<i>ZFPM2</i> rs4734879, <i>SLC19A2</i> rs2038024, <i>CNTN6</i> rs6764623, <i>OTUD7A</i> rs7164569, <i>F11</i> rs4253417 and <i>PROCR</i> rs10747514	<i>ZFPM2</i> rs4734879 [AA genotype; aHR=2.67; 95% CI, 1.12-6.39; $P=0.027$], <i>SLC19A2</i> rs2038024 [CC genotype; aHR=13.78; 95% CI, 2.89-65.71; $P=0.001$] and <i>CNTN6</i> rs6764623 [CC genotype; aHR=5.05; 95% CI, 1.41-18.18; $P=0.013$] were associated with tumour recurrence in FIGO I/II stages. These variants were associated also with overall survival ($P<0.05$)

** In addition to *TFPI* SNP haplotypes, *TFPI* rs5940, rs10153820 and rs10179730 were individually associated with clinicopathological parameters (normal-like tumour subtype, T status and N status, respectively).

5. Conclusions and future perspectives

Cancer-related VTE, also known as Trousseau syndrome, was initially recognised by Jean-Baptiste and Armand Trousseau in the 19th century. Despite being a well-known condition, the underlying biological mechanisms that link tumour with thrombogenesis are poorly understood [109]. From a clinical perspective, factors related to the patient (including both acquired and genetic determinants), cancer biology and antineoplastic treatments are recognised to act synergically in venous thrombogenesis [11]. Remarkably, the deregulation in the haemostatic system in cancer patients seems to not only lead to thrombogenesis but also benefit tumour growth and dissemination, meaning that cancer-associated coagulopathy may not be an epiphenomenon [110].

Given the detrimental impact of VTE on patient prognosis, disease prevention among cancer patients is crucial. However, the development of risk assessment models with adequate sensitivity and specificity has been challenging. Nevertheless, as studies in the general population showed that VTE is highly heritable, genetic markers and particularly genetic polymorphisms constitute attractive candidates as predictors of cancer-related VTE. Furthermore, given the roles of haemostatic components in carcinogenesis, these genetic markers might be also potential predictors of cancer susceptibility and/ or progression [11].

To the best of our knowledge, this is the first review of existing studies assessing the role of haemostatic gene polymorphisms in cancer-related VTE, cancer susceptibility and cancer progression. Previous reviews have focused mainly on the two most studied VTE-related polymorphisms, *F5* rs6025 and *F2* rs1799963, dismissing other potentially relevant polymorphisms in the setting of malignancy, and describing their impact either on cancer-related VTE or cancer susceptibility and tumour progression [21, 43]. Regarding cancer-related VTE, briefly, 25 out of 44 studies have reported significant associations with haemostatic gene polymorphisms, with reports of a total of 33 variants with predictive value. In general, most of these studies had a small cohort of participants and were performed in heterogeneous populations, dismissing potential confounding factors, which may partially explain the somewhat conflicting results. Furthermore, although some well-known VTE genetic markers were validated (*F5* rs6025, for instance), the existing data suggests that thrombogenesis among oncological patients might be a different entity from VTE in the general population, with some biological mechanisms being more relevant than others depending on tumour model. Thus, additional studies with larger datasets are needed, particularly to analyse less frequent variants (including *F5* rs6025 and *F2* rs1799963) and perhaps common gene variations not directly associated with haemostasis given the complexity of VTE pathogenesis.

As for cancer susceptibility and progression, few studies were conducted to this day (14 and six, respectively) and they are mostly focused on OSCC, breast cancer and gastrointestinal cancer. A total of 12 and nine polymorphisms associated with cancer susceptibility and progression were reported respectively. To be noted, most of these variants have yet to be validated as predictors of cancer-related VTE. Nevertheless, according to the scarce data, haemostatic gene polymorphisms seem to have an effect on carcinogenesis, which appears to be cancer model-specific but also tumour stage-dependent.

The scientific basis of these observations needs further investigation, given the implications for thromboprophylaxis, cancer screening, prognosis assessment and the development of potential antineoplastic therapies targeting coagulation [11]. In this context, studies of gene expression profiling may help fulfil the current gaps in our knowledge of pathophysiological mechanisms underlying cancer-related VTE and the interplay between haemostatic components and tumour cells even in the absence of VTE [23].

Author Contributions

Literature search and collection, Valéria Tavares; writing, Valéria Tavares; revision, Valéria Tavares, Beatriz Vieira Neto, Maria Isabel Vilas-Boas, Deolinda Pereira and Rui Medeiros; supervision, Rui Medeiros; funding holder, Valéria Tavares. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest. The funder had no role in the writing of the manuscript, or in the decision to publish it.

Data availability

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CHAPTER IE – Long Non-coding RNAs: Roles in Venous Thromboembolism Pathogenesis

This chapter includes the following paper:

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

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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.

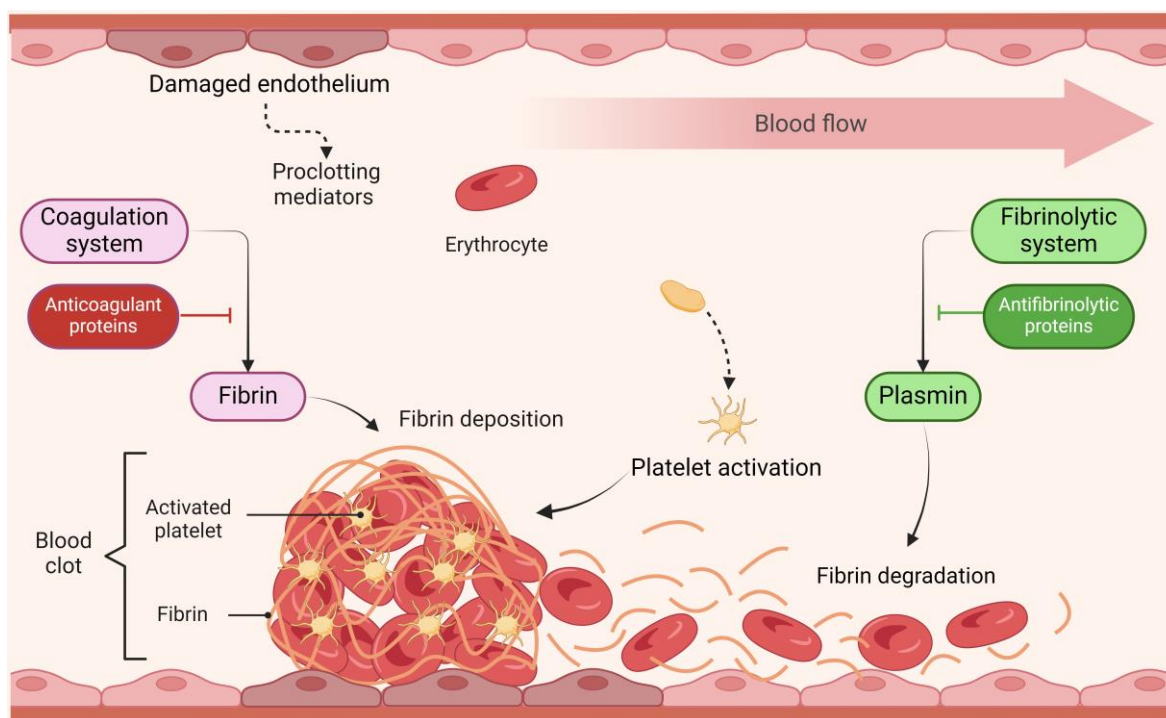
Keywords: RNA; long non-coding; venous thromboembolism; deep vein thrombosis; tissue-factor-pathway inhibitor 2

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 stabilise 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].



Chapter IE - Figure 1. Interplay between the components of the haemostatic system. The figure was created with 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].

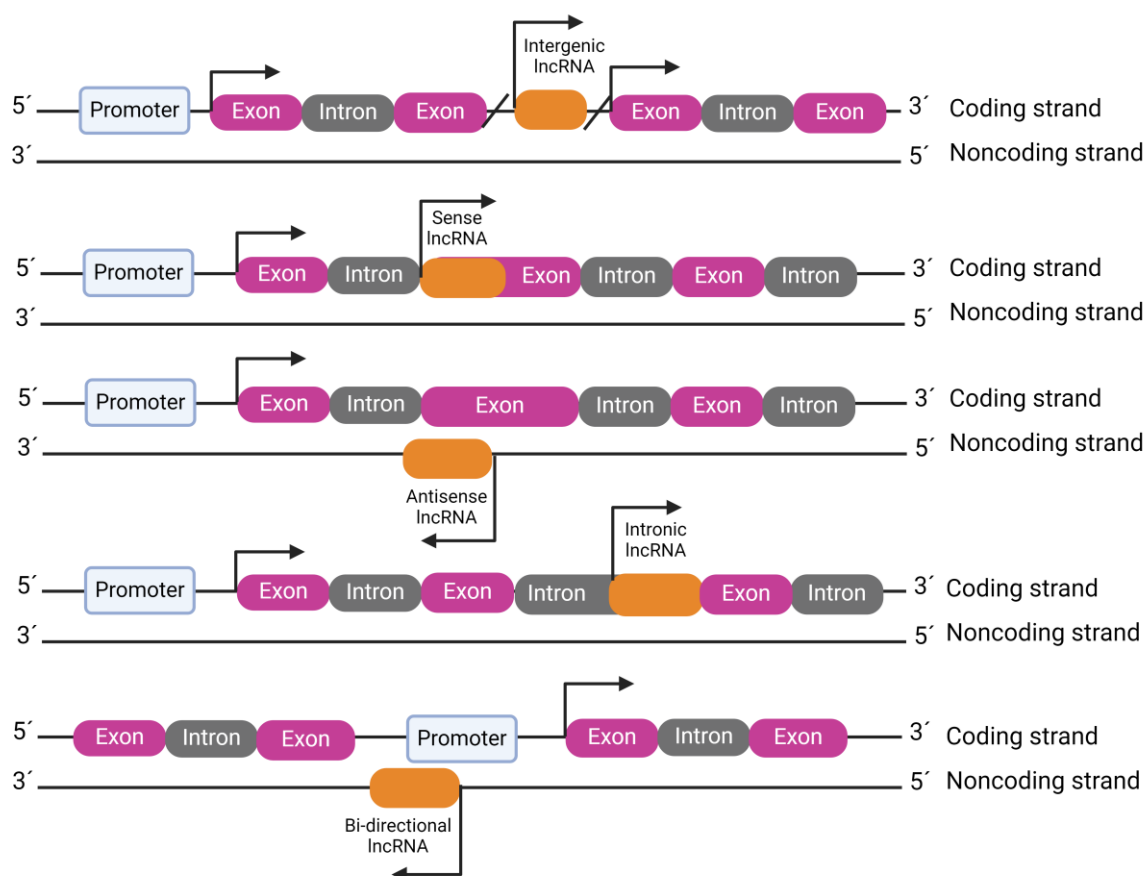
The most clinically used biomarker for VTE risk prediction and diagnosis is D-dimer (DD), which is a product resultant from the breakdown of cross-linked fibrin (i.e., fibrinolysis) [17]. In addition to the first VTE events, DD is also associated with the rate of recurrent events and mortality [18]. 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].

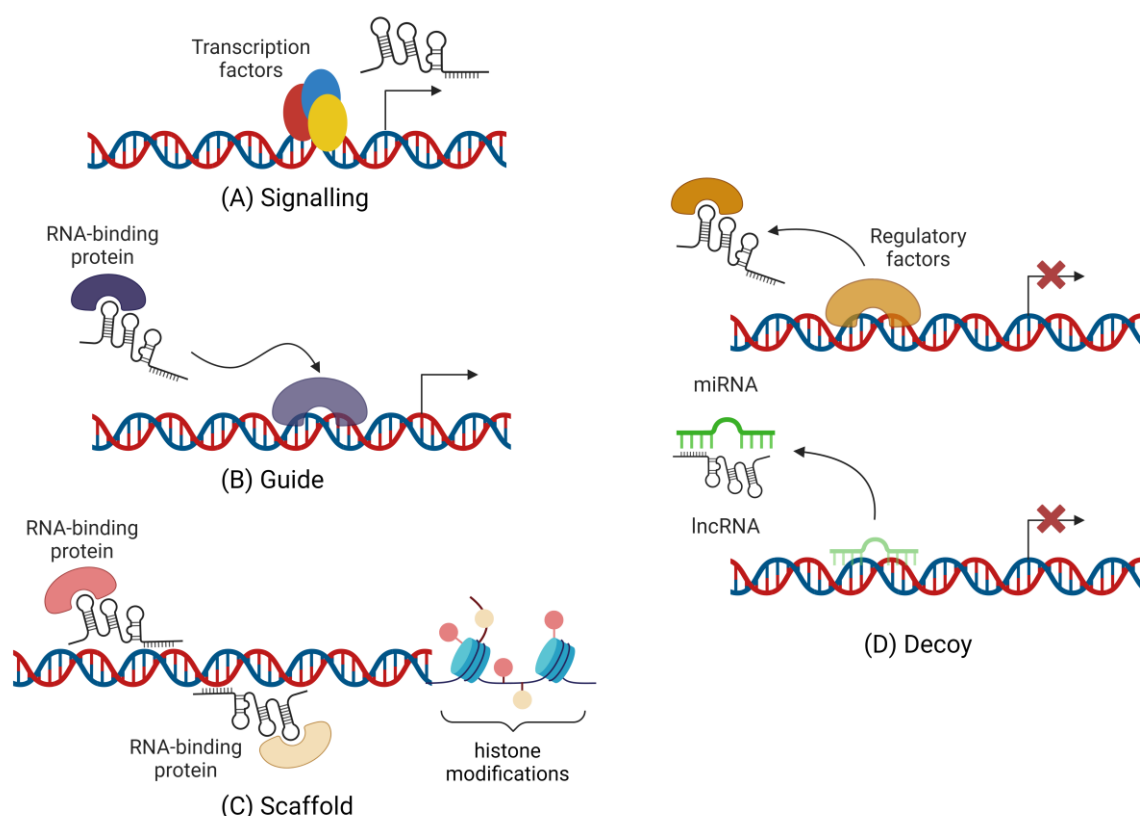
Similarly to messenger RNAs (mRNAs), lncRNAs are also transcribed by RNA Polymerase II and are often capped and polyadenylated, also presenting evolutionarily conserved patterns and a high level of tissue specificity [28]. According to the NONCODE database (version 6.0) (<http://www.noncode.org>, accessed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



Chapter IE - Figure 2. Genomic localization of lncRNAs. The figure was created with 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 localised 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 promoter-associated binding sites, lncRNAs can induce changes in promoter 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 promoter [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].

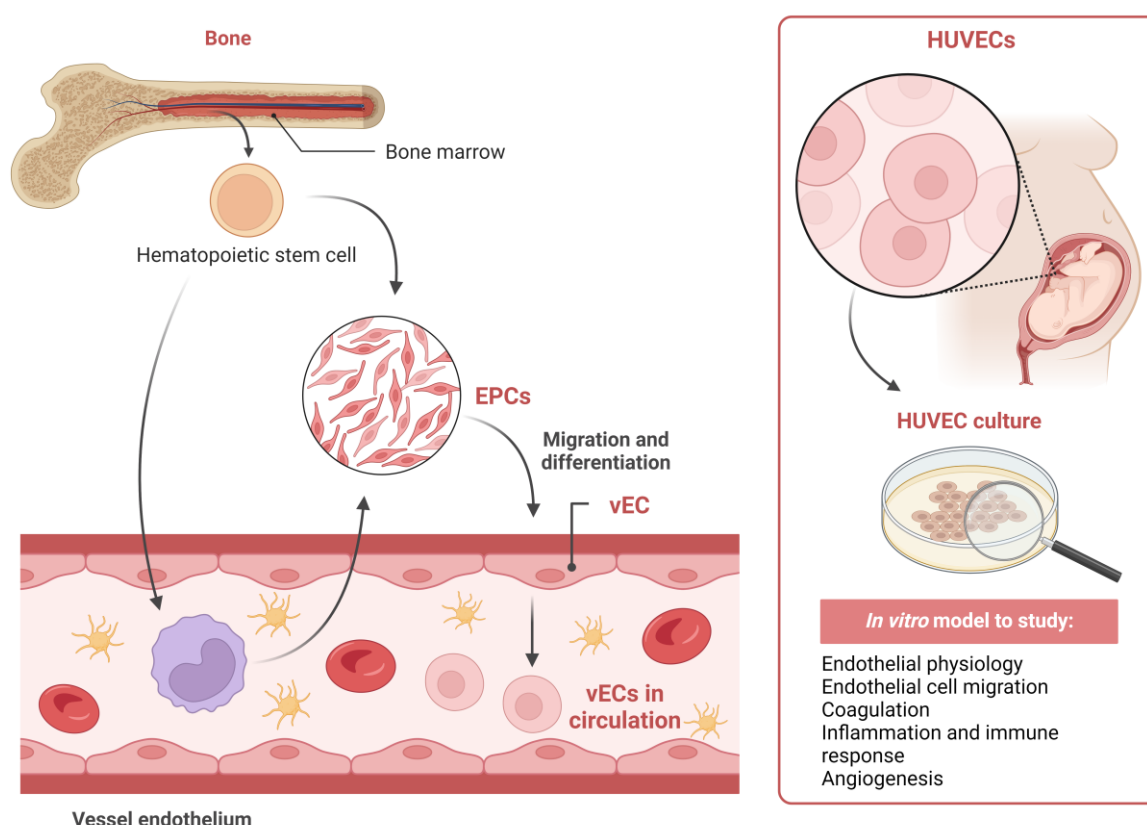


Chapter IE - Figure 3. Four archetypes of lncRNAs functions. The figure was created with BioRender.com (accessed on 23 July 2023).

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 [59,53]. 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].



Chapter IE - 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 (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 lncRNA 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 hypothesised 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].

Chapter IE - 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
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 Pcyox1l expression and aggravating thrombus formation in DVT	Promotes DVT

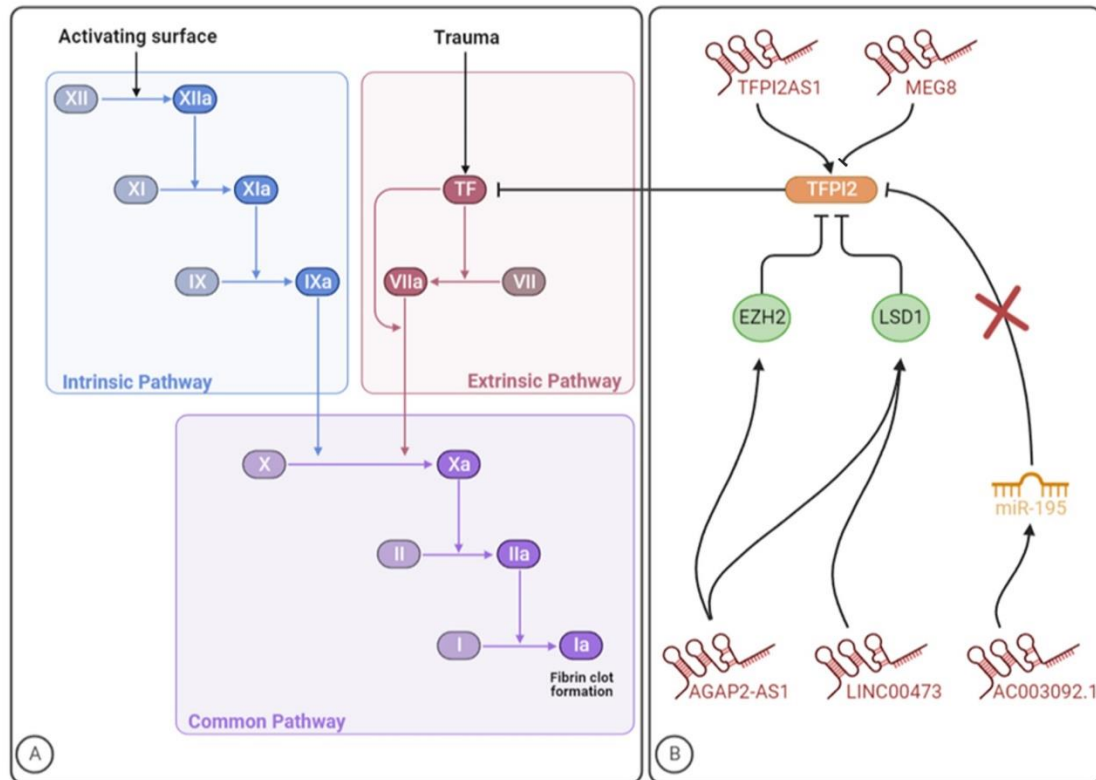
Abbreviations: DVT, deep vein thrombosis; ECs, endothelial cells; EPCs, endothelial progenitor cells, HUVECs, human umbilical vein endothelial cells; IVC: inferior vena cava; LEDVT; lower extremity deep vein thrombosis; lncRNA; long non-coding ribonucleic acid; n.a, not available; VTE, venous thromboembolism. 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 generates 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 (FXII), 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].



Chapter IE - 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 (accessed on 10 July 2023).

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].

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 summarised 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 characterised 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 lncRNA was found to be downregulated in patients' placenta, while *in vitro*, the lncRNA 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 lncRNA 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 lncRNAs 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 lncRNA 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 lncRNA 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].

Chapter IE - 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	↑

Abbreviations: 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 can 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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CHAPTER IF – MicroRNAs: Roles in Venous Thromboembolism Pathogenesis

This chapter includes the following paper:

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

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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. However, 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.

Keywords: venous thromboembolism; neoplasms; microRNAs; tissue factor; tissue factor pathway inhibitor; prognosis.

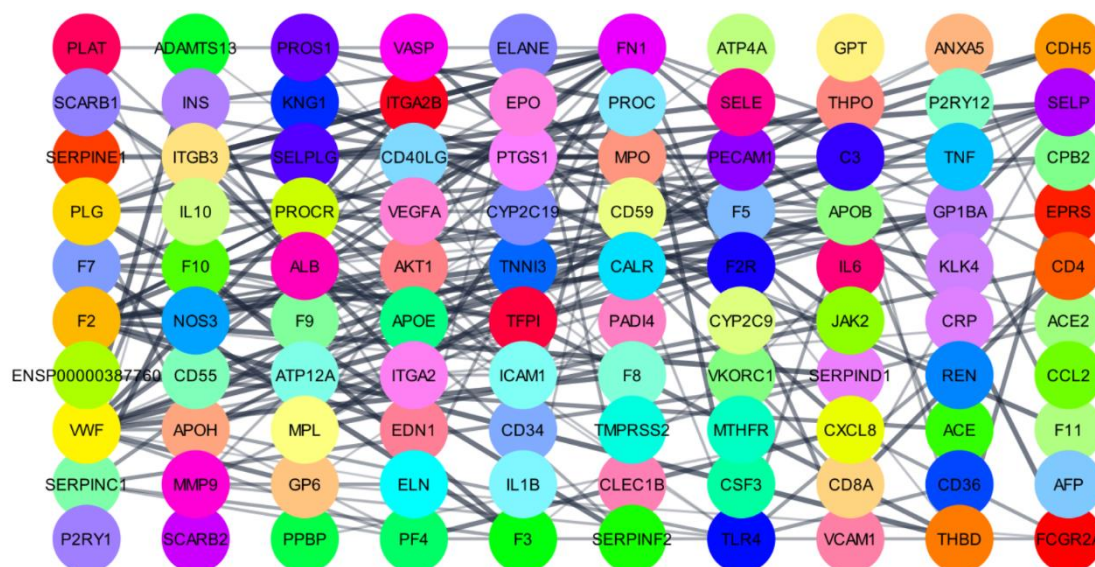
1. Introduction

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].

Thromboembolism is a common cardiovascular disorder characterised 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].



Chapter IF - Figure 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)).

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 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).

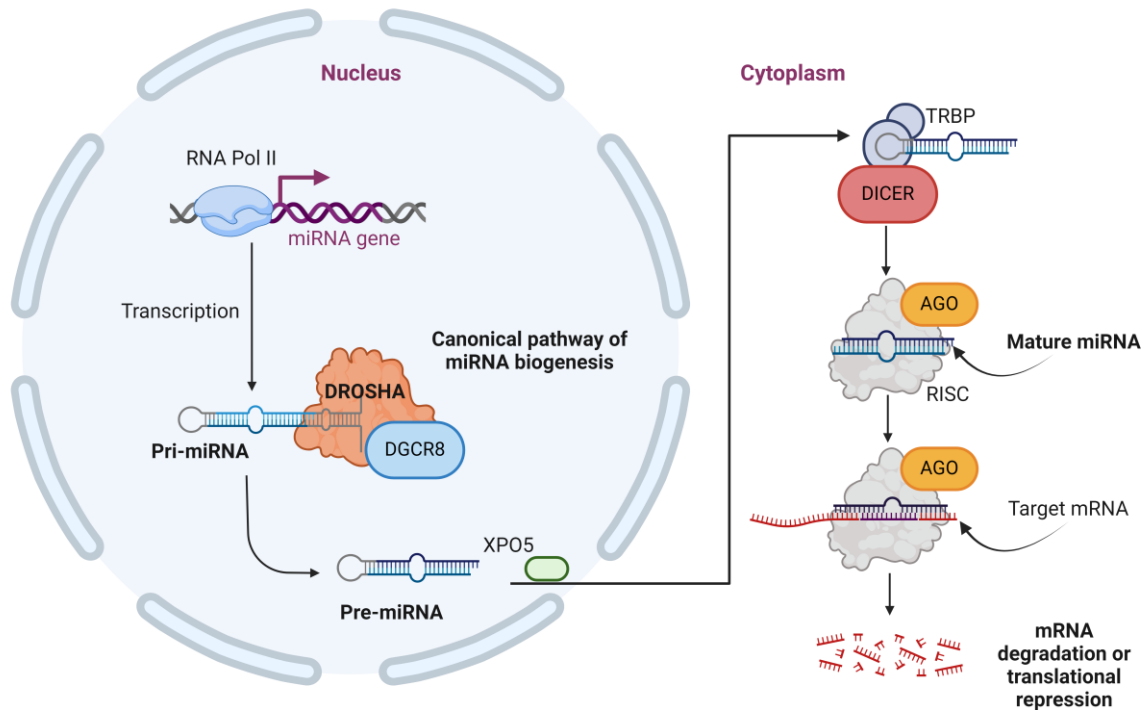
2. MiRNAs

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 cytoplasmatic 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].



Chapter IF - Figure 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.

2.1. Role of MiRNAs in Venous Thromboembolism

Like other biological processes, haemostasis is thought to be 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-characterised 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 synthesised 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**).

Chapter IF - 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

Chapter IF - 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
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.

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**). 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 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 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.

Chapter IF - Table 2. miRNAs associated with VTE in the cancer setting.

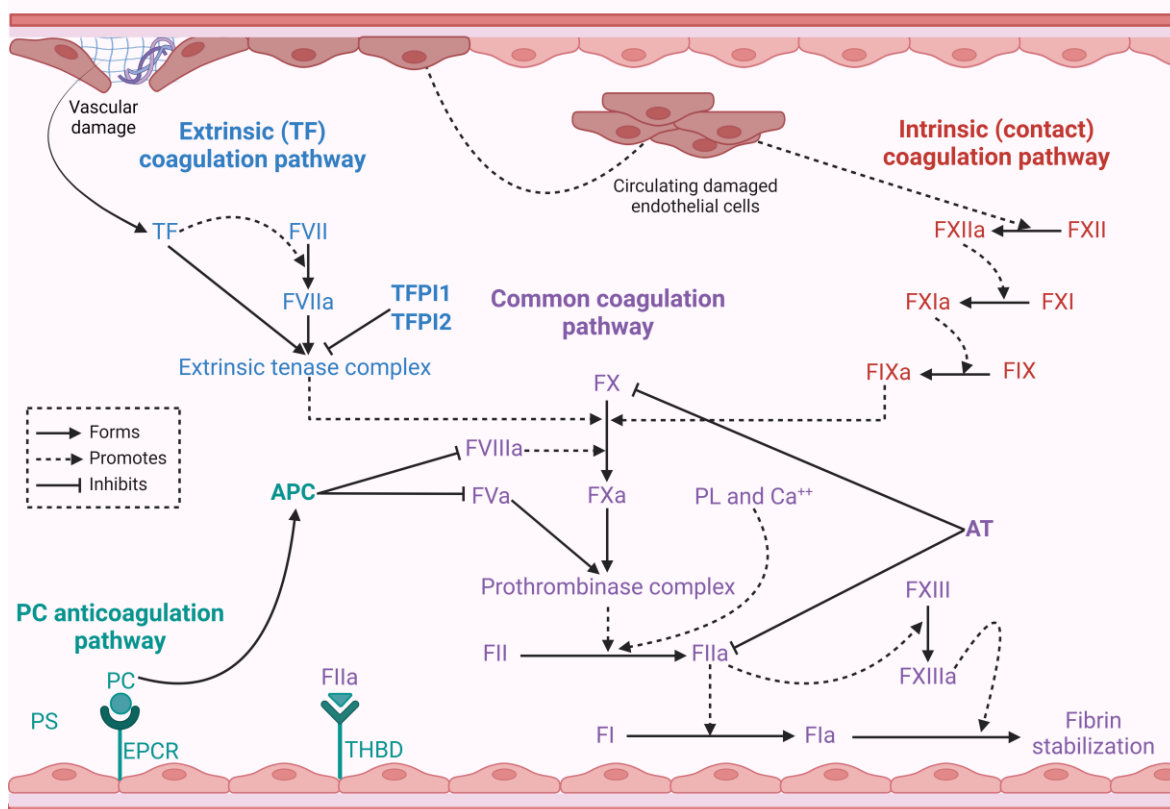
Study	Country	Study design	Sample/ Methods	Deregulated miRNAs in patients with cancer-associated VTE
Kim et al. (2019) [72]	United States of America	Prospective study with sex and age matching: 7 CCR patients with VTE 14 CCR patients without VTE	Plasma miRNA microarray	miR-4451, miR-942-3p, miR-8063, 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.

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 recognised as the key initiator of the extrinsic coagulation pathway (**Figure 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 vascularised 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 (FXIa), which subsequently triggers a series of events towards fibrin deposition at the injury site [20,77].



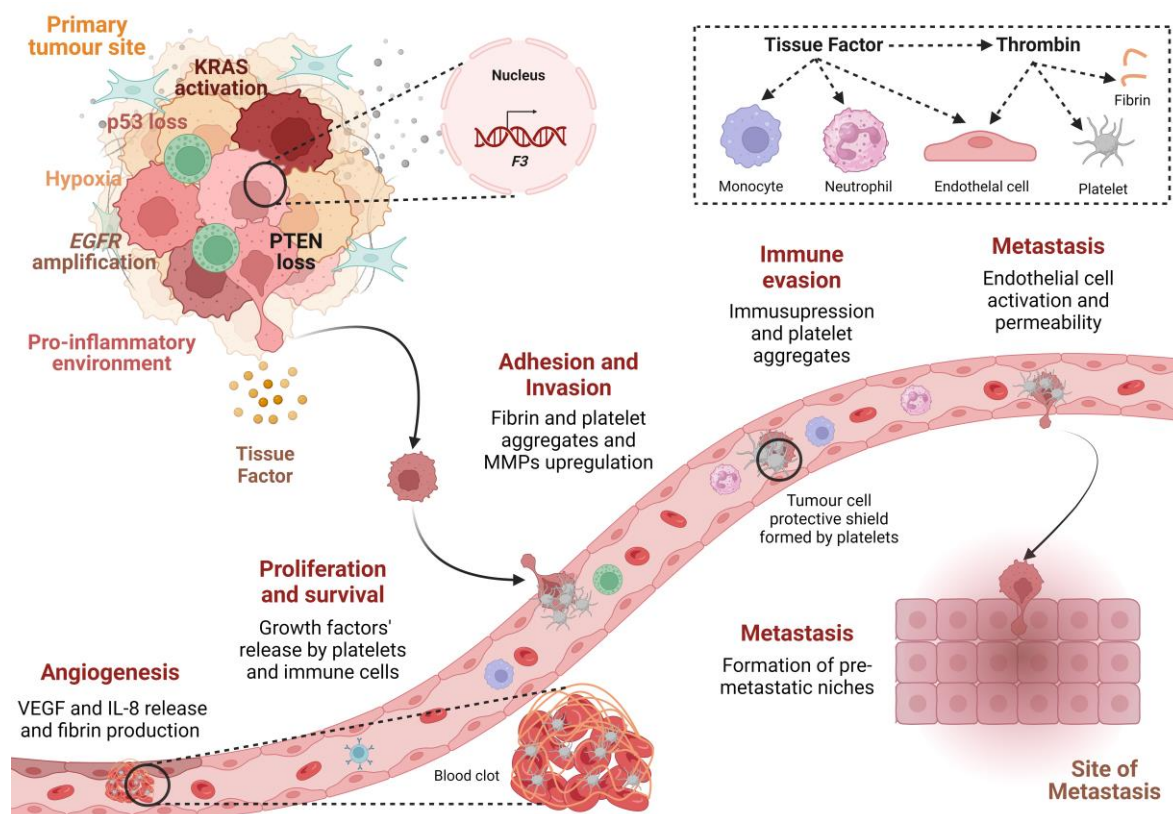
Chapter IF - Figure 3. Pathways of blood coagulation according to the waterfall/cascade model. The figure was created with BioRender.com (accessed on 10 July 2023). The coagulation cascade involves three major pathways: extrinsic (or TF coagulation pathway), intrinsic (or contact

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 (FIa) 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.

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-characterised mechanism through which these cells initiate pro-thrombotic 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]. 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.



Chapter IF - Figure 4. Simplistic representation of the roles of tissue factor in tumour progression. The figure was created with 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.

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-520g, 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.

Chapter IF - 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-520g	Luciferase reporter assay
Balia et al. (2017) [100]	Italy	TF expression in PBMCs	PBMCs	miR-19a and miR-20a	qRT-PCR

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

Study	Country	Pathophysiological condition	Sample/compartment	MiRNA	Technique to analyse miRNA-mRNA interaction**
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

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

3.3. Involvement of Tissue Factor Pathway Inhibitors in Cancer

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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, 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 TFPI α 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 personalised 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.

Chapter IF - 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 Luciferase reporter assay
Ali et al. (2016) [133]	Norway	Breast cancer and estrogenic-regulation of TFPI	Breast cancer cells	miR-27a/b and miR-494/TFPI α	Luciferase reporter assay
Cai et al. (2017) [135]	China	Prostate cancer	Prostate cancer tissue and cells	miR-500/TFPI1	Luciferase reporter assay qRT-PCR
Xu et al. (2018) [129]	China/United States of America	Preeclampsia	Preeclampsia placental tissues	miR-616-3p/TFPI2	Luciferase reporter assay
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

** Methods that provide strong evidence. Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction.

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-520g, 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. 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 available on request.

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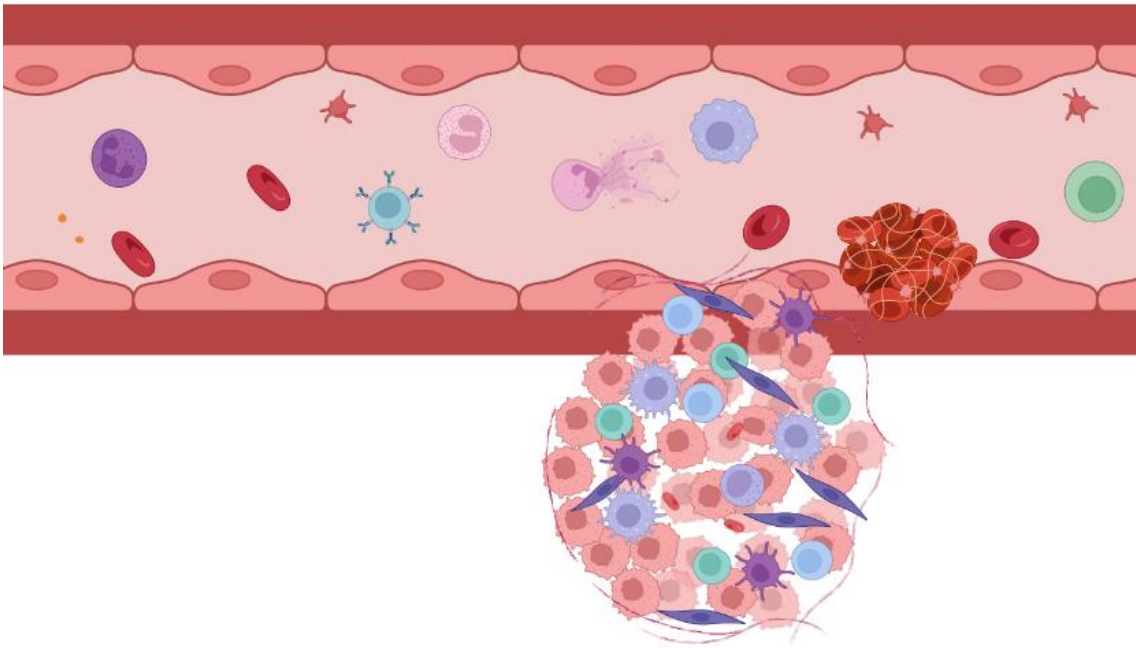
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Chapter II



CHAPTER II – Aims

Haemostatic components involved in venous thromboembolism (VTE) pathogenesis have been demonstrated to promote tumour progression. Consequently, investigating thrombosis-related biomarkers represents a promising strategy for enhancing prognostication in ovarian cancer (OC) patients, extending beyond their direct role in predicting cancer-associated thrombosis (CAT). Furthermore, integrating studies of the tumour coagulome could potentially reveal novel therapeutic targets for cancer management. Therefore, the **primary aim** of this thesis project was to identify clinically relevant prognostic biomarkers for OC by examining the tumour coagulome and CAT pathogenesis in patients with this malignancy, utilising liquid biopsies. To this end, the following **specific aims** and tasks were established:

1. Evaluate the incidence of CAT occurrence among OC patients and study its implications on patients' clinical outcomes (**Chapters III-VI**):

Tasks:

- A. Based on an established CAT definition, assess the frequency of events in a cohort of OC patients and perform their characterisation;
- B. Explore the impact of CAT on time to first progression of OC and patients' survival;
- C. Assess the performance of the Khorana score (KS) and evaluate the predictive value for CAT occurrence of baseline coagulation assays routinely used in clinical routine - prothrombin time (PT), international normalised ratio (INR) and activated partial thromboplastin time (aPTT) - as a surrogate for coagulation protein activity;
- D. Explore potential associations of KS and baseline coagulation assays with the VTE-related biomarkers studied in this project, including genetic variations (**Chapter III**), haemostatic gene expression (**Chapter IV**), and levels of long non-coding RNAs (lncRNAs; **Chapter V**) and microRNAs (miRNAs; **Chapter VI**);
- E. Evaluate the impact of KS and coagulation assays on time to first progression of OC and patients' survival.

2. Since VTE is a highly heritable disease, investigate the role of haemostatic gene variations in tumour coagulome and their impact on the clinical outcomes of OC patients (**Chapter III**).

Tasks:

- A. Conduct a literature review on genetic variants underlying CAT pathogenesis and their implications in tumourigenesis;
 - B. Select the most appropriate genetic variants to be studied;
 - C. Explore their functional consequences and assess their clinical implications in a cohort of OC patients.
3. Considering the inflammatory processes underlying CAT pathogenesis and the pivotal role of the coagulation system in venous thrombogenesis, decode the expression of coagulation system-related genes in peripheral blood components (PBCs) in the setting of immunothrombosis and their clinical significance for OC patients (**Chapter IV**).

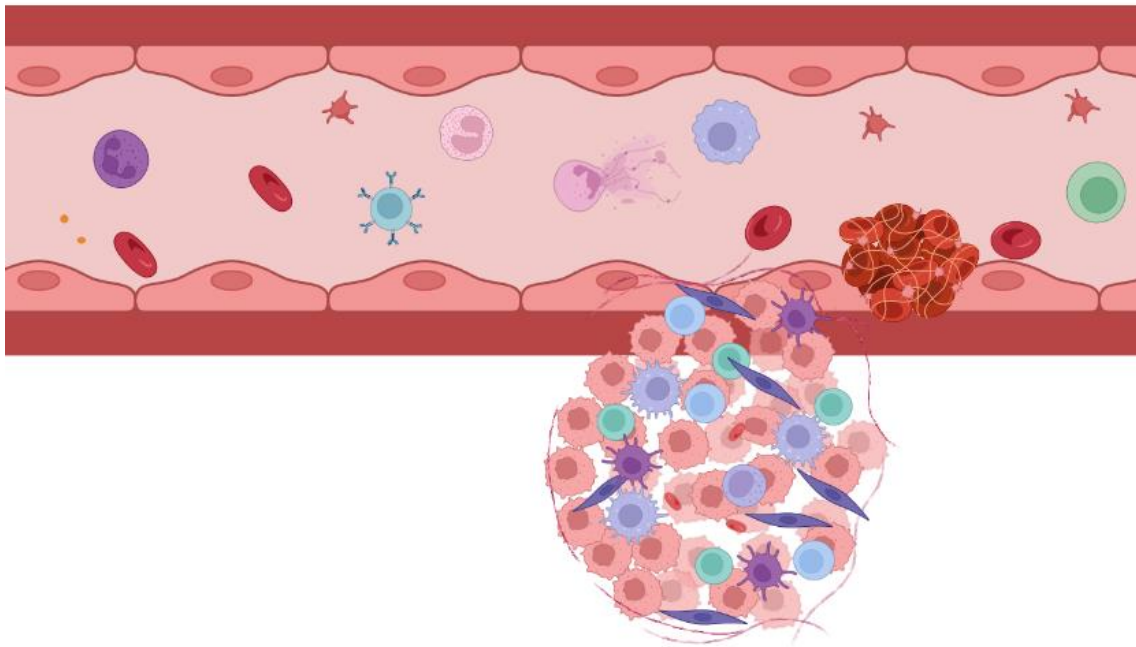
Tasks:

- A. Conduct a literature review on the interaction between the immune and haemostatic systems and its contributions to CAT pathogenesis;
 - B. Select the most relevant haemostasis-related genes to be studied and evaluate their clinical implications in a cohort of OC patients.
4. Examine the biomarker potential of lncRNAs (**Chapter V**) and miRNAs (**Chapter VI**) and their contribution to CAT pathogenesis and the clinical outcomes of OC patients.

Tasks:

- A. Conduct a literature review on the current knowledge of lncRNAs and miRNAs in venous thrombogenesis;
- B. Select the most pertinent lncRNAs and miRNAs and evaluate their clinical implications in a cohort of OC patients.

Chapter III



CHAPTER III – Unveiling the Role of Haemostatic Gene Variations in Tumour Coagulome and Ovarian Cancer Aggressiveness

This chapter includes the following unpublished paper:

Valéria Tavares, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Joana Assis, Deolinda Pereira, Rui Medeiros. **Heritable Genetic Variability in Ovarian Tumours: Exploring Venous Thromboembolism Susceptibility and Cancer Prognosis in a Hospital-Based Study.** (Submitted).

Heritable Genetic Variability in Ovarian Tumours: Exploring Venous Thromboembolism Susceptibility and Cancer Prognosis in a Hospital-Based Study

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Abstract

Venous thromboembolism (VTE) is a frequently encountered paraneoplastic syndrome in patients with ovarian cancer (OC), an inflamm-aging entity. VTE is known to exacerbate their already poor prognosis, which is partially attributed to the contribution of the haemostatic system to ovarian tumourigenesis. In the past decade, numerous single-nucleotide polymorphisms (SNPs) implicated in VTE pathways have been proposed to influence tumour susceptibility and progression. These SNPs represent potential tools to improve the prognosis accuracy of OC patients. Hence, this study explored the influence of 12 haemostasis-associated SNPs on the risk for VTE, risk of OC progression and related death among 98 OC patients. The findings revealed a 20.5% incidence of VTE, which was associated with more rapid disease progression and shorter survival times (log-rank test, $p<0.05$). *PROCR* rs10747514 (AA/AG vs. GG; odds ratio (OR)=3.67, $p=0.037$) and *SERPINE1* rs2070682 (CC/CT vs. TT; OR=9.28, $p=0.040$) were predictors of OC-related VTE development. Regarding patients' prognosis regardless of venous thrombogenesis, *RGS7* rs2502448, *F3* rs1361600, *FGG* rs2066865, and *SERPINE1* rs2070682 were the most relevant biomarkers in different patient groups. These genetic variants might constitute attractive prognostic indicators among OC patients, offering insights to refine disease management strategies.

Keywords: Ovarian Neoplasms; Venous Thromboembolism; Prognosis; Genes; Polymorphism, Single Nucleotide.

1. Introduction

Genetic polymorphisms, present in at least 1% of a population, represent the most common form of DNA variation. These variations encompass single-nucleotide polymorphisms (SNPs), insertions and deletions (Indels), copy number variations (CNVs), and tandem repeats. Among these, SNPs are the most prevalent, occurring approximately once in every 800 base pairs [1,2]. Functional SNPs influence human susceptibility to multiple conditions, specifically common and complex diseases such as cardiovascular diseases [3,4].

Haemostasis is a dynamic process that gateways the integrity of blood vessels, ensuring continuous blood circulation [5]. It is tightly regulated to prevent both haemorrhage and unnecessary clotting (thrombosis), relying on a delicate balance between clotting and anti-clotting signals. Four key players are implicated: 1) blood vasculature, which typically provides an anti-clotting signal, unless dysfunctional; 2) platelets, responsible for the initial

plug to stop blood loss following vascular damage (primary haemostasis); 3) the coagulation system, consisting of coagulation factors and anticoagulant proteins regulating fibrin clot formation (secondary haemostasis); and 4) the fibrinolytic system, tasked with clot breakdown or fibrinolysis (tertiary haemostasis) [6,7]. Under physiological conditions, these components limit blood clotting to where and when it is needed. Consequently, upon haemostatic abnormalities, individuals are predisposed to coagulopathies, such as venous thromboembolism (VTE) [8]. This cardiovascular disease, encompassing mainly deep venous thrombosis (DVT) and pulmonary embolism (PE), affects approximately 10 million people worldwide each year [9]. In Western countries, 1 in 12 people will be diagnosed with VTE during their lifetime. Approximately 20% of affected individuals die within a year, and survivors frequently encounter various complications [10].

Malignancy is a major risk factor for VTE, with cancer patients having up to a nine-fold risk increase compared to the general population [11]. Several risk-assessment models for cancer-associated thrombosis (CAT) have been studied, the most validated being the Khorana Score (KS). However, it can present a poor performance, which can be partially explained by the complexity of CAT pathogenesis [12]. The disease is attributed to patient-related factors (e.g., advanced age and genetic factors), the characteristics of the malignant disease (e.g., primary tumour site) and antineoplastic treatments (e.g., chemotherapy) [13]. In the tumour microenvironment, cancer cells release multiple pro-clotting factors such as coagulation factor III (tissue factor, TF), along with heparanases and fibrinolytic regulators like plasminogen activator inhibitor-1 (PAI-1), which shifts the haemostatic balance towards tumour coagulome [13,14]. Also, these cells can activate endothelial cells, platelets and immune cells leading to thrombo-inflammation [15]. Inversely, disturbed haemostasis enhances cancer cells' ability to survive, create new blood vessels, evade the immune system, invade neighbouring tissues, and metastasise to distant sites - a phenomenon known as Trousseau syndrome [13].

Women with gynaecological cancers are particularly susceptible to Trousseau syndrome [16,17]. Among these, ovarian cancer (OC), the most lethal, stands out as having the most significant evidence linking coagulopathy defects to negative survival outcomes [18,19]. The reported incidence of CAT in OC patients ranges from 10% to 30%, a variation likely due to differences in diagnostic methods, the timing of VTE occurrence, and the size and characteristics of patient cohorts [20-28]. Given the poor prognosis of OC, ongoing research seeks to improve the treatment of the disease by identifying biomarkers that could enhance early detection, treatment outcomes, and overall patient care [20]. Thus, OC, an inflamm-aging entity, is an ideal candidate to study CAT and the role of haemostasis in cancer progression.

Several SNPs have been linked to VTE susceptibility, the most relevant being *coagulation factor 2 (F2)* rs1799963 and *coagulation factor 5 (F5)* rs6025 [4]. Although initially identified in the general population, these genetic variants are increasingly recognised for their role in CAT development and tumourigenesis [29]. Given the potential for biomarker discovery and/or validation, the present study was conducted to evaluate VTE-related SNPs as potential CAT predictors and prognostic biomarkers (regardless of thrombosis) in OC patients.

2. Methods

2.1. Population description and data collection

A retrospective cohort study included Caucasian patients diagnosed with epithelial ovarian cancer, who were admitted for initial treatment at the Gynecology Clinic of the Portuguese Oncology Institute of Porto (IPO Porto) between March 2017 and September 2023. The standard treatment protocol involved cytoreductive surgery and chemotherapy with platinum-based agents typically in combination with taxanes, administered every 21 days. The chemotherapy strategy - neoadjuvant, adjuvant, or standalone for those not eligible for surgery - determined the number of treatment cycles. Patients were excluded if they (1) were under 18 years of age, (2) were pregnant or breastfeeding at the time of their OC diagnosis, (3) declined chemotherapy, or (4) refused to provide informed consent. With these criteria, 98 patients from the North region of Portugal were recruited, all of whom had biological material derived from blood samples available in the institutional biobank. The median follow-up period was 25.5 months, with a range of 1.0 to 85.0 months. Each patient provided written informed consent following the principles of the Helsinki Declaration.

Information on patients' demographic and clinicopathological factors was revised and retrieved from their electronic medical records (**Table 1**). Given their potential relevance for CAT, data on baseline full blood count and coagulation assays - prothrombin time (PT), international normalised ratio (INR) and activated partial thromboplastin time (aPTT) - were also retrieved and KS was computed (**Table 1**) [12]. Also, monocyte-to-lymphocyte ratio (MLR), neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) were determined using data on baseline full blood count. In this study, cancer-associated VTE was defined as an event occurring from six months prior to two years after an OC diagnosis [30]. Active screening was not conducted as it is not part of the established clinical protocols at IPO Porto.

This study has been approved by the ethics committee at the research centre of IPO Porto (CI-IPOP) (CES. 69/021).

Chapter III - Table 1. Demographic and clinicopathological features of OC patients at first-line treatment (N=98)

Variable	Mean $\pm$ SD Median [min; max]	N (%)
Age at OC diagnosis (years) *	63.2 $\pm$ 11.8	
≥ 63		57 (58.2)
Hormonal status at OC diagnosis		
Postmenopausal		79 (80.6)
ECOG PS at OC diagnosis 		
>1		13 (13.3)
Baseline BMI (kg/m ²) *	26.0 $\pm$ 4.5	
≥ 26.0		48 (49.0)
Baseline haemoglobin levels (U/mL) *	12.2 $\pm$ 1.7	
<12.3		45 (45.9)
Baseline platelet count ($\times 10^9/L$) **	310.5 [135.0; 572.0]	
≥ 310.5		46 (46.9)
Baseline leucocyte count ($\times 10^9/L$) **	7.8 [4.3; 31.7]	
≥ 7.8		46 (46.9)
Baseline neutrophil count ($\times 10^9/L$) **	5.1 [2.2; 30.2]	
≥ 5.1		46 (46.9)
Baseline monocyte count ($\times 10^9/L$) **	0.6 [0.2; 1.4]	
≥ 0.6		46 (46.9)
Baseline lymphocyte count ($\times 10^9/L$) **	1.5 [0.5; 7.2]	
≥ 1.5		45 (45.9)
Baseline PT (s) **	14.2 [10.9; 31.2]	
≥ 14.2		41 (41.8)
Baseline INR **	1.1 [1.0; 2.2]	
≥ 1.1		41 (41.8)
Baseline aPTT (s) **	27.1 [21.2; 51.2]	
≥ 27.1		38 (38.8)
KS ***		
≥ 2		40 (40.8)
≥ 3		9 (9.2)
Platelet anti-aggregation therapy at OC diagnosis		10 (10.2)
Anticoagulation therapy at OC diagnosis 		9 (9.2)
History of previous and concurrent tumours		12 (12.2)
OC-related germline mutations		9 (9.2)
Tumour histology		
Serous		82 (83.7)
Clear cell		6 (6.1)
Endometrioid		4 (4.1)
Mixed		3 (3.1)
Unusual		2 (2.0)
Mucinous		1 (1.0)

Histological grade		
	High	88 (89.8)
	Borderline	3 (3.1)
	Low	4 (4.1)
Tumour stage #		
	III/IV	74 (75.5)
Baseline CA-125 levels (U/mL) **		
	541.3 [7.6; 10184.0]	
	≥541.3	48 (49.0)
Upfront treatment		
	Surgery and adjuvant chemotherapy	41 (41.8)
	Neoadjuvant chemotherapy, surgery and adjuvant chemotherapy	18 (18.4)
	Neoadjuvant chemotherapy and surgery	13 (13.3)
	Chemotherapy only	25 (25.5)
Platinum sensitivity δ		
		70 (71.4)
Maintenance therapy		
	PARPi	24 (24.5)
	bevacizumab	11 (11.2)

Baseline values deemed those preceding cancer treatment. * Variables with a normal distribution (Kolmogorov-Smirnov test, $p \geq 0.05$) were presented as mean $\pm$ (SD). The categories for the respective nominal variable were defined using the mean as a cut-off. † In addition to ECOG PS, the G-8 screening tool - recommended for older cancer patients - was not computed due to lack of data and the retrospective nature of this study [31]. ** Non-normally distributed variables (Kolmogorov-Smirnov test, $p < 0.05$) were presented as median [minimum; maximum]. The median value was used as a cut-off to define the categories for the nominal variable. *** Computed using as parameters: primary tumour site and baseline haemoglobin levels, BMI, platelet and leukocyte count. A cut-off of 2 and 3 were considered. A high KS is associated with a high CAT risk [12]. § Included patients with CAT events before OC diagnosis. # According to FIGO Cancer Report 2021 [32]. δ The disease was classified as platinum-sensitive if the tumour progressed at least six months following chemotherapy. Some patients lack information: 21 for aPTT, 19 for INR, 17 for PT, 11 for KS, nine for baseline monocyte and lymphocyte count, seven for baseline leukocyte and neutrophil count, six for baseline haemoglobin levels and platelet counts and platinum sensitivity, three for the histological grade, two for germline mutations and baseline CA-125 levels and one for BMI. One patient died before starting the upfront treatment. Abbreviations: aPTT, activated partial thromboplastin time; BMI, body mass index; CA-125, cancer antigen 125; ECOG PS; Eastern Cooperative Oncology Group Performance status; FIGO, International Federation of Gynecology and Obstetrics; INR, international normalised ratio; KS, Khorana score; OC, ovarian cancer; PT, prothrombin; SD, standard deviation.

2.2. Sample processing and genomic DNA isolation

Using a conventional venipuncture technique, peripheral venous blood samples were collected from the patients and placed into EDTA-coated tubes. Genomic DNA was isolated from these samples using the Qiagen® QIAmp DNA Blood Mini Kit (Qiagen® 51106), following the manufacturer's protocol. DNA purity and concentration were evaluated using the NanoDrop Lite spectrophotometer (Thermo Scientific®, Waltham, MA, USA). After extraction, the DNA samples were stored at -20°C until use.

2.3. SNP selection and genotyping

Previous reviews were conducted to identify haemostatic gene SNPs linked to VTE and CAT development, tumour susceptibility and progression [1,4,29,33]. The most suitable DNA variations were selected to be studied by prioritising those with a predictive value for CAT and/or those associated with cancer patient's prognosis, regardless of thrombogenesis. Given the complexity of CAT pathogenesis, the different components of haemostasis - blood vasculature, platelets and coagulation/fibrinolytic systems - were covered. Except for *F2* rs1799963 and *F5* rs6025, only variants with a minimum minor allele frequency (MAF) of 10% within the Iberian population were considered to ensure comprehensive coverage of SNP genotypes. Following these criteria, 12 SNPs were selected to be evaluated (**Table 2**).

Chapter III - Table 2. Selected single-nucleotide polymorphisms (SNPs) related to haemostasis

Role	Gene	Genetic variant	Location	Functionality
Endothelium activity	<i>Nitric oxide synthase 3</i>	<i>NOS3</i> rs1799983 (G>T)	7q36.1	Missense
Platelet function	<i>Integrin subunit beta 3</i>	<i>ITGB3</i> rs5918 (T>C)	17q21.32	Missense
Platelet function	<i>Prostaglandin-endoperoxide synthase 2</i>	<i>PTGS2</i> rs20417 (C>G)	1q31.1	Non-coding transcript exon
Platelet function	<i>Regulator of G protein signalling 7</i>	<i>RGS7</i> rs2502448 (T>C)	1q43	Intronic
Coagulation system	<i>Coagulation factor 2</i>	<i>F2</i> rs1799963 (G>A)	11p11.2	3 prime UTR
Coagulation system	<i>Coagulation factor 3</i>	<i>F3</i> rs1361600 (T>C)	1p21.3	Non-coding transcript exon
Coagulation system	<i>Coagulation factor 5</i>	<i>F5</i> rs6025 (C>T)	1q24.2	Missense
Coagulation system	<i>Fibrinogen gamma chain</i>	<i>FGG</i> rs2066865 (G>A)	4q32.1	Intergenic
Coagulation system	<i>Protein C receptor</i>	<i>PROCR</i> rs10747514 (G>A)	20q11.22	Intronic
Coagulation system	<i>Tissue Factor Pathway Inhibitor 1</i>	<i>TFPI</i> rs10153820 (G>A)	2q32.1	Intronic
Fibrinolytic system	<i>Serpin family E member 1</i>	<i>SERPINE1</i> rs2070682 (T>C)	7q22.1	Intronic
Multifunctional	<i>Zinc finger protein, FOG family member 2</i>	<i>ZFPM2</i> rs4734879 (A>G)	8q23.1	Intronic

Data on SNP characteristics was retrieved from the Ensembl database, which uses the forward strand as a reference [34]. Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; UTR, untranslated region.

Polymorphism genotyping was conducted using a StepOnePlus™ Real-time Polymerase Chain Reaction (RT-PCR) system (Applied Biosystems®, Carlsbad, CA, USA) and applying the TaqMan® Allelic Discrimination method. The PCR reactions were performed using 2.5 µL of TaqPath™ ProAmp™ Master Mix (1x), 2.375 µL of DNA and RNA-free water, 0.125 µL of predesigned TaqMan® Genotyping assays for *NOS3* rs1799983 (C__3219460_20), *ITGB3* rs5918 (C__818008_30), *PTGS2* rs20417 (C__11997909_40), *RGS7* rs2502448 (C__26887460_10), *F2* rs1799963 (C__8726802_20), *F3* rs1361600 (C__8384809_10), *F5* rs6025 (C__11975250_10), *FGG* rs2066865 (C__11503414_10), *PROCR* rs10747514 (C__1825060_10), *TFPI*

rs10153820 (C__11514522_10), *SERPINE1* rs2070682 (C__2620939_1_) and *ZFPM2* rs4734879 (C__1315535_10), as well as 1.0 μ L of genomic DNA, making a total volume of 6 μ L. In each PCR run, negative controls (lacking DNA) were incorporated to assess false positives. The cycling conditions for DNA amplification were the following: 10 min at 95 °C (activation of Taq DNA polymerase), 45 cycles at 95 °C for 15 s (denaturation of DNA chains), and 60 s at 60 °C (pairing and extension of primers). Over 10% of the DNA samples were randomly selected for a double sampling and complete concordance was confirmed. The StepOne Software version 2.3 (Applied Biosystems) was used to analyse the data on DNA amplification. Genotyping results were evaluated by two researchers with no previous knowledge concerning the demographic and clinical data of the patients.

2.4. Statistical analysis

Statistical analysis and graphing were done employing the IBM SPSS Statistics for Windows (version 29, IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 9.0.0, GraphPad Software Inc., La Jolla, CA, USA), respectively.

Data distribution was evaluated using the Kolmogorov-Smirnov test ($N > 50$). Continuous variables were dichotomised using the mean or median value as a cut-off for normally and non-normally distributed variables, respectively.

Patients were classified as VTE-free if they did not develop the condition or passed away without presenting it during the two-year follow-up period. Patients who were still alive but had a follow-up of less than two years were not included in the VTE analysis.

Genotype distribution in this study was compared with that reported for the Iberian population in the Ensembl database (<https://www.ensembl.org/index.html>, accessed on 8th October 2023). The Hardy-Weinberg equilibrium (HWE) was evaluated using the chi-square test (χ^2). Associations between the SNPs and patients' clinical and demographic characteristics (categorical variables), including CAT status, were also assessed using χ^2 across different groups.

The area under the curve (AUC) score was calculated for SNPs that had a predictive role in OC-related VTE development. To confirm these findings, logistic regression analysis was performed. Additionally, bootstrapping was used to assess the stability of the results.

The evaluated clinical outcomes - progression-free survival (PFS) and overall survival (OS) - were described elsewhere. The former was defined as the duration from the start of treatment to the earliest event of tumour progression, relapse, patient death or the last clinical assessment. Tumour progression was evaluated using the Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 [35]. OS, on the other hand, was the period between the cancer diagnosis and death from any cause or the patient's last recorded follow-up. The Kaplan-Meier (K-M) method and log-rank test were utilised to generate

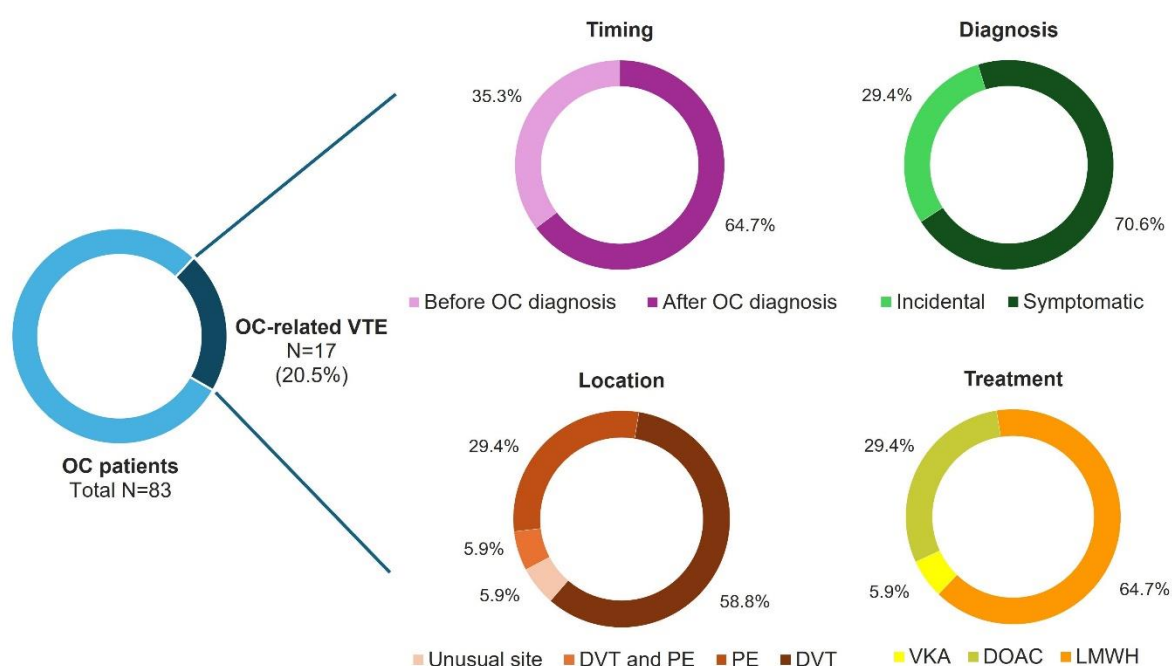
survival curves and calculate survival probabilities, respectively. The impact of VTE and the SNPs on these clinical outcomes was investigated across distinct groups (only the subgroups with significant findings were shown). The most suitable genetic model for the SNPs - dominant or recessive - was determined by evaluating the K-M curves under the additive model. The impact of the SNPs on the risk of OC progression and mortality was confirmed with multivariable Cox regression analyses. Harrell's concordance (C)-index was computed to compare the predictive ability of the models for disease progression and death [36].

All applied tests were two-sided, with a 5% significance level considered. Additionally, *p*-values between 0.05 and 0.06 were deemed to have marginal significance.

3. Results

3.1. Impact of VTE on patients' prognosis

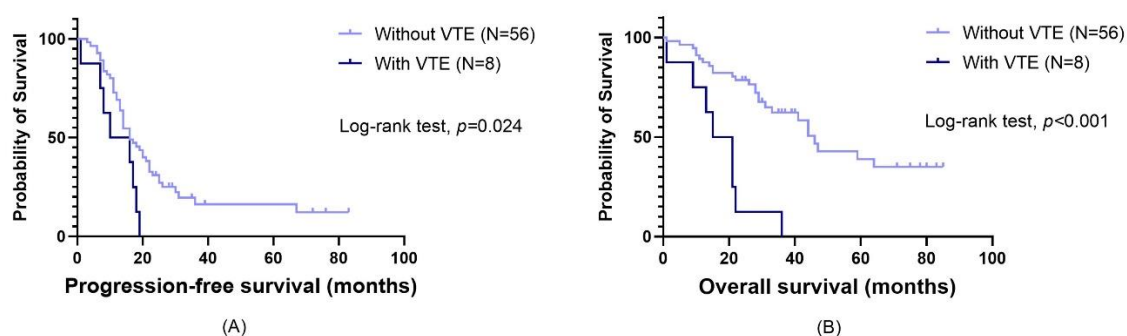
In the cohort with at least a two-year follow-up (N=83), 17 patients (20.5%) developed CAT (**Figure 1**). Patients with an event preceding tumour diagnosis (N=6) presented a median interval between CAT and OC diagnosis of 3.5 months (minimum=0; maximum=6 months), while for their counterparts (N=11), the median time was 9.0 months (minimum=0; maximum=24 months).



Chapter III - Figure 1. Incidence and characteristics of ovarian cancer (OC)-related venous thromboembolism (VTE) in the study cohort. Abbreviations: DOAC, direct oral anticoagulants;

DVT, deep venous thrombosis; LMWH, low-molecular-weight heparin; OC, ovarian cancer; PE, pulmonary embolism; VKA, vitamin K antagonists; VTE, venous thromboembolism.

The impact of VTE on OC patients' PFS and OS was analysed under four conditions: 1) the entire cohort (N=83), 2) excluding those on anticoagulation therapy at OC diagnosis (encompassing patients with CAT events before OC diagnosis), 3) excluding those with a history of previous or concurrent tumours, and 4) excluding both groups. Only in the latter condition did VTE significantly impact the time to cancer progression (log-rank test, $p=0.024$; **Figure 2A**), with patients having a two-fold increased risk of progression (hazard ratio (HR)=2.34, 95% confidence interval (95% CI), 1.07-5.11, $p=0.033$). Concerning OS, VTE had a significant influence across all conditions (log-rank test, $p<0.001$). Excluding both anticoagulation at cancer diagnosis and tumour history groups, VTE patients showed lower survival times (log-rank test, $p<0.001$; **Figure 2B**) with a five-fold increased risk of death (HR=5.34, 95% CI, 2.28-12.50, $p<0.001$). Across all subgroup analyses, none of the patient characteristics, including data on KS, baseline coagulation assays, full blood count and its derivatives (MLR, NLR and PLR), showed any association with OC-related VTE development (χ^2 , $p\geq 0.05$). Interestingly, most of the patients with VTE after OC diagnosis had a low KS score [KS<2, N=7 (63.6%); KS<3, N=9 (81.8%)].

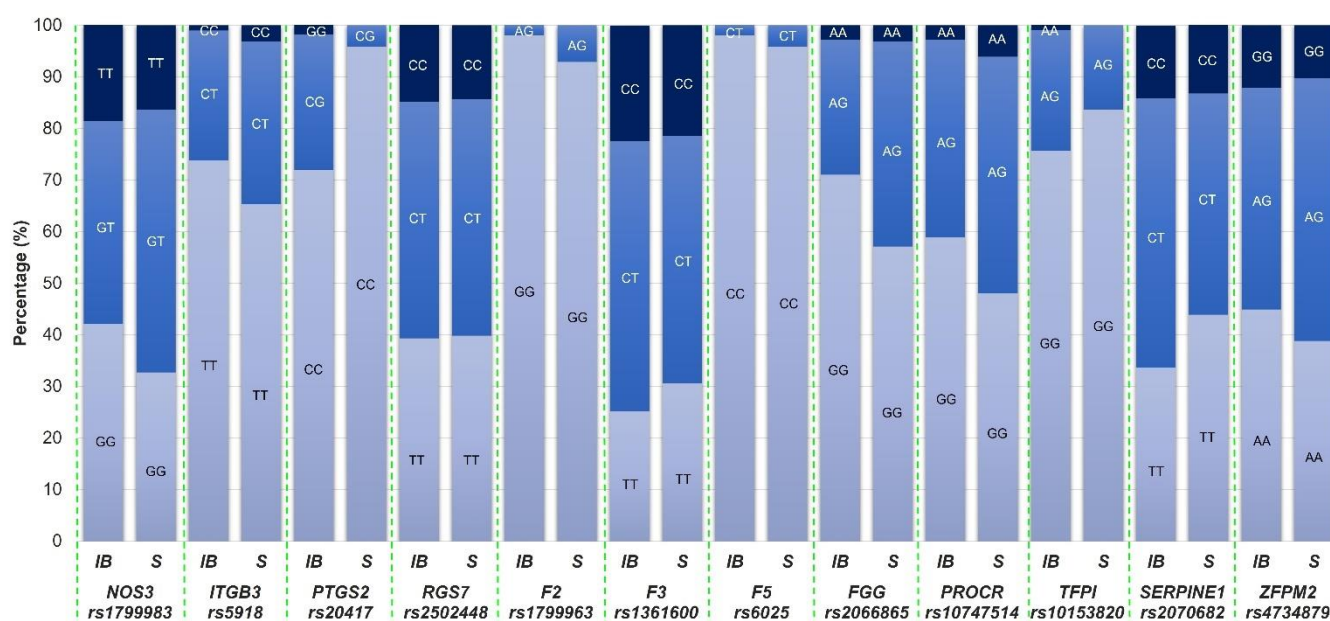


Chapter III - Figure 2. Progression-free survival (PFS) (A) and overall survival (OS) (B) by Kaplan-Meier and log-rank test among ovarian cancer (OC) patients, according to cancer-associated thrombosis (CAT) occurrence post-tumour diagnosis. (A) CAT patients had an inferior PFS than their counterparts (mean PFS of 12.0 ± 3.2 months and 26.4 ± 3.6 months, respectively; log-rank test, $p=0.024$). **(B)** Likewise, they also presented a lower OS (mean OS of 17.3 ± 3.7 months and 50.6 ± 4.5 months, respectively; log-rank test, $p<0.001$).

3.2. Genotype distribution

The distribution of the SNPs' genotypes is represented in **Figure 3**. There was a significant difference in the distribution of *PTGS2* rs20417 genotypes in the Iberian

population and study cohort (χ^2 , $p < 0.001$), while the remaining variants were in HWE (χ^2 , $p \geq 0.05$). Thus, *PTGS2* rs20417 was not included in the further analyses.



Chapter III - Figure 3. Genotype distribution of the evaluated single-nucleotide polymorphisms (SNPs) in the Iberian population (IB) and the study cohort (S).

3.3. Genetic variants and patients' characteristics

In the entire cohort, significant associations were identified for *ITGB3* rs5918, *RGS7* rs2502448, *F3* rs1361600, *F5* rs6025 and *ZFPM2* rs4734879. The *ITGB3* rs5918 C allele was more common in patients with elevated baseline INR (CC/CT vs. TT; χ^2 , $p = 0.004$). *RGS7* rs2502448 T allele genotypes were more prevalent in post-menopausal patients (TT/CT vs. CC; χ^2 , $p = 0.006$) and those not eligible for bevacizumab therapy (TT/CT vs. CC; χ^2 , $p = 0.007$). The *F3* rs1361600 CC genotype was more frequent among patients with a body mass index (BMI) < 26.0 kg/m² (CC vs. TT/CT; χ^2 , $p = 0.004$). The *F5* rs6025 CC genotype was more common in patients with KS < 3 (CC vs. CT; χ^2 , $p = 0.052$). The *ZFPM2* rs4734879 GG genotype was more common in patients with KS < 2 (GG vs. AA/AG; χ^2 , $p = 0.010$). These associations were further confirmed across different subgroups: patients without a history of previous or concurrent tumours, those not under anticoagulation therapy at OC diagnosis, those not receiving platelet anti-aggregation therapy at diagnosis, and those not receiving either treatment at diagnosis.

3.4. Genetic variants and OC-related VTE susceptibility

The χ^2 test detected no significant associations between the SNPs and CAT ($p \geq 0.05$). However, *PROCR* rs10747514 (AA/AG vs. GG) showed a marginal link to disease

susceptibility (χ^2 , $p=0.057$) with an AUC score of 0.65. Specifically, the A allele genotypes were the most prevalent among VTE patients. Moreover, excluding patients on anticoagulation at OC diagnosis, a significant association between *SERPINE1* rs2070682 (CC/CT vs. TT) and VTE development was revealed (χ^2 , $p=0.038$), with an AUC score of 0.70. Among those with VTE, the rs2070682 C allele genotypes were more common than the TT genotype. Both SNPs' associations with CAT were validated through logistic regression analysis (**Table 3**).

Chapter III - Table 3. *PROCR* rs10747514 and *SERPINE1* rs2070682 genotype frequencies according to venous thromboembolism (VTE) status and associated odds ratios.

SNP	VTE patients N (%)	VTE-free patients N (%)	OR	95% CI	p-Value
N=83					
<i>PROCR</i> rs10747514					
GG	4 (23.5)	35 (53.0)	Reference	-	
AA/AG	13 (76.5)	31 (47.0)	3.67	1.08-12.44	0.037
					0.023 *
N=75					
<i>SERPINE1</i> rs2070682					
TT	1 (10.0)	33 (50.8)	Reference	-	
CC/CT	9 (90.0)	32 (49.2)	9.28	1.11-77.51	0.040
					0.028 *

* Bootstrap analysis. Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism; VTE, venous thromboembolism.

3.5. Impact of genetic variants on patients' prognosis

Significant findings were observed for *F3* rs1361600 (TT/CT vs. CC), *FGG* rs2066865 (GG/AG vs. AA), *RGS7* rs2502448 (CC/CT vs. TT) and *SERPINE1* rs2070682 (TT/CT vs. CC) in different cohort groups (log-rank test, $p<0.05$; **Table 4**).

Chapter III - Table 4. Progression-free survival (PFS) and overall survival (OS) by log-rank test among ovarian cancer (OC) patients across different subgroups.

SNP	N	PFS Mean ± SD (months)	p-Value	N	OS Mean ± SD (months)	p-Value
Entire cohort regardless of VTE (N=98)						
FGG rs2066865						
GG/AG	94	28.4 ± 3.2	0.011			
AA	3	10.0 ± 1.5				
No tumour history (N=86)						
FGG rs2066865						
GG/AG	83	26.2 ± 3.2	0.008	84	46.5 ± 4.0	0.035
AA	2	9.0 ± 2.0		2	19.0 ± 9.0	
RGS7 rs2502448						
CC/CT	52	19.8 ± 2.5	0.021			
TT	33	39.6 ± 6.2				
No anticoagulation therapy at OC diagnosis (N=89)						
FGG rs2066865						
GG/AG	85	29.1 ± 3.4	0.035			
AA	3	10.0 ± 1.5				
No platelet anti-aggregation therapy at OC diagnosis (N=88)						
FGG rs2066865						
GG/AG	85	28.9 ± 3.4	0.028			
AA	2	9.5 ± 2.5				
Absence of both anticoagulation therapy at OC diagnosis and a tumour history (N=77)						
RGS7 rs2502448						
CC/CT	43	19.3 ± 2.7	0.021			
TT	33	39.6 ± 6.2				
No platelet anti-aggregation therapy at OC diagnosis and no tumour history (N=78)						
RGS7 rs2502448						
CC/CT	47	20.8 ± 2.8	0.047			
TT	30	39.7 ± 6.6				
Absence of both antithrombotic therapies at OC diagnosis (N=80)						
F3 rs1361600						
TT/CT				64	44.6 ± 4.5	0.040
CC				16	63.2 ± 7.9	
FGG rs2066865						
GG/AG	77	29.4 ± 3.7	0.029			
AA	2	9.5 ± 2.5				
SERPINE1 rs2070682						
TT/CT	69	25.9 ± 3.6	0.041			
CC	10	46.2 ± 10.0				
Absence of both antithrombotic therapies at OC diagnosis and no tumour history (N=70)						
RGS7 rs2502448						
CC/CT	39	20.3 ± 3.0	0.046			
TT	30	39.7 ± 6.6				

Abbreviations: OC, ovarian cancer; SNP, single-nucleotide polymorphism; VTE, venous thromboembolism.

The entire cohort, patients who were not receiving any antithrombotic therapy at OC diagnosis and those who additionally had no history of other tumours yielded distinct and significant findings, highlighting the unique characteristics of these patient groups (**Table 4**). Multivariate Cox analyses, adjusted for relevant patient characteristics, were conducted for the SNPs considering the entire cohort and the two subgroups. Predictors of OC

progression in the entire cohort (N=98) included patients' age (≥ 63 years, HR=1.64, $p=0.049$) and Eastern Cooperative Oncology Group Performance Status (ECOG PS >1 , HR=2.89, $p=0.001$) at tumour diagnosis, International Federation of Gynecology and Obstetrics (FIGO) staging (III/IV stages, HR=2.94, $p=0.001$), baseline INR (≥ 1.1 , HR=2.09, $p=0.008$) and first treatment (surgery, HR=0.23, $p<0.001$). These predictors were further validated in the two subgroups. Subsequently, in a multivariate Cox model using the backward Wald method, patients' age at OC diagnosis and first treatment emerged as the most significant predictors of disease progression across all three groups.

Regarding the risk of death, predictors in the overall cohort included patients' age (≥ 63 years, HR=3.62, $p<0.001$), ECOG PS (>1 , HR=2.80, $p=0.004$) and hormonal status (post-menopause, HR=3.91, $p=0.010$) at tumour diagnosis, FIGO stages (III/IV stages, HR=2.44, $p=0.031$), metastasis at diagnosis and/or during cancer management (yes, HR=4.62, $p=0.001$), baseline haemoglobin levels (<12.3 U/mL, HR=2.14, $p=0.015$), lymphocyte count ($\geq 1.5 \times 10^9/L$, HR=0.43, $p=0.010$) and INR (≥ 1.1 , HR=2.01, $p=0.037$), KS (≥ 3 , HR=2.49, $p=0.044$), first treatment (surgery, HR=0.30, $p<0.001$), surgery (no, HR=5.33, $p<0.001$) and platinum sensitivity (no, HR=5.05, $p<0.001$). Notably, the variable surgery was defined as whether or not the intervention was included in the patient's first-line treatment. Among these factors, ECOG PS, baseline haemoglobin levels, INR, and KS did not exhibit a predictive capability in the cohort subgroups. Focusing on the remaining factors, the multivariable Cox analysis using the backward Wald method identified patients' age at OC diagnosis, baseline lymphocyte count, surgery and platinum sensitivity as the most relevant predictors of the risk of death across all cohort subgroups.

Considering the important predictors for the risk of OC progression and death (clinic models), multivariate Cox analyses incorporating the evaluated SNPs (integrative models) were conducted for the three patient groups, as summarised in **Table 5**. In terms of predictive ability, as measured by the C-index, the integrative models demonstrated superior performance compared to their clinical counterparts.

Chapter III - Table 5. Multivariable Cox analysis on the risk of tumour progression and risk of death

Patient group (N)	Variable	aHR	95%CI	p- Value	C-index		Event
					Clinic model	Integrative model	
Entire cohort (98)	Patients' age at OC diagnosis (≥63 vs. <63 years ¹)	1.66	0.99-2.77	0.054	0.703	0.713	Risk of disease progression
	Primary treatment (Surgery vs. chemotherapy ¹)	0.22	0.13-0.40	<0.001			
	FGG rs2066865 (AA vs. GG/AG ¹)	3.79	1.14-12.66	0.030			
Absence of antithrombotic therapy at OC diagnosis (80)	Patients' age at OC diagnosis (≥63 vs. <63 years ¹)	2.49	1.34-4.62	0.004	0.692	0.729	
	Primary treatment (Surgery vs. chemotherapy ¹)	0.27	0.14-0.51	<0.001			
	FGG rs2066865 (AA vs. GG/AG ¹)	9.44	2.05-43.49	0.004			
	RGS7 rs2502448 (TT/CT vs. CC ¹)	3.12	1.29-7.57	0.012			
	SERPINE1 rs2070682 (TT/CT vs. CC ¹)	2.79	1.02-7.64	0.046			
	ZFPM2 rs4734879 (GG vs. AA/AG ¹)	3.08	1.30-7.27	0.010			
Absence of antithrombotic therapy at OC diagnosis and a history of other tumours (70)	Patients' age at OC diagnosis (≥63 vs. <63 years ¹)	3.91	1.86-8.21	<0.001	0.690	0.719	
	Primary treatment (Surgery vs. chemotherapy ¹)	0.43	0.22-0.85	0.015			
	F3 rs1361600 (TT/CT vs. CC ¹)	2.90	1.10-7.68	0.032			
Entire cohort (84)	Patients' age at OC diagnosis (≥63 vs. <63 years ¹)	6.37	2.72-14.92	<0.001	0.855	-	Risk of death
	Baseline lymphocyte count (≥1.5 vs. <1.5 ×10 ⁹ /L ¹)	0.35	0.1-0.73	0.005			
	Surgery (No vs. yes ¹)	3.46	1.74-6.86	<0.001			
	Platinum sensitivity (No vs. yes ¹)	4.60	2.63-8.04	<0.001			
Absence of antithrombotic therapy at OC diagnosis (68)	Patients' age at OC diagnosis (≥63 vs. <63 years ¹)	14.48	4.49-46.66	<0.001	0.862	0.883	
	Baseline lymphocyte count (≥1.5 vs. <1.5 ×10 ⁹ /L ¹)	0.27	0.11-0.67	0.005			
	Surgery (No vs. yes ¹)	2.20	0.97-4.99	0.059			
	Platinum sensitivity (No vs. yes ¹)	6.98	3.39-14.39	<0.001			
	F3 rs1361600 (TT/CT vs. CC ¹)	4.45	1.23-16.03	0.023			
Absence of antithrombotic therapy at OC diagnosis and a history of other tumours (58)	Patients' age at OC diagnosis (≥63 vs. <63 years ¹)	21.41	5.69-80.59	<0.001	0.834	0.874	
	Baseline lymphocyte count (≥1.5 vs. <1.5 ×10 ⁹ /L ¹)	0.21	0.08-0.56	0.002			
	Platinum sensitivity (No vs. yes ¹)	6.65	3.06-14.43	<0.001			
	F2 rs1799963 (GG vs. AG ¹)	6.02	0.76-47.84	0.090			
	F3 rs1361600 (TT/CT vs. CC ¹)	4.35	0.96-19.81	0.057			

¹ Reference group. Clinic models incorporated only demographic and clinicopathological factors, while integrative models also encompassed the genetic variants. Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; N, number of participants; OC, ovarian cancer.

4. Discussion

As the population ages, cancer incidence is expected to rise [37]. Concurrently, complications like CAT may increase, also driven by new cancer treatments [13,29]. Genetic factors, including mutations and polymorphisms, are key modulators of thrombogenic potential. From a population perspective, genetic polymorphisms like SNPs exert a more significant role as they are more prevalent and have a stronger association with common diseases compared to genetic mutations - common variant-common disease (CVCD) theory [4]. Drawing upon the concept of Trousseau syndrome, this study explored the implications of haemostatic DNA variations in CAT occurrence and OC patients' prognosis, regardless of VTE. The results revealed a CAT incidence of nearly 21%, which is consistent with the literature [20]. The disease was found to affect OC patients' prognosis adversely. However, unlike OS, PFS was significantly impacted only when excluding patients who were on anticoagulation therapy at OC diagnosis and those with a history of previous or concurrent tumours. Several factors could be in play, including variations in CAT pathogenesis based on whether it occurs before or after OC diagnosis/treatment, potential benefits of using anticoagulants before cancer treatment for patients with CAT preceding tumour diagnosis, and the influence of a complex medical history with multiple tumours and an underlying pro-tumourigenic microenvironment. In this study, neither patients' characteristics nor KS were predictors of OC-related VTE development, which requires confirmation in larger cohorts.

The SNP rs5918 involves a thymidine (T) to cytosine (C) substitution (T>C) in exon 2 of the *ITGB3*. The gene encodes for the integrin $\beta 3$ subunit, also known as glycoprotein (GP) IIIa, a member of the integrin complex found at the platelet's surface. This complex, composed of GP IIb/IIIa receptors, binds to fibrinogen and other ligands, thereby regulating platelet activity [38]. In addition to coagulation, which promotes tumourigenesis, GP IIIa is involved in many signalling pathways that fuel tumour cell proliferation, migration and invasion [39]. As a missense variant, rs5918 leads to a leucine-to-proline substitution at amino acid 33 of GP IIIa, altering the protein's three-dimensional structure and its affinity for fibrinogen [40,41]. This variant has been associated with platelet reactivity, aspirin resistance, and both venous and arterial thrombotic events [38,42-44]. Also, in a previous study of our research group conducted with an independent cohort, *ITGB3* rs5918

modulated the prognosis of OC patients at early disease stages [45]. In the present study, except for a link to baseline INR, (χ^2 , $p=0.004$), no significant association was detected for this SNP. Further investigation in larger and more diverse cohorts is needed to explore the implications of *ITGB3* rs5918 in OC patients.

The intronic SNP rs2502448 (T>C) is within the *RGS7* gene. This gene encodes for a protein with the same name, which modulates G-protein-coupled receptor (GPCR) signalling pathways by blocking signal transduction [34,46]. *RGS7* seems to modulate platelet activity [47]. Consistently, like *ITGB3* rs5918, *RGS7* rs2502448 was previously linked to aspirin resistance [48,49]. Although its functional consequence is unclear, intronic variants such as *RGS7* rs2502448 can influence gene expression by disrupting transcription factor binding sites or altering protein function by interfering with constitutive splicing [50]. Indeed, RGS proteins' expression is thought to be dynamically modulated by several mechanisms, from epigenetic to post-translation regulation [51]. Regarding the role of rs2502448 in CAT and tumour pathogenesis, our research group found that the SNP C allele had a pro-thrombotic effect in cervical cancer patients, whereas the T allele was associated with a higher risk of death when excluding those with VTE [52]. Consistently, our previous study on OC patients has also identified a negative effect of the rs2502448 T allele on survival time [45]. The present study found no association between the SNP and CAT development in OC patients. Regarding patient prognosis, rs2502448 did not influence the risk of death, while its impact on tumour progression varied depending on the context of the analysis. Among patients with a history of tumours, the rs2502448 C allele was associated with a negative effect (log-rank, $p<0.05$). Conversely, in multivariable Cox analysis dismissing patients on antithrombotic therapy at OC diagnosis, the T allele genotypes were linked to a higher risk of disease progression. These genotypes were more prevalent in post-menopausal patients and those not eligible for bevacizumab therapy. However, none of these factors predicted OC progression. Although these findings should be interpreted carefully given the small cohort size, they collectively suggest a context-dependent role for *RGS7* rs2502448, aligning with the multifunctional nature of RGS proteins [51].

The polymorphism rs1799963 (G>A) is located within the 3' untranslated region (UTR) of the *F2* gene, which encodes for prothrombin, also known as coagulation factor II (FII) [34]. The prothrombinase complex (formed by activated coagulation factors V (FV) and X (FX)) converts prothrombin into thrombin, which subsequently converts fibrinogen into fibrin and activates platelets, while also initiating anticoagulation and fibrinolytic pathways to reestablish haemostasis [53,54]. In addition to blood clotting, thrombin impacts tumourigenesis by interacting with the protease-activated receptor (PAR-1) [55-57]. The 3' UTR variants, such as SNP rs1799963, can influence gene expression by interfering with

the binding sites for RNA-binding proteins (RBPs) and microRNAs [58]. Studies indicate that the rs1799963 A allele is linked to prothrombin upregulation. Recognised as one of the most relevant SNPs in VTE pathogenesis in the general population, alongside *F5* rs6025, *F2* rs1799963 has been extensively studied in cancer patients. Although no association with CAT has been found, likely due to a lack of genotype representation, this SNP was linked to the risk for several tumours [29]. Additionally, our previous study with cervical cancer patients revealed that the rs1799963 A allele negatively impacts OS, consistent with its pro-thrombotic effect. [59]. Contrariwise, in the present study, no significant association of rs1799963 with patients' characteristics, CAT development or clinical outcomes was detected, a finding that the underrepresentation of the SNP A allele could influence. Notably, in the multivariable Cox analysis excluding patients with a history of other tumours and those on antithrombotic therapy at OC diagnosis, the SNP was retained in the integrative model for the risk of death with an improved predictive ability compared to its clinical counterpart. However, rs1799963 was not an independent predictor ($p \geq 0.05$).

The non-coding transcript exon SNP rs1361600 (T>C) is within the *F3* gene, responsible for encoding TF [34]. This protein triggers the extrinsic coagulation pathway, which has a central role in CAT development and favours cancer cell growth, angiogenesis, invasion and metastasis [60,61]. Inclusively, TF upregulation in several cancer types, including OC, is linked to an unfavourable prognosis [20]. The protein levels are influenced by rs1361600, though the findings are controversial. For example, Shi and colleagues (2014) demonstrated that, in a sepsis context, C allele carriers had significantly lower *F3* expression in peripheral blood cells compared to TT carriers [62]. Conversely, Kishor et al. (2018) found that the C allele was significantly more prevalent in DVT patients than healthy controls and associated with higher TF levels than the TT genotype [63]. In the context of cancer, this SNP is poorly investigated, despite the crucial role of TF in tumour development [29]. In this study, the rs1361600 CC genotype was more prevalent among patients with a lower BMI (χ^2 , $p=0.004$). Notably, BMI was not a predictor of OC progression or mortality risk. Moreover, no association between the SNP and CAT was found. Conversely, the SNP T allele was associated with decreased OS in the patient subgroup not receiving antithrombotic therapy at OC diagnosis (log-rank test, $p=0.040$). The T allele's negative effect was confirmed in multivariable Cox analyses for both OC progression and mortality risk. In the former, the SNP was an independent predictor only when additionally excluding patients without a history of other tumours. For the risk of death, in both conditions, the rs1361600 T allele was shown to have a negative effect. However, in the subgroup with the absence of both a history of other tumours and antithrombotic therapy at OC diagnosis, the association was only marginally significant (aHR=4.35, $p=0.057$), which could be explained

by the small subgroup size. Collectively, these findings pinpoint *F3* rs1361600 as an important prognostic indicator of OC.

The missense SNP *F5* rs6025 (C>T) causes an arginine-to-glutamine substitution at position 506 of FV, leading to impaired clearance of the activated protein by protein C. This results in a condition known as activated protein C (APC) resistance, which diminishes the anticoagulant effect and enhances the risk of thrombosis [29,34]. As previously mentioned, rs6025 is one of the most studied SNPs in the broader population [4,33]. As such, its impact on CAT development has been evaluated, with numerous studies linking the SNP T to a significantly elevated risk of CAT. The potential role of this SNP in both tumour susceptibility and progression has also been reported, although the underlying mechanisms are poorly understood [29]. At least partially, FV may contribute to cancer pathways through the generation of thrombin, which itself has recognised roles in tumourigenesis [55-57]. This study observed a marginally significant association between *F5* rs6025 and the KS, with the CC genotype being more prevalent among patients with KS<3 (χ^2 , $p=0.052$). Since a lower KS indicates a reduced risk of venous thrombogenesis, this finding suggests that individuals with the CC genotype may have a lower susceptibility, consistent with existing literature [29,33]. However, no association between *F5* rs6025 and CAT development was detected, nor did it impact the prognosis of OC patients, which could be partially explained by the underrepresentation of the SNP T alleles in the cohort.

The intergenic variant rs2066865 is near the *FGG* gene, which encodes for the gamma chain of fibrinogen [34]. Fibrinogen, which is converted into fibrin for blood clotting, has a prognostic value in various cancers, including ovarian, colorectal, renal, and gastric malignancies [64,65]. Elevated levels of this haemostatic protein are thought to correlate with poor prognosis of ovarian carcinoma [66]. Among other functions, fibrinogen can promote metastasis by enhancing the adhesion of tumour cells to platelets [64,65]. Regarding the SNP, while implicated in CAT development, with the AA genotype having a pro-thrombotic risk, its impact on cancer is poorly explored [29]. Mechanistically, the SNP A allele is thought to affect the composition of fibrinogen, thereby changing its function [67]. While no link to CAT occurrence was observed in this study, *FGG* rs2066865 emerged as a significant predictor of tumour progression. Namely, the SNP AA genotype was found to have a detrimental effect on OC progression, which was corroborated by multivariable analysis. Although these results should be carefully interpreted given the underrepresentation of the AA genotype, they align with the literature attributing a pro-thrombotic effect to the AA genotype [29]. As for patients' survival time, the effect observed in the univariable analysis was not sustained in the multivariable Cox analyses.

The intronic variant rs10747514 (G>A) is found within *PROCR*, which encodes for the human endothelial protein C receptor (EPCR), a serine protease involved in the protein C

anticoagulant pathway [68-70]. Although with an unclear functional consequence, *PROCR* rs10747514 is thought to affect EPCR expression and/or activity, with implications in VTE. According to Ensembl (last accessed on 24 July 2024), the SNP A allele is in complete linkage disequilibrium ($r^2=1$, meaning coinheritance) with the *PROCR* rs34234989 deletion (D) allele in several populations, including the Iberians. The D allele of this intronic indel was previously linked to an increased VTE risk in the general population in a genome-wide association study (GWAS) [71]. Thus, the rs10747514 A allele could presumably exert a prothrombotic effect, which was corroborated in a previous study of our research group with cervical cancer patients [52]. Consistently, in the current study, the rs10747514 AA and AG genotypes were associated with nearly a four-fold increase in the risk for CAT development ($p=0.037$). Thus, this SNP could be a valuable tool to personalise thromboprophylaxis among OC cancer patients. Notably, it remains unclear whether rs10747514 or rs34234989 is the functional polymorphism, requiring further investigation. Beyond thrombogenesis, EPCR is implicated in multiple tumourigenic-related processes [52]. However, no association between rs10747514 and OC patients' prognosis was observed.

The gene *SERPINE1* encodes for PAI-1, a protein that inhibits plasminogen activators and restricts fibrinolysis [72]. Elevated PAI-1 is linked to thrombosis and tumourigenesis, with the protein overexpressed in several tumours [72-74]. This antifibrinolytic protein has been shown to favour cell growth in OC and promote cisplatin resistance [75,76]. The intronic variant *SERPINE1* rs2070682 (T>C) may affect the protein expression and/or activity, thereby influencing VTE susceptibility and tumourigenic processes. In the CAT setting, this SNP was implicated in venous thrombogenesis among myeloma patients [77]. Our research group has observed a pro-thrombotic effect of the *SERPINE1* rs2070682 C allele in cervical cancer patients [59]. Presumably, those with CC and CT genotypes may express higher PAI-1 levels and/or activity, thus inhibiting fibrinolysis. Consistently, in the present study, those with these genotypes also presented a higher risk for CAT development (OR=9.28, $p=0.040$). Intriguingly, by dismissing those under antithrombotic therapy at OC diagnosis, *SERPINE1* rs2070682 T allele was found to negatively impact tumour progression (log-rank test, $p=0.041$), which was confirmed by multivariable Cox analysis. Putatively, excluding these patients (including those with CAT events before OC diagnosis) may have revealed a coagulation-independent mechanism associated with the SNP. Although further research is needed, *SERPINE1* rs2070682 could be a promising biomarker for OC patients aiding in thromboprophylaxis and tumour management.

The intronic SNP rs4734879 (A>G) is located within the *ZFPM2* gene, which encodes for a transcriptional factor that interacts with GATA transcriptional factors, influencing the expression of multiple genes [78]. According to Ensembl (last accessed on 24 July 2024), the variant is in strong linkage disequilibrium with *ZFPM2* rs4602861 ($r^2=0.94$) in the Iberian

population. This intronic SNP was associated with VTE susceptibility in the general population in a GWAS [33]. Yet, to our knowledge, both variants remain unexplored in the context of CAT [29]. In our previous study with an independent cohort of OC patients, rs4734879 was a prognostic factor. Specifically, the G allele genotypes were associated with prolonged disease-free survival (DFS) and OS among patients at early cancer stages [78]. Notably, subgroup analysis on OC stages was not conducted in the present study given the lack of sufficient statistical power. In this study, the SNP GG genotype was more prevalent in the KS<2 group (χ^2 , $p=0.010$), suggesting an antithrombotic effect associated with this genotype. However, no association with CAT was observed. In univariable analysis, no influence of the SNP on patients' clinical outcomes was detected (log-rank test, $p\geq 0.05$). Contrariwise, in the multivariable Cox analysis dismissing patients under antithrombotic therapy at OC diagnosis, those with the GG genotype presented a three-fold increase in the risk of OC progression compared to AA/AG carriers (aHR=3.08, $p=0.010$). Like *SERPINE1* rs2070682, excluding these patients and adjusting for clinicopathological factors may have revealed a coagulation-independent mechanism associated with rs4734879.

As for the remaining SNPs, specifically *NOS3* rs1799983 (G>T), *TFPI* rs10153820 (G>A) and *PTGS2* rs20417 (C>G), for the former two variants no significant association was detected, while for the latter, no analysis was conducted given the absence of HWE. Briefly, *NOS3* rs1799983 is a missense SNP and its minor allele is linked to a significant reduction in NOS3 enzyme activity, contributing to endothelial dysfunction – a key factor for VTE pathogenesis. Both the protein and the SNP have been linked to tumour development [1]. The intronic SNP rs10153820 is found within the *TFPI*, a gene that encodes for the main inhibitor of the TF coagulation pathway. This anticoagulant protein also possesses tumour suppressor roles [59,79-81]. The SNP has been previously associated with TFPI levels and clinicopathological parameters in breast cancer [82,83]. As for rs20417, this non-coding transcript exon SNP is located with the *PTGS2* promoter, potentially influencing the gene expression [34,46]. *PTGS2* is an inducible enzyme implicated in inflammation and platelet formation and activity, impacting aspirin resistance and tumourigenesis [38,84-86]. In our previous study, *PTGS2* rs20417 was associated with the prognosis of OC patients [45].

The study has limitations. First, the absence of active screening for CAT may have led to an underestimation of asymptomatic events. Secondly, due to the study's retrospective nature, it was unfeasible to evaluate VTE-related factors, such as baseline D-dimer and fibrinogen levels, which are not routinely assessed at our institution. Additionally, the small cohort size, driven by the relatively low incidence of OC and the need to control for multiple confounders, may have limited the statistical power to detect smaller effects. Therefore, investigation in larger and more diverse cohorts is needed to provide more insights and

enhance the generalizability of the findings. Despite these limitations, a major strength of the study lies in its comprehensive consideration of significant risk factors for VTE and CAT.

5. Conclusions

This study shows that VTE negatively impacts tumour progression and survival of OC patients. Although caution is needed when interpreting these findings due to the small cohort size, the SNPs *PROCR* rs10747514 and *SERPINE1* rs2070682 were identified as predictors of CAT, while *RGS7* rs2502448, *F3* rs1361600, *FGG* rs2066865 and *SERPINE1* rs2070682 emerged as the most relevant prognostic markers in different cohort subgroups. Among them, *SERPINE1* rs2070682 and *F3* rs1361600 stand out. The former was a predictive biomarker for both CAT and OC progression, while the latter was a predictor of both OC progression and mortality. These SNPs seem to have a context-dependent role in OC patients, reflecting the functional complexity of their related proteins and the intricate pathogenesis of CAT and OC. Hence, further research is required, particularly in larger cohorts with different populations considering the low MAF associated with some of these SNPs and the complexity of the underlying mechanisms. More functional studies are also necessary to elucidate their biological implications, specifically how they influence the expression and/or activity of their respective proteins. Furthermore, big data, machine learning and artificial intelligence algorithms may be valuable tools to help understand the effects of these SNPs in specific disease contexts. These advanced technologies can help identify patterns that are not apparent, thereby enhancing the predictive models [87]. Overall, genetic polymorphisms could be appealing for assessing VTE risk and conducting initial prognosis evaluation in OC patients as, unlike other biomarkers, they remain stable and unaffected by tumour progression, treatments or related complications [88]. While further studies are required for validation, these genetic variations hold promise for improving thromboprophylaxis, prognosis accuracy and therapeutic protocols for OC patients.

Author Contributions

All authors made a significant contribution to the study. Conceptualization, VT, JA and RM; Patient recruitment and ethical approval, JSB; Funding acquisition, VT and RM; Investigation, VT; Review of medical files: VT, MR and JLP; Formal analysis: VT and RM; writing—original draft preparation, VT; Writing—review and editing, VT, JA, JLP, MR, JSB, DP and RM; Supervision: JA, DP and RM. All authors have read and agreed to the published version of the manuscript.

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Declarations:

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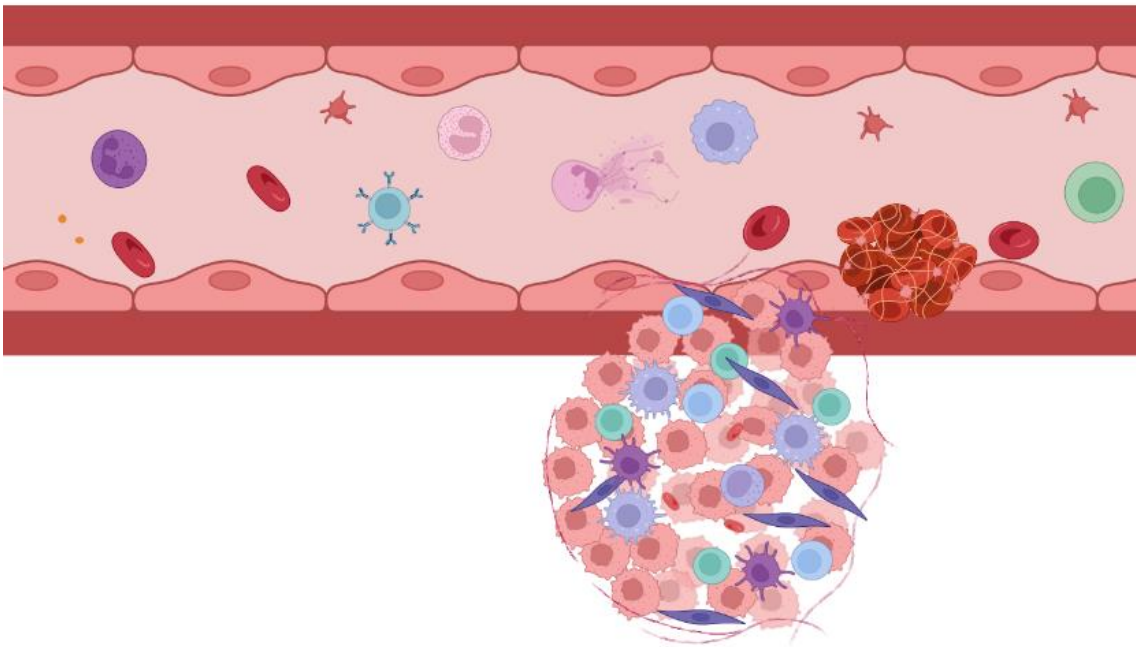
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Chapter IV



CHAPTER IV – Decoding Haemostatic Gene Expression in Immunothrombosis: Clinical Insights for Ovarian Cancer Patients

This chapter includes the following paper:

Valéria Tavares, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Joana Assis, Deolinda Pereira, Rui Medeiros. **Haemostatic Gene Expression in Cancer-Related Immunothrombosis: Contribution for Venous Thromboembolism and Ovarian Tumour Behaviour.** *Cancers*. **2024**; 16 (13): 2356. <https://doi.org/10.3390/cancers16132356>. (Impact factor and quartile reported in 2023: **4.5** and **Q1**).

Haemostatic Gene Expression in Cancer-Related Immuno-thrombosis: Contribution for Venous Thromboembolism and Ovarian Tumour Behaviour

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Abstract: Ovarian cancer (OC) is the deadliest gynaecological cancer. Identifying new prognostic biomarkers is an important research field. Haemostatic components together with leukocytes can drive cancer progression while increasing the susceptibility to venous thromboembolism (VTE) through immunothrombosis. Unravelling the underlying complex interactions offers the prospect of uncovering relevant OC prognostic biomarkers, predictors of cancer-associated thrombosis (CAT), and even potential targets for cancer therapy. Thus, this study evaluated the expression of *F3*, *F5*, *F8*, *F13A1*, *TFPI1*, and *THBD* in peripheral blood components (PBCs) of 52 OC patients. Those with VTE after tumour diagnosis had a worse overall survival (OS) compared to their counterparts (mean OS of 13.8 ± 4.1 months and 47.9 ± 5.7 months, respectively; log-rank test, $p = 0.001$). Low pre-chemotherapy *F3* and *F8* expression levels were associated with a higher susceptibility for OC-related VTE after tumour diagnosis (χ^2 , $p < 0.05$). Regardless of thrombogenesis, patients with low baseline *F8* expression had a shorter progression-free survival (PFS) than their counterparts (adjusted hazard ratio (aHR) = 2.54; $p = 0.021$). Among those who were not under platelet anti-aggregation therapy, low *F8* levels were also associated with a shorter OS (aHR = 6.16; $p = 0.006$). Moving forward, efforts should focus on external validation in larger cohorts.

Keywords: ovarian neoplasms; prognosis; thrombosis; inflammation; genes; liquid biopsy

1. Introduction

Worldwide, ovarian cancer (OC) stands as the eighth most common and deadliest cancer among women, with approximately 324,000 new cases and 206,000 reported deaths in 2022 [1]. The disease is regarded as the most lethal gynaecological malignancy, with a 5-year survival rate lower than 50% in most countries [2]. Over the last few years, significant advancements have been made in OC treatment, with the development of new therapeutic approaches. However, given the disease heterogeneity, more and better predictive and/or prognostic biomarkers are needed to personalise disease management, reduce side effects, and prolong patient survival [3].

Like other solid tumours, OC is known to modulate the activity of platelets, endothelial cells, and leukocytes at the tumour microenvironment (TME) to fuel tumourigenesis [4]. Under physiological conditions, these components have an active role in haemostasis, driving continuous blood circulation and preventing coagulopathies, while gatekeeping vascular integrity. However, up to 50% of all cancer patients and 90% of those with metastases exhibit haemostatic abnormalities, including haemorrhage and thrombosis

events [5]. Cancer-associated thrombosis (CAT), comprising both arterial and venous events, is a common paraneoplastic syndrome and a major cause of morbimortality [6,7]. Although arterial thrombosis may occur, most CAT events involve the veins [8]. Patients with OC have an incidence of venous thromboembolism (VTE) events, including deep venous thrombosis (DVT) and pulmonary embolism (PE), ranging from 10% to 30%, placing them among the cancer populations most affected by VTE [9]. This is critical given that VTE stands as the second leading cause of mortality among oncological patients [10].

Leukocytes (particularly monocytes and neutrophils), platelets, and endothelial cells are known to establish a complex interplay within TME, leading to a process known as immunothrombosis or thromboinflammation. This novel concept links inflammation and thrombogenesis to cancer progression [11,12]. In settings of inflammation (e.g., infection and cancer), leukocytes express numerous haemostatic proteins, including platelet activators, procoagulant factors, anticoagulant proteins, and fibrinolytic modulators [13]. Thus, the gene expression profile of these cells in OC patients may inform their thrombotic potential, helping anticipate VTE events. Importantly, given the contribution of haemostatic components in tumour growth and dissemination, this could lead to the identification of prognostic biomarkers of OC regardless of VTE, as well as potential therapeutic targets for cancer management. This is relevant considering the increasing recognition of liquid biopsies as non-invasive tests for assessing cancer patient prognosis and monitoring [14]. Hence, this study evaluated the expression of haemostasis-related genes in peripheral blood components (PBCs) of OC patients, exploring their impact on OC-related VTE development and patients' clinical outcomes.

2. Materials and Methods

2.1. Patient Enrolment

Adult Caucasian patients with confirmed ovarian carcinoma, who were admitted for frontline treatment at the Clinic of Gynaecology of the Portuguese Oncology Institute of Porto (IPO Porto) between March 2017 and August 2022, were enrolled in a retrospective cohort study. The standard treatment protocol consisted of cytoreductive surgery and chemotherapy using platinum-based agents (cisplatin or carboplatin), typically combined with taxanes (paclitaxel or docetaxel), administered every 21 days. The treatment approach (neoadjuvant, adjuvant, or chemotherapy alone for non-surgical candidates) determined the number of chemotherapy cycles, tailored to individual patient requirements and therapeutic responses. Exclusion criteria encompassed patients that (1) had synchronous and

metachronous tumours, (2) were immunosuppressed and/or had autoimmune diseases, (3) had an acute infection at the time of cancer diagnosis, (4) were pregnant or in postpartum (here defined as lasting six weeks following childbirth) at OC diagnosis, (5) were receiving anticoagulation therapy because of other conditions rather than VTE, (6) had prothrombin G20210A (*F2* rs1799963) or factor V Leiden (*F5* rs6025) polymorphisms, and (7) did not provide informed consent. Applying these criteria, 52 OC patients were recruited, for whom biological material derived from peripheral blood samples before first-line chemotherapy was available in our biobank.

The demographical and clinical data of the patients were revised using their electronic medical records. Data on baseline full blood count and coagulation tests, namely prothrombin time (PT), international normalised ratio (INR), and activated partial thromboplastin time (aPTT), were also retrieved. The most validated CAT risk assessment model (RAM)—the Khorana score (KS)—was determined for all patients with available data for the following parameters: the cancer site, baseline haemoglobin levels, leukocyte and platelet count, and body mass index (BMI). A cut-off of 2 was considered [8]. Cancer-associated VTE was deemed an event taking place six months before to two years after OC diagnosis [15]. Active screening was not performed since it is not integrated into the standard clinical procedures at IPO Porto. The median follow-up period was 26.5 months, with a range of 1.0 to 82.0 months.

Each patient signed a written informed consent following the principles of the Helsinki Declaration. The study protocol has received approval from the ethics committee at the research centre of IPO Porto (CI-IPOP) (CES. 69/021).

2.2. Blood Sample Collection and Processing

Before and after first-line chemotherapy, peripheral blood samples were collected in EDTA-coated tubes using a standard venipuncture technique. Erythrocytes in the samples were lysed using a 1 × ammonium–chloride–potassium (ACK) solution. Subsequently, the samples were frozen at –20 °C for 20 min and then centrifugated for 10 min at 2500 rpm at room temperature. After removing the supernatant, the samples were sequentially washed with 1 × ACK solution and 1 × phosphate-buffered saline (PBS) solution. Each wash was followed by rounds of centrifugation for 10 min at 2500 rpm and room temperature to separate and remove the supernatant. The resulting pellet of PBCs was diluted with TriPure® Isolation Reagent (Roche Applied Science, Penzberg, Germany) and conserved at –80 °C until use.

2.3. Gene Selection

One of the most studied mechanisms underlying CAT among OC patients is the tumour overexpression of the coagulation factor III (FIII), also known as tissue factor (TF). This coagulation factor triggers the extrinsic coagulation pathway, which along with the intrinsic pathway, converges into the common pathway leading to fibrin deposition [16]. Thus, haemostatic genes to be evaluated in this study were selected focusing on those related to coagulation factors and anticoagulants of the extrinsic and common coagulation pathways that (1) are expressed by platelets (here defined as messenger RNA (mRNA) presence) and leukocytes (**Table 1**), (2) are associated with VTE, (3) were previously implicated in tumour progression, and (4) had available TaqMan® gene expression assays. Based on these criteria, *coagulation factor 3 (F3)*, *coagulation factor 5 (F5)*, *coagulation factor 8 (F8)*, *coagulation factor 13 A chain (F13A1)*, *tissue factor pathway inhibitor 1 (TFPI1)*, and *thrombomodulin (THBD)* were selected.

Chapter IV - Table 1. Selected haemostasis-related genes and their expression profile.

Gene	Genomic Location	Main Source	Expression in Platelets and Immune Cells *
<i>F3</i>	1p21.3	Extravascular tissue cells (pericytes, fibroblasts, smooth and epithelial cells) [17]	Monocytes [18] Platelets [19]
<i>F5</i>	1q24.2	Hepatocytes [20]	Monocytes [18,20] Neutrophils [21] T cells [21] Platelets [20]
<i>F8</i>	Xq28	Liver sinusoidal endothelial cells [22] Hepatocytes [23] Megakaryocytes [24,25]	Monocytes [18] Macrophages [20] Platelets [25]
<i>F13A1</i>	6p25.1	Cells of bone marrow and the mesenchymal lineage [26]	Monocytes [18,20] Macrophages [20] Platelets [20,27]
<i>TFPI1</i>	2q32.1	Vascular endothelial cells [28] Platelets [18]	Monocytes [18] Macrophages [28]
<i>THBD</i>	20p11.21	Vascular endothelial cells [20]	Monocytes [18,20] Macrophages [20] Neutrophils [29] Dendritic cells [30]

* Defined as messenger RNA presence and protein synthesis.

2.4. Total RNA Extraction

Total RNA from the peripheral blood fraction was isolated using the GRS RNA kit—Blood & cultured cells (#GK08.0100) (Grisp Research Resolutions®, Porto, Portugal). Importantly, the DNase treatment (DNase I set (#GKC01.0100), Grisp Research Resolutions®, Porto, Portugal) duration was extended to guarantee the complete removal of genomic DNA, which would interfere with downstream applications. RNA purity and

quantity were assessed using the NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Post extraction, RNA samples were frozen at -80°C until use.

2.5. cDNA Synthesis

The RNA samples (150 ng) were reverse-transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Carlsbad, CA, USA) according to the manufacturer's protocol. All the conversions were carried out in a Mycycler™ Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following cycling conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Negative controls (lacking RNA) were integrated into all reactions to assess false positives.

2.6. Gene Relative Quantification

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to evaluate gene expression levels using a StepOnePlus™ qPCR system (Applied Biosystems®, Foster City, CA, USA). Each PCR reaction utilised 5 μL of $2 \times$ TaqMan™ Fast Advanced Master Mix (Applied Biosystems®, Foster City, CA, USA), 3.0 μL of RNA and DNA-free water, 0.5 μL of $20 \times$ TaqMan™ Gene Expression Assays for *F3* (Hs01076029_m1), *F5* (Hs00914120_m1), *F8* (Hs00252034_m1), *F13A1* (Hs01114178_m1), *TFPI1* (Hs00409206_m1, which quantify both *TFPI* isoforms), *THBD* (Hs00264920_s1), *GAPDH* (Hs03929097_g1), and *ACTB* (Hs99999903_m1) as well as 1.5 μL of the cDNA sample, in a total volume of 10 μL . *GAPDH* and *ACTB* were tested as endogenous controls (housekeeping genes) due to their reported expression stability in human blood cells [31–33]. The cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles of 15 s at 95°C , and 60°C for 60 s. The expression levels of each sample's target and housekeeping genes were quantified on the same plate. Quantification was carried out in triplicate, with negative controls (without cDNA) incorporated in all reactions for quality control. Cycle threshold (Ct) values with a standard deviation (SD) greater than 0.5 were excluded. The Thermo Fisher Connect platform (Thermo Fisher Scientific, Waltham, MA, USA) was employed to set the same baseline and threshold values in all plates, ensuring uniform generation of Ct values for all the target and housekeeping genes in each sample.

2.7. Statistical Analysis

Statistical analysis and graphing were carried out using IBM SPSS Statistics for Windows version 29 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 9.0.0 (GraphPad Software Inc., La Jolla, CA, USA), respectively.

The normalised relative expression of the target genes was assessed using the Livak method. Among the endogenous controls, *GAPDH* showed a more stable expression (meaning with the lowest SD values) than *ACTB*. Thus, *GAPDH* was used to perform the normalisation of gene expression. The interquartile range (IQR) method was employed to pinpoint severe outliers, which were excluded. For each gene, four profiles of expression were created as follows: profile 1 (low versus vs. high expression using the median value of gene expression level as cut-off), profile 2 (low vs. intermediate vs. high expression using terciles), profile 3 (low vs. high, where low included the combination of the first and second tercile and the latter was the third tercile), and profile 4 (low vs. high, where the first tercile was classified as low and the remaining terciles as the high expression). All these profiles were evaluated in analyses employing gene-normalised relative expression as a nominal variable.

Patients were regarded as VTE-free if they remained without VTE or died without presenting the condition during a two-year follow-up period. The live patients with less than two years of follow-up were excluded from the VTE analysis.

Data normality was assessed by employing the Kolmogorov–Smirnov test or the Shapiro–Wilk test depending on the cohort size ($N > 50$ and $N \leq 50$, respectively). Depending on the distribution, Pearson's correlation coefficient test (P) or Spearman's rank correlation coefficient test was computed to assess the relationship between the genes' expression before first-line chemotherapy. Results were deemed relevant if $p < 0.05$ and the coefficient ≥ 0.500 . The coefficient of determination (R^2) was also reported.

Associations of VTE development and pre-chemotherapy (baseline) gene expression levels with patients' characteristics (**Table 2**) were evaluated using the Chi-Square test (χ^2), excluding patients with VTE before OC diagnosis. In this analysis, subgroup evaluations according to primary treatment (surgery vs. chemotherapy) were also conducted.

Statistical differences in the baseline gene expression levels according to VTE status (without VTE vs. VTE before OC diagnosis vs. VTE after OC diagnosis) were analysed using the Kruskal–Wallis test or one-way ANOVA followed by Dunnett's test for multiple comparisons, depending on data normality. In this analysis, those with VTE before and after OC diagnosis were compared with VTE-free patients, respectively. χ^2 was additionally employed for confirmation. Depending on data distribution, the paired *t*-test or Wilcoxon's matched-pairs signed-rank test was used to assess the impact of first-line chemotherapy on gene expression levels.

The study evaluated progression-free survival (PFS) and overall survival (OS) as clinical outcomes. PFS was defined as the time from the treatment initiation to the first occurrence of tumour progression or relapse, the patient's death, or last clinical examination. The former was assessed following the Response Evaluation Criteria in Solid

Tumours (RECIST) criteria version 1.1 (RECIST 1.1) [34]. Regarding OS, it was the period between cancer diagnosis and death related to all causes or the patient's last follow-up date. Survival curves were generated using the Kaplan–Meier method, and survival probabilities were evaluated using the log-rank test. The influence of gene expression on the risk of OC progression and mortality was confirmed using the Cox regression model. A multivariate Cox analysis was performed for the relevant genes, adjusting for patient characteristics with prognostic value according to univariate Cox analyses. In these analyses, patients with VTE before tumour diagnosis were excluded.

Chapter IV - Table 2. Characteristics of ovarian cancer (OC) patients (N = 52).

Variable	N (%)	
Age at OC diagnosis (years) *		63.6 ± 12.0
≥64	27 (51.9)	
Hormonal status at OC diagnosis		
Postmenopausal	40 (76.9)	
Baseline BMI (kg/m²) *		26.8 ± 4.9
≥27.0	21 (40.4)	
ECOG PS at OC diagnosis		
>1	7 (13.5)	
Baseline haemoglobin levels (U/mL) *		12.4 ± 1.4
<12.4	25 (48.1)	
Baseline platelet count (×10⁹/L) **		296.0 [164.0; 572.0]
≥296.0	25 (48.1)	
Baseline leucocyte count (×10⁹/L) *		7.9 ± 2.3
≥7.9	25 (48.1)	
Baseline neutrophil count (×10⁹/L) *		5.1 ± 2.1
≥5.1	25 (48.1)	
Baseline monocyte count (×10⁹/L) **		0.6 [0.3; 1.4]
≥0.6	24 (46.2)	
Baseline lymphocyte count (×10⁹/L) **		1.5 [0.6; 4.3]
≥1.5	24 (46.2)	
Baseline PT (s) **		14.2 [11.4; 31.2]
≥14.2	22 (42.3)	
Baseline INR **		1.1 [1.0; 2.2]
≥1.1	21 (40.4)	
Baseline aPTT (s) *		27.1 ± 2.4
≥27.1	21 (40.4)	
KS		
≥2	22 (42.3)	
Platelet anti-aggregation therapy at OC diagnosis	8 (15.4)	
Anticoagulation therapy at OC diagnosis ***	3 (5.8)	
OC-related inherited mutations	5 (9.6)	
Tumour histology		
Serous	44 (84.6)	
Clear cell	3 (5.8)	
Endometrioid	1 (1.9)	
Mixed	2 (3.8)	
Unusual	2 (3.8)	
Histological grade		
High	49 (94.2)	
FIGO stage #		
I/II	10 (19.2)	
III/IV	42 (80.8)	
Baseline CA-125 levels (U/mL) **		1067.0 [7.7; 10,184.0]
≥1067	26 (50.0)	
Upfront treatment		
Surgery and adjuvant chemotherapy	23 (44.2)	
Neoadjuvant chemotherapy and surgery	2 (3.8)	
Neoadjuvant chemotherapy, surgery and adjuvant chemotherapy	14 (26.9)	
Chemotherapy only	13 (25.0)	
Platinum sensitivity δ	40 (76.9)	
Maintenance therapy		
PARPi	16 (30.8)	
bevacizumab	6 (11.5)	

Baseline values were defined as those at tumour diagnosis, preceding treatment.* Presented as mean $\pm$ standard deviation since the variable had a normal distribution (Kolmogorov–Smirnov, $p > 0.05$). ** The variable was presented as median [minimum; maximum] due to its non-normally distributed nature. *** Patients with OC-related venous thromboembolism before OC diagnosis. # According to FIGO Cancer Report 2021 [35]. δ Those with disease progression six months after the completion of first-line platinum-based chemotherapy were deemed platinum-sensitive [3]. Some patients had missing information: 10 for aPTT, PT, and INR, five for monocyte and lymphocyte counts, four for KS, three for haemoglobin levels, platelet, leukocyte, and neutrophil counts, two for histological grade, and one for BMI, OC-related inherited mutations, and CA-125 levels. Abbreviations: aPTT, activated partial thromboplastin time; BMI, body mass index; CA-125, cancer antigen 125; ECOG PS; Eastern Cooperative Oncology Group Performance Status; FIGO, International Federation of Gynecology and Obstetrics; INR, international normalised ratio; KS, Khorana score; OC, ovarian cancer; PARPi, Poly (ADP-ribose) polymerase inhibitors; PT, prothrombin time.

In all analyses, a p -value lower than 0.05 was considered statistically significant. Furthermore, a p -value ranging between 0.05 and 0.06 was deemed marginally significant.

3. Results

3.1. Patients Characteristics

Demographic and clinical factors of the OC patients are presented in **Table 2**.

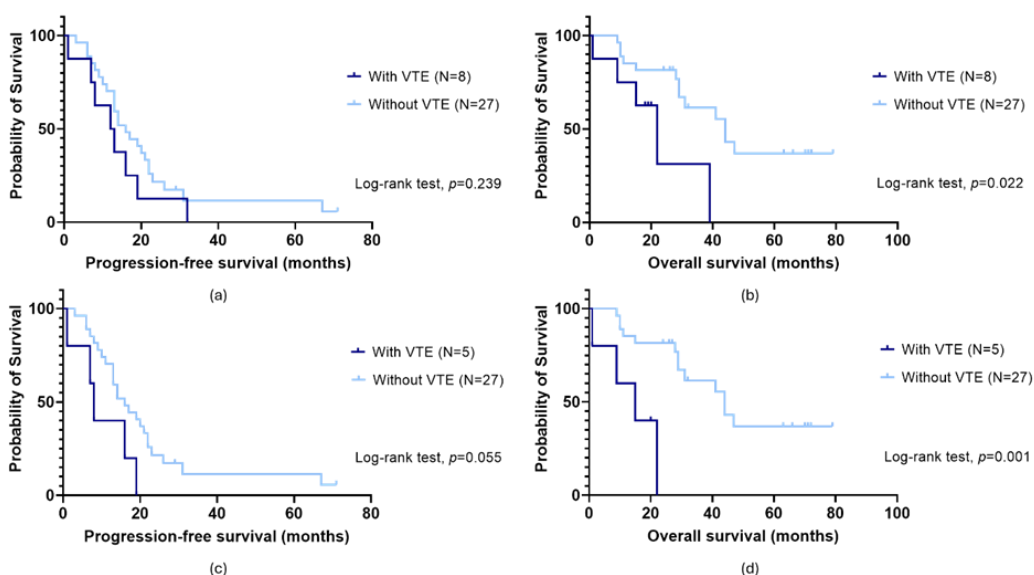
3.2. Impact of VTE on Patients' Prognosis

Considering the patients with a two-year follow-up ($N = 35$), eight (22.9%) presented OC-related VTE, the majority being symptomatic (6 (75.0%)). Among the events, seven were DVT and one was PE. Those with thrombotic events before OC diagnosis ($N = 3$) present a mean period between VTE and tumour diagnosis of 2.3 ± 2.5 months. As for those with the condition after OC diagnosis ($N = 5$), the mean time to VTE occurrence was 4.6 ± 3.9 months.

Except for baseline leucocyte count, no significant association between VTE incidence and patients' characteristics, including KS, was observed regardless of VTE timing (χ^2 , $p > 0.05$). Patients with venous thrombotic events more often presented a high baseline leucocyte count compared to their counterparts (7 vs. 1; χ^2 , $p = 0.043$).

No significant impact of VTE on patients' PFS was observed (log-rank test, $p = 0.239$; **Figure 1a**). In opposition, those with OC-related VTE demonstrated a lower survival time than their counterparts (mean OS of 22.2 ± 6.2 months and 47.9 ± 5.7 months, respectively; log-rank test, $p = 0.022$; **Figure 1b**). Excluding those with the condition before OC diagnosis, a marginally significant association was observed between VTE and patients' PFS (long-rank test, $p = 0.055$; **Figure 1c**). Specifically, those with OC-related VTE had a

faster disease progression compared to their counterparts (mean PFS of 10.2 ± 3.2 months and 21.6 ± 3.8 months, respectively). In the same subgroup, a negative impact of VTE on OS was also observed (mean OS of 13.8 ± 4.1 months and 47.9 ± 5.7 months, respectively; log-rank test, $p = 0.001$; **Figure 1d**).

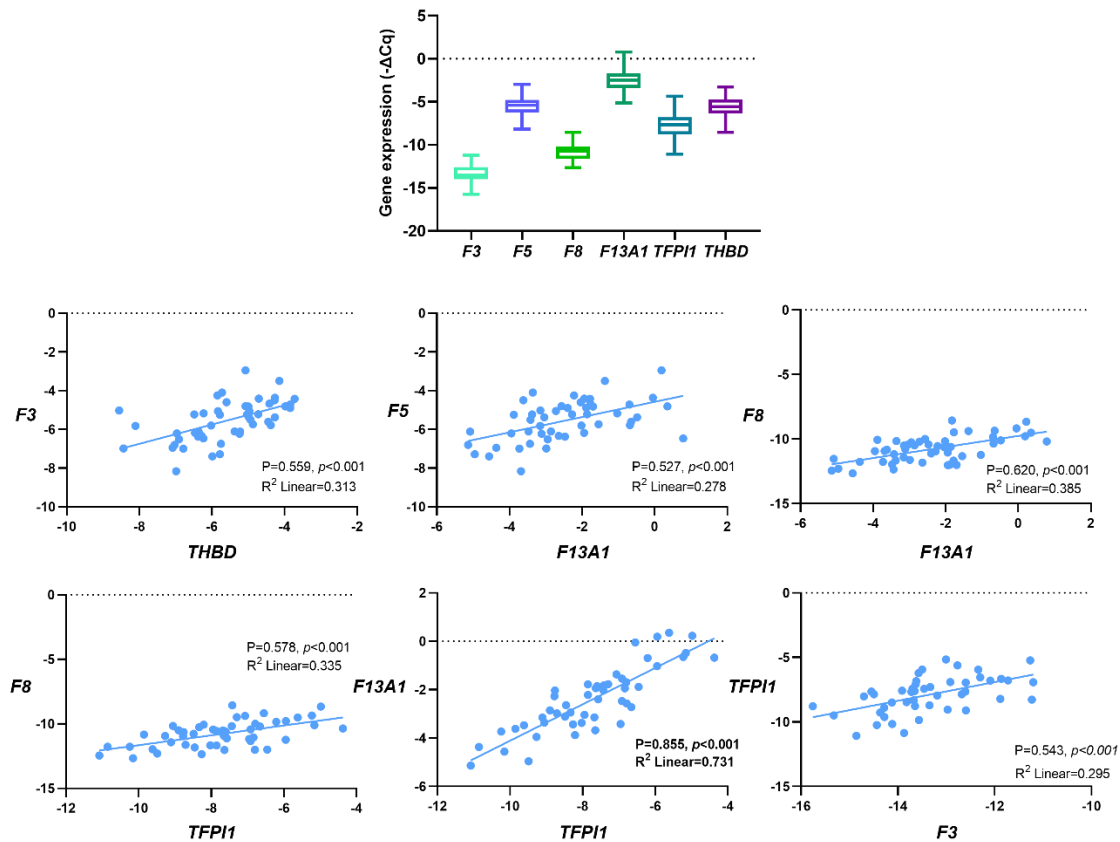


Chapter IV - Figure 1. Progression-free survival (PFS) (a, c) and overall survival (OS) (b, d) by Kaplan–Meier and log-rank test for ovarian cancer (OC) patients according to venous thromboembolism (VTE) status. (a) No association between PFS and VTE (log-rank test, $p = 0.239$) was observed in the overall cohort ($N = 35$). (c) When patients with VTE before OC diagnosis were dismissed, a marginally significant impact was detected (long-rank test, $p = 0.055$). Specifically, those with OC-related VTE had a faster disease progression compared to their counterparts (mean PFS of 10.2 ± 3.2 months and 21.6 ± 3.8 months, respectively). (b) Considering the entire cohort ($N = 35$), a significant association between OS and VTE was observed (log-rank test, $p = 0.022$). Those with the condition had a lower survival time than their counterparts (mean OS of 22.2 ± 6.2 months and 47.9 ± 5.7 months, respectively). (d) The same was observed excluding those with VTE before OC diagnosis (log-rank test, $p = 0.001$). Specifically, patients with OC-related VTE and those without had a mean OS of 13.8 ± 4.1 months and 47.9 ± 5.7 months, respectively.

3.3. Correlation between Baseline Gene Expression

Given the normal distribution (Kolmogorov–Smirnov, $p > 0.05$), Pearson's correlation coefficient (P) test was employed to evaluate the relationship between the genes' expression levels before first-line chemotherapy (**Figure 2**). Considering the entire cohort, positive correlations were detected between genes encoding for coagulation factors (*F5* and *F13A1*; *F8* and *F13A1*) and genes encoding for anticoagulants and coagulation factors (*F3* and *THBD*, *F8* and *TFPI1*, *F13A1* and *TFPI1* and *F3* and *TFPI1*). The strongest correlation was detected between *F13A1* and *TFPI1* expression ($P = 0.855$, $p < 0.001$, R^2

= 0.731). All the correlations were also observed when excluding those with VTE before OC diagnosis.



Chapter IV - Figure 2. Correlation between baseline haemostatic genes' expression in peripheral blood components (PBCs) in a cohort of ovarian cancer (OC) patients (N = 52) by Pearson's correlation coefficient (P) test.

3.4. Baseline Gene Expression and Patients' Characteristics

For *F5* and *F13A1*, no significant association with patients' characteristics was detected (χ^2 , $p > 0.05$), regardless of the expression profile and primary treatment (surgery vs. chemotherapy).

High pre-chemotherapy *F3* levels were more common among those with a high PT (≥ 14.2 s; profile 1, χ^2 , $p = 0.032$; profile 2, χ^2 , $p = 0.040$; profile 4, χ^2 , $p = 0.040$). In the subgroup analysis, according to primary treatment (surgery vs. chemotherapy), this association was confirmed among those first treated with surgery (≥ 14.2 s; profile 2, χ^2 , $p = 0.006$; profile 4, χ^2 , $p = 0.024$). In the same subgroup, high pre-chemotherapy levels of the gene were predominant among those at FIGO I/II stages compared to their counterparts (profile 2, χ^2 , $p = 0.016$; profile 3, χ^2 , $p = 0.036$).

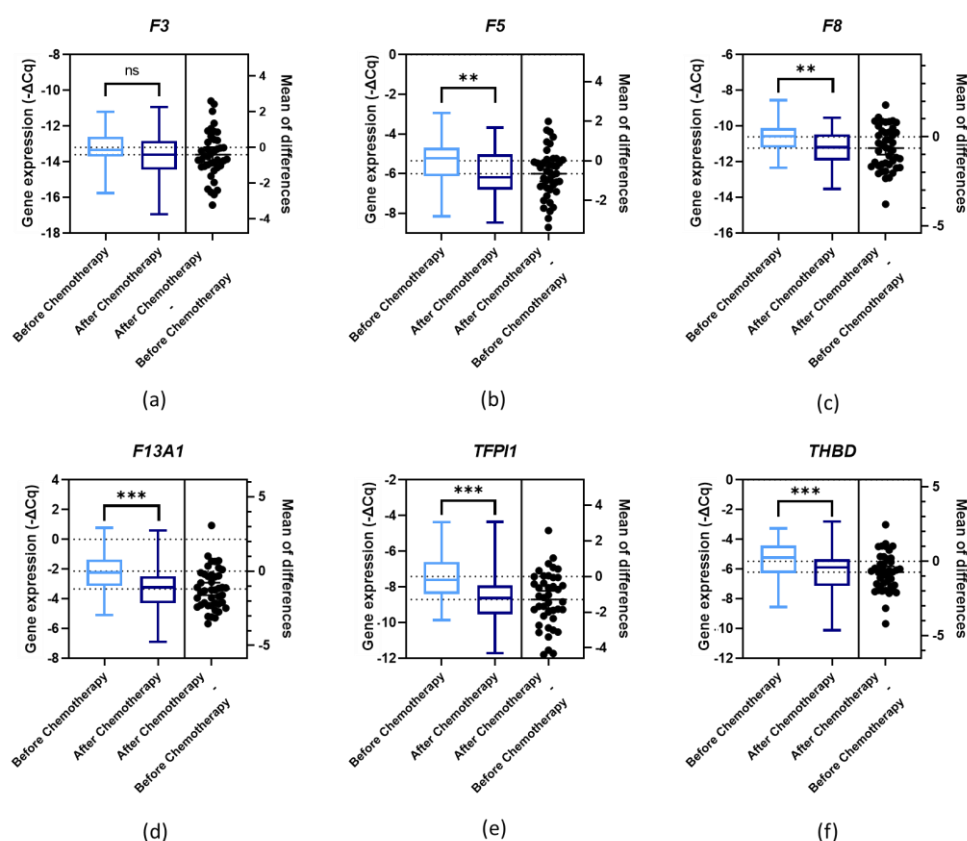
High baseline *F8* levels were more common among patients with low INR (<1.1 ; profile 2, χ^2 , $p = 0.004$; profile 4, χ^2 , $p = 0.031$). Furthermore, its high levels were also more prevalent in the surgery group (profile 2, χ^2 , $p = 0.042$; profile 4, χ^2 , $p = 0.039$). In the chemotherapy subset (patients who were treatment-naïve upon sample collection), high pre-chemotherapy *F8* levels were predominant among younger patients (<64 years, profile 4, χ^2 , $p = 0.043$), those with high leucocyte count ($\geq 7.9 \times 10^9/L$; profile 3, χ^2 , $p = 0.019$), high platelet count ($\geq 296.0 \times 10^9/L$; profile 4, χ^2 , $p = 0.025$) and with a high KS (≥ 2 ; profile 4, χ^2 , $p = 0.008$) compared to their counterparts.

High pre-chemotherapy *TFPI1* levels were more common among patients with a high PT (≥ 14.2 s; profile 2, χ^2 , $p = 0.026$; profile 3, χ^2 , $p = 0.019$). In the chemotherapy group, high gene expression levels were predominant in patients with KS ≥ 2 (profile 1, χ^2 , $p = 0.019$).

Patients with elevated baseline *THBD* levels commonly presented a high KS (≥ 2 ; profile 2, χ^2 , $p = 0.054$; profile 3, χ^2 , $p = 0.041$). The subgroup analysis confirmed this association in the chemotherapy group (≥ 2 ; profile 2, χ^2 , $p = 0.046$; profile 3, χ^2 , $p = 0.040$).

3.5. Gene Expression and First-Line Chemotherapy

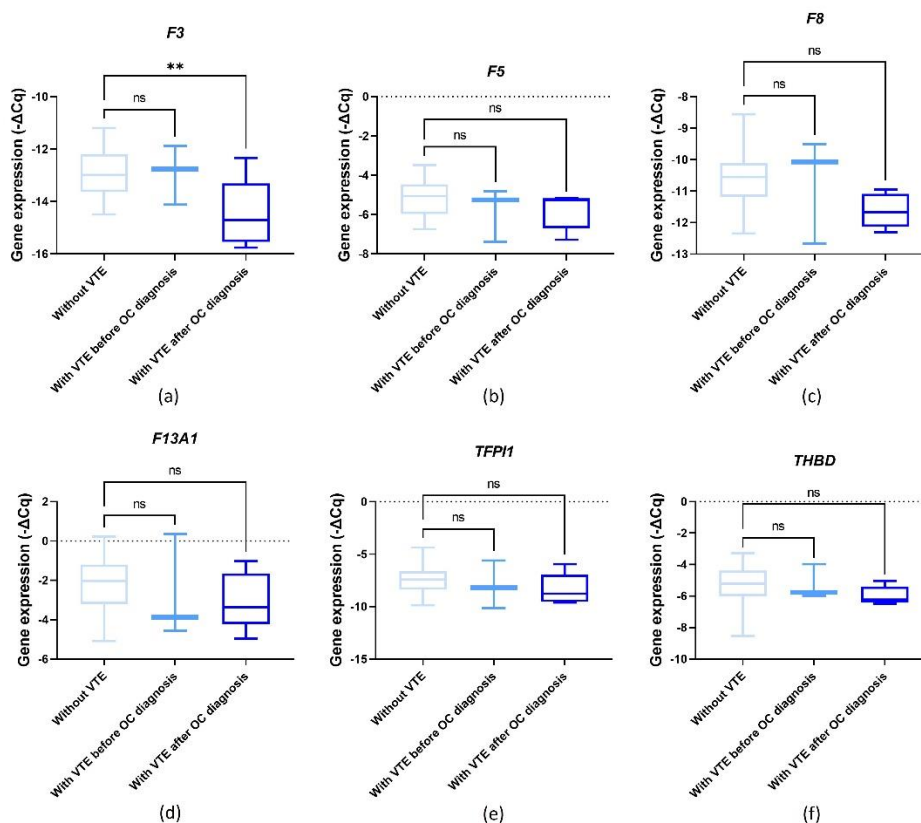
Gene expression levels were evaluated according to first-line chemotherapy. Significant differences were observed (paired *t*-test, $p < 0.05$) between the genes' expression levels before and after chemotherapy for *F5*, *F8*, *F13A1*, *TFPI1* and *THBD* (**Figure 3b** ($p = 0.001$), **Figure 3c** ($p = 0.001$), **Figure 3d** ($p < 0.001$), **Figure 3e** ($p < 0.001$) and **Figure 3f** ($p < 0.001$), respectively). As for *F3*, the difference was only marginally significant (paired *t*-test, $p = 0.056$; **Figure 3a**). In the subgroup analysis, according to primary treatment, except for *F3* (paired *t*-test, $p = 0.445$) and *F5* (paired *t*-test, $p = 0.100$), the expression levels of all genes significantly diminished after chemotherapy among those treated first with surgery. Regarding the chemotherapy group, only *F8* did not present a significant difference in expression levels after the treatment (paired *t*-test, $p = 0.189$).



Chapter IV - Figure 3. Normalised relative expression levels of the evaluated genes (–ΔCq) in peripheral blood components (PBCs) in a cohort of ovarian cancer (OC) patients before and after first-line chemotherapy: (a) *F3* expression; (b) *F5* expression; (c) *F8* expression; (d) *F13A1* expression; (e) *TFPI1* expression; and (f) *THBD* expression; paired t-test, ** $p < 0.01$, *** $p < 0.001$; ns, non-significant.

3.6. Baseline Gene Expression and OC-Related VTE

The influence of pre-chemotherapy genes' expression levels on OC-related VTE incidence was assessed. No significant differences were detected for *F5*, *F13A1*, *TFPI1* and *THBD* comparing VTE and VTE-free patients (**Figure 4b,d-f**; one-way ANOVA followed by Dunnett's test, $p > 0.05$). Regarding *F3*, its pre-chemotherapy expression levels were significantly decreased in patients who later presented OC-related VTE compared to those without venous thrombogenesis (one-way ANOVA followed by Dunnett's test, $p = 0.008$; **Figure 4a**). This finding was confirmed by χ^2 (profile 2, $p = 0.028$; profile 4, $p = 0.016$). A marginal association was detected for *F8*. Namely, its pre-chemotherapy expression levels were decreased among those who later had a diagnosis of OC-related VTE compared to those without CAT (one-way ANOVA followed by Dunnett's test, $p = 0.057$; **Figure 4c**). This finding was also corroborated by χ^2 (profile 1, $p = 0.051$; profile 2, $p = 0.028$).



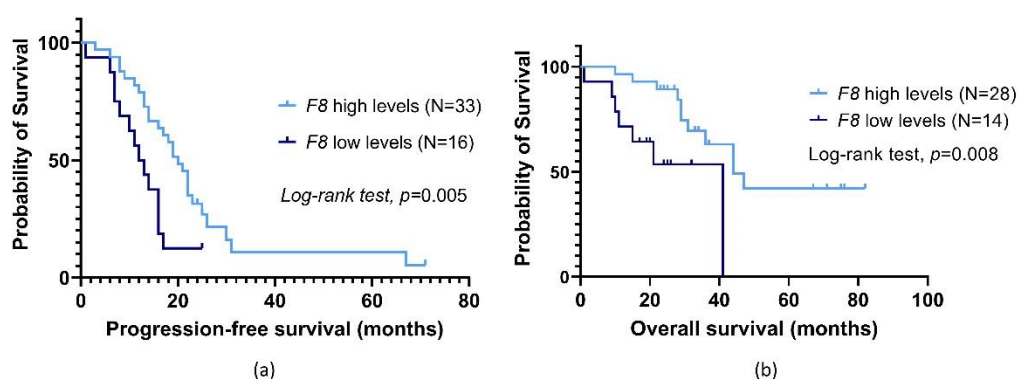
Chapter IV - Figure 4. Normalised relative expression levels of the evaluated genes ($-\Delta Cq$) in peripheral blood components (PBCs) in a cohort of ovarian cancer (OC) patients before first-line chemotherapy and in the context of venous thromboembolism (VTE): (a) *F3* expression; (b) *F5* expression; (c) *F8* expression; (d) *F13A1* expression; (e) *TFPI1* expression; and (f) *THBD* expression; one-way ANOVA followed by Dunnett's test for multiple comparisons, ** $p < 0.01$; ns, non-significant.

3.7. Impact of Baseline Gene Expression on Patients' Prognosis

For *F3*, *F5*, *F13A1*, *TFPI1* and *THBD*, the baseline expression of these haemostatic genes had no significant impact on the patient's clinical outcomes, regardless of the expression profile (log-rank test and univariable and multivariable Cox analyses; $p > 0.05$).

Regarding *F8*, its low baseline expression was found to be associated with a lower time to OC progression compared to their counterparts (profile 4; mean PFS of 12.8 ± 1.6 months and 23.7 ± 3.5 months, respectively, log-rank test, $p = 0.005$; **Figure 5a**). The patients with a low expression of this haemostatic gene had almost a three-fold increase in the risk of disease progression (profile 4, hazard ratio (HR) = 2.56, 95% confidence interval (95%CI), 1.28–5.15, $p = 0.008$). This association was observed regardless of primary treatment (profile 4, adjusted HR (aHR) = 2.23, 95%CI, 1.08–5.59, $p = 0.030$). The negative effect of low *F8* baseline expression was corroborated in a multivariable Cox analysis adjusted for patients' age at OC diagnosis and presence of metastasis at disease diagnosis and/or

during treatment (profile 4; aHR = 2.54, 95%CI, 1.15–5.58, $p = 0.021$; **Table 3**). As for patient survival, no significant association between baseline *F8* expression and patients' OS was detected (log-rank test, $p > 0.05$). However, among those who were not under platelet anti-aggregation therapy at OC diagnosis, low pre-chemotherapy *F8* levels were associated with a decreased survival time compared to their counterparts (profile 4; mean OS of 27.5 ± 4.5 months and 53.7 ± 5.9 months, respectively; log-rank test, $p = 0.008$; **Figure 5b**). In this subgroup, patients with low gene expression had almost a four-fold increase in the risk of mortality (profile 4, HR = 3.80, 95%CI, 1.31–10.98, $p = 0.014$). This negative influence was also observed regardless of primary treatment (profile 4, aHR = 3.36, 95%CI, 1.09–10.29, $p = 0.034$). A multivariable Cox analysis adjusted for surgery and platinum sensitivity also corroborated the negative contribution of low *F8* baseline expression (profile 4, aHR = 6.16, 95%CI, 1.68–22.52, $p = 0.006$; **Table 3**).



Chapter IV - Figure 5. Progression-free survival (PFS) (a) and overall survival (OS) (b) by Kaplan–Meier and log-rank test for ovarian cancer (OC) patients according to *F8* baseline expression in peripheral blood components (PBCs). (a) Patients with low expression levels had a lower PFS than their counterparts (profile 4; mean PFS of 12.8 ± 1.6 months and 23.7 ± 3.5 months, respectively; log-rank test, $p = 0.005$). (b) Dismissing patients under platelet anti-aggregation therapy at OC diagnosis, those with low expression had an inferior OS compared to their counterparts (profile 4; mean OS of 27.5 ± 4.5 months and 53.7 ± 5.9 months, respectively; log-rank test, $p = 0.008$).

Chapter IV - Table 3. Multivariable Cox regression analysis on the risk of disease progression (N = 48) and risk of death (N = 41) among OC patients according to pre-chemotherapy *F8* levels (profile 4) in PBCs.

Variable	aHR	95%CI	p-Value	Event
<i>F8</i> baseline expression (Low vs. high ¹)	2.54	1.15–5.58	0.021	Risk of disease progression
Age at OC diagnosis (≥64 vs. <64 years ¹)	1.99	0.96–4.10	0.063	
Metastatic disease (Yes vs. no ¹)	10.65	2.46–46.12	0.002	
<i>F8</i> baseline expression (Low vs. high ¹)	6.16	1.68–22.52	0.006	Risk of death
Surgery (No vs. yes ¹)	2.64	0.77–9.10	0.124	
Platinum sensitivity (Others vs. sensitive ¹)	13.72	3.35–56.22	<0.001	

Bold values were deemed statistically significant. ¹ Reference group. Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; OC, ovarian cancer; PBCs, peripheral blood cells.

4. Discussion

Haemostatic components in the OC microenvironment shape cancer growth and dissemination while heightening susceptibility to cancer-related VTE, which complicates patient management and negatively impacts prognosis [36]. Numerous mechanisms have been proposed for the hypercoagulable state seen in cancer patients [10]. Intriguingly, tumour cells can instigate a thrombo-inflammatory cascade by activating endothelial cells, platelets, and immune cells. Platelets and endothelial cells recruit neutrophils to the TME, while concurrently neutrophils activate platelets by generating neutrophil extracellular traps (NETs) and releasing pro-thrombotic mediators [4,37,38]. Moreover, platelets and endothelial cells interact with activated monocytes, contributing to immunosuppressive macrophage polarization. These immune cells also play a role in thrombus formation by expressing and releasing pro-thrombotic mediators, including TF [4,39].

The liver and vascular endothelium are deemed the primary sites for the synthesis of most coagulation factors and anticoagulant proteins (except for TF, FIV (calcium) and FVIII) [40]. However, platelets and immune cells can also produce these haemostatic proteins, especially in pathological states [18,20]. Indeed, platelets carry a pool of mRNA from megakaryocytes during thrombopoiesis, which could allow them to biosynthesise proteins [41]. Given the complex interplay between tumour cells, platelets, and immune and endothelial cells in the OC microenvironment, this study explored the implications of haemostatic gene expression patterns in PBCs among OC patients.

First, this study demonstrates a CAT incidence of 22.9%. Consistently, the literature reports an incidence of 10–30% for patients with this malignancy [9,42–48]. When focusing on those with the condition after OC diagnosis, a marginally significant association between VTE and patients' PFS was detected (long-rank test, $p = 0.055$). Specifically, those with VTE presented a shorter PFS. Regardless of VTE timing, CAT patients exhibit a lower OS (log-rank test, $p < 0.05$). Regarding clinical characteristics with predictive impact on VTE, high leucocyte count was prevalent among VTE subjects (χ^2 , $p = 0.043$), supporting the involvement of these immune cells in OC-related VTE pathogenesis [49,50]. Notably, the pre-chemotherapy leucocyte count is one of the parameters of KS. This VTE RAM showed unfavourable performance in this study, as previously described [9]. These findings highlight the detrimental role of CAT in OC patients and underscore the need for better RAMs to improve primary thromboprophylaxis [51–53].

Correlations between the pro-clotting and anti-clotting gene expression were detected in this study, the strongest being between *F13A1* and *TFPI1* expression ($P = 0.855$, $p < 0.001$, $R^2 = 0.731$). This suggests that PBCs might have a coordinated regulation of haemostatic genes to gatekeep the delicate balance of haemostasis. However, the precise dynamics of haemostatic gene regulation in these entities, mainly in pathological settings, represent an area of ongoing research.

The *F3* gene encodes for TF. Mainly expressed by extravascular tissue cells, TF is released into the bloodstream following vascular damage, binding to FVII [17]. The TF-activated FVII complex—the tenase complex—activates coagulation factor X (FX), which triggers thrombin generation [16]. Tumour overexpression of TF, a main promotor of OC-related VTE, is a common event and is associated with an unfavourable prognosis [16,54]. Beyond thrombosis, TF facilitates tumour growth and dissemination via numerous biological pathways, including the protease-activated receptor (PAR) 2 signalling [55,56]. Tumour cells can release TF, alone or within microvesicles, increasing the thrombogenic potential and aggravating cancer aggressiveness [54]. Furthermore, although with some debate, *F3* expression has been reported in platelets and immune cells, particularly monocytes, contributing to immunothrombosis in cancer and other pathological conditions [18,19,57,58].

In this study, high *F3* expression was more common among those with high PT (χ^2 , $p < 0.05$), suggesting a link to haemostatic abnormalities [59]. Likewise, patients at FIGO I/II stages presented higher *F3* levels (χ^2 , $p < 0.05$). Our research group's previous studies suggest that certain VTE-related biomarkers may have a greater influence before metastasis, potentially facilitating this process [60]. Regarding the implications of first-line chemotherapy, a significant difference was only detected when focusing on those who underwent chemotherapy as the first therapy (paired *t*-test, $p < 0.05$). Multiple mechanisms

can explain the difference in the expression. First, chemotherapy may have reduced the cancer-induced stimulation of PBCs by lowering the tumour burden. The therapeutic approach can also cause leukopenia, as well as negatively impact platelets and endothelial cells [61]. This could have influenced the interaction between these entities, decreasing *F3* expression in PBCs. However, further exploration is needed. Concerning thrombosis, lower baseline *F3* expression was associated with a higher risk for a later CAT event (one-way ANOVA followed by Dunnett's test, $p = 0.008$). Although it seems counterintuitive, this association could be due to complex interactions within the haemostatic system or even intrinsic mechanisms underlying *F3* mRNA expression, processing, and storage in these cells, for which there are currently scarce data. Additionally, monocytes can release TF-bearing microvesicles that bind to activated platelets, endothelial cells, and neutrophils, which could also influence the kinetics of *F3* expression [62,63]. In-depth functional studies are required to dissect the dynamics of *F3* expression in PBCs. As for patient prognosis, no significant impact was observed.

The *F5* gene encodes for coagulation factor V (FV). When activated, this glycoprotein together with activated FX forms the prothrombinase complex [64]. This complex in the presence of a phospholipid surface and calcium (FIV) catalysed the conversion of prothrombin (coagulation factor II (FII)) to thrombin (activated FII) in the common coagulation pathway [65]. Beyond its pro-clotting function, FV also inhibits coagulation in an intriguing process involving TFPI1 [66]. Regarding tumourigenesis, although not entirely comprehended, *F5* expression appears to have an oncogenic function [67–69]. In addition, FV generates thrombin, which has been associated with several tumourigenic processes [70]. Concerning its expression in PBCs, *F5* has been reported in leukocytes in pathological conditions [18,20,21]. As for platelets, it is unclear whether these cell fragments synthesise FV or the platelet-derived protein originated solely from megakaryocytes [20,71]. In this study, no association of *F5* levels with patients' characteristics was detected (χ^2 , $p < 0.05$). Except for those who had surgery as the primary intervention, first-line chemotherapy significantly impacted *F5* expression levels (paired *t*-test, $p < 0.05$), which could be due to the previously described mechanisms. Lastly, no association of baseline *F5* levels with OC-related VTE development and clinical outcomes was observed ($p > 0.05$). Given the small cohort size, further studies are needed to gather more evidence on the role of this coagulation factor in PBCs among OC patients.

The *F8* gene encodes for FVIII. Once activated by thrombin, this glycoprotein accelerates the activation of FX in conjunction with activated coagulation factor IX (FIX), FIV and phospholipids [23,40]. In the bloodstream, FVIII is typically bound to the von Willebrand factor (vWF), which stabilises and protects it from premature proteolysis, while also facilitating its transportation to sites of endothelial damage [23]. The relationship

between coagulation factors and VTE is thought to be largely attributed to deregulated FVIII and vWF levels, indicating a central role of this coagulation factor in venous thrombogenesis [72]. Although mainly synthesised in the liver, FVIII is also expressed by megakaryocytes/platelets and monocytes/macrophages as an additional strategy to restore haemostasis [18,20,22–25]. Several epidemiological studies have shown elevated FVIII circulating levels in cancer patients, with this expression having prognostic significance and being an independent predictor of VTE [73–75].

In the present study, high *F8* levels were more common among those with a lower INR, suggesting a potential link to haemostatic abnormalities (χ^2 , $p < 0.05$). Among those first treated with chemotherapy, high baseline *F8* levels were predominant among younger patients (profile 4, χ^2 , $p = 0.043$), those with high leucocyte count (profile 3, χ^2 , $p = 0.019$), high platelet count (profile 4, χ^2 , $p = 0.025$) and with a high KS (profile 4, χ^2 , $p = 0.008$). Concordantly, ageing has been shown to influence FVIII expression [76]. Concerning the association with leukocyte and platelet counts, it was expected given the reported expression profile in these entities [18,20,25,77]. As for the relationship between *F8* levels and KS, this finding also suggests a potential role in OC coagulome. Regarding the influence of first-line chemotherapy, the gene expression was significantly decreased after treatment (paired *t*-test, $p < 0.001$). However, no difference was detected when focusing on the patients who underwent chemotherapy as their first therapeutic intervention (paired *t*-test, $p > 0.05$). Concerning thrombosis, lower baseline *F8* expression was associated with a higher risk for a later OC-related VTE event (one-way ANOVA followed by Dunnett's test, $p = 0.057$; χ^2 , profile 1, $p = 0.051$; profile 2, $p = 0.028$). Like *F3*, functional studies are required to dissect the dynamics of *F8* expression in PBCs as current knowledge remains limited, particularly in malignancy [78].

Considering clinical outcomes, patients with low baseline *F8* had almost a three-fold increase in the risk of disease progression (profile 4; aHR = 2.54, $p = 0.021$), supporting the finding that FVIII impacts tumourigenesis [73,74,78,79]. As for patient survival, only when focusing on patients who were not under platelet anti-aggregation therapy at OC diagnosis (in addition to excluding those receiving anticoagulation therapy upon tumour diagnosis) was a significant association between baseline *F8* expression and patients' OS detected. Specifically, those with low gene expression had a six-fold increase in the risk of mortality (profile 4; aHR = 6.16, $p = 0.006$). Antiplatelet agents limit platelet aggregation and vasoconstriction, potentially reducing FVIII release from platelet alpha-granules [80]. Additionally, antiplatelet agents, such as acetylsalicylic acid (ASA), possess anti-inflammatory properties, which could affect leukocyte activity, potentially influencing their expression of *F8* [81]. To our knowledge, this is the first study linking *F8* expression in PBCs to OC patients' prognosis. Overall, low baseline *F8* expression was associated with a high

risk of CAT and an unfavourable prognosis regardless of thrombosis. This suggests that its expression by PBCs could be an attractive OC biomarker. Yet, more data are required.

Coagulation factor XIII (FXIII) is a transglutaminase that once activated crosslinks fibrin molecules to ensure blood clot stability [82]. In the plasma, FXIII circulates as a zymogen with two catalytic A subunits (FXIII-A₂) and two non-catalytic B-subunits (FXIII-B₂) [83,84]. The A subunit of FXIII (FXIIIA) is encoded by *F13A1*, which is expressed by cells of bone marrow and the mesenchymal lineage leading to three main pools of circulating FXIIIA: plasma, platelets and monocytes/macrophages [18,20,26]. In addition to coagulopathies, abnormal expression and/or activity of FXIII is proposed to influence cancer susceptibility and behaviour. Intriguingly, monocytes have been implicated in this mechanism [85,86]. In this study, no association between *F13A1* expression and patients' characteristics was observed (χ^2 , $p > 0.05$). Concerning the influence of first-line chemotherapy, the intervention had a significant impact on the gene expression in all groups (paired *t*-test, $p < 0.05$). Contrariwise, no link to CAT and clinical outcomes was detected. Whether this finding is related to the small sample size or whether FXIIIA has a context-dependent role needs to be clarified.

The *TFPI1* (or *TFPI*) gene encodes for a protein with the same name (TFPI1/TFPI), known as the primary inhibitor of the extrinsic or TF coagulation pathway. There are two TFPI1 isoforms—TFPI α and TFPI β . While the former is released by endothelial cells and activated thrombocytes, inhibiting both the prothrombinase and tenase complexes, the latter is presented at the endothelium surface and it is known to block the tenase complex more effectively [16]. In cancer settings, TFPI1 levels have been associated with VTE risk, metastatic potential, and all-cause mortality [87,88]. Indeed, this anticoagulant seems to act as a tumour suppressor [89]. In this study, first-line chemotherapy impacted *TFPI1* expression in all groups (paired *t*-test, $p < 0.05$). High *TFPI1* levels were more common among those with high PT and KS ≥ 2 , the latter only among those that were first treated with chemotherapy (χ^2 , $p < 0.05$). Although these findings pinpoint a potential role in CAT, no significant contribution of *TFPI1* expression in PBCs was detected, neither in thrombosis nor in patients' prognosis. More studies are needed to solidify these data.

The *THBD* gene encodes for thrombomodulin (TM), a thrombin receptor mainly expressed by endothelial cells, which reduces thrombin activity, preventing it from activating platelets and converting fibrinogen to fibrin [90]. Likewise, the TM-thrombin complex can terminate excessive coagulation by activating the protein C anticoagulation pathway, which further inactivates FV and FVIII [90,91]. This anticoagulant has been implicated in VTE in the broader population and cancer settings [92]. Overall, tumour expression of TM is linked to a favourable prognosis, decreasing the tumourigenic and metastatic potential, which can be explained by its anticoagulant and anti-inflammatory properties [93–97]. In this study,

initial chemotherapy notably influenced *THBD* expression levels across all cohorts (paired *t*-test, $p < 0.05$). Those with elevated *THBD* levels commonly presented a high KS (χ^2 , $p < 0.05$), suggesting a potential role in the tumour coagulome. However, no association with OC-related VTE nor a prognostic significance of *THBD* expression in PBCs was observed, which requires further confirmation.

Overall, this research provides preliminary insights into the expression of haemostatic genes in PBCs and their potential implications for OC patients. While the study yielded promising results, it is essential to recognise its limitations. The main one was the small cohort size, which may have limited the statistical power. This was due to the relatively low incidence of OC and the necessity to control for major confounders associated with CAT and gene expression analysis in PBCs. Additionally, it would be important to assess gene expression in other settings, including healthy conditions and close to VTE occurrence in cancer and cancer-free individuals to study the expression dynamics in PBCs. An analysis close to thrombotic events was not possible given the retrospective nature of the study. Also, no active VTE screening was conducted, which could have led to the underestimation of asymptomatic events. Nevertheless, this study also has its strengths. Specifically, most of the major risk factors linked to VTE were accounted for. Furthermore, the findings could open avenues for liquid biopsies to predict OC-related VTE and assess patients' prognosis.

5. Conclusions

Among gynaecological tumours, OC is deemed the most lethal. Due to active investigation, new therapeutic approaches have emerged to overcome OC resistance to treatment, paralleling the increased demand for disease biomarkers. Conversely, exploring the contributions of haemostasis deregulation to cancer progression is a potential avenue for identifying OC biomarkers and therapeutic targets. In the present research, the expression of haemostasis-related genes in PBCs was evaluated in a cohort of OC patients for their implications in CAT and patients' prognosis (regardless of VTE). To our knowledge, this is the first study to do so. According to the findings, OC patients have a high tendency towards VTE, which negatively affects their prognosis. Furthermore, pre-chemotherapy *F3* and *F8* expression was found to predict OC-related VTE development. Although this should be analysed carefully given the small cohort size, these preliminary data point out the expression of haemostasis-related genes in platelets and leukocytes as a potential source of CAT predictors. The low expression of *F8* was also linked to shorter patients' PFS and OS, supporting the two-way relationship between thrombosis and ovarian tumourigenesis. All in all, the expression of these genes in PBCs in a setting of cancer immunothrombosis

could be an attractive tool to assess OC patient's thromboembolic risk profile and prognosis non-invasively. This might pave the way for personalised thromboprophylaxis and better oncological treatment strategies to improve clinical outcomes. However, future studies should clarify the clinical applicability of these potential OC biomarkers in larger and more diverse cohorts. Also, it will be important to perform single-cell RNA sequencing to identify the specific cells responsible for the expression of these genes and their dynamics in physiological and pathological conditions.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Portuguese Institute of Oncology of Porto (CES. 69/021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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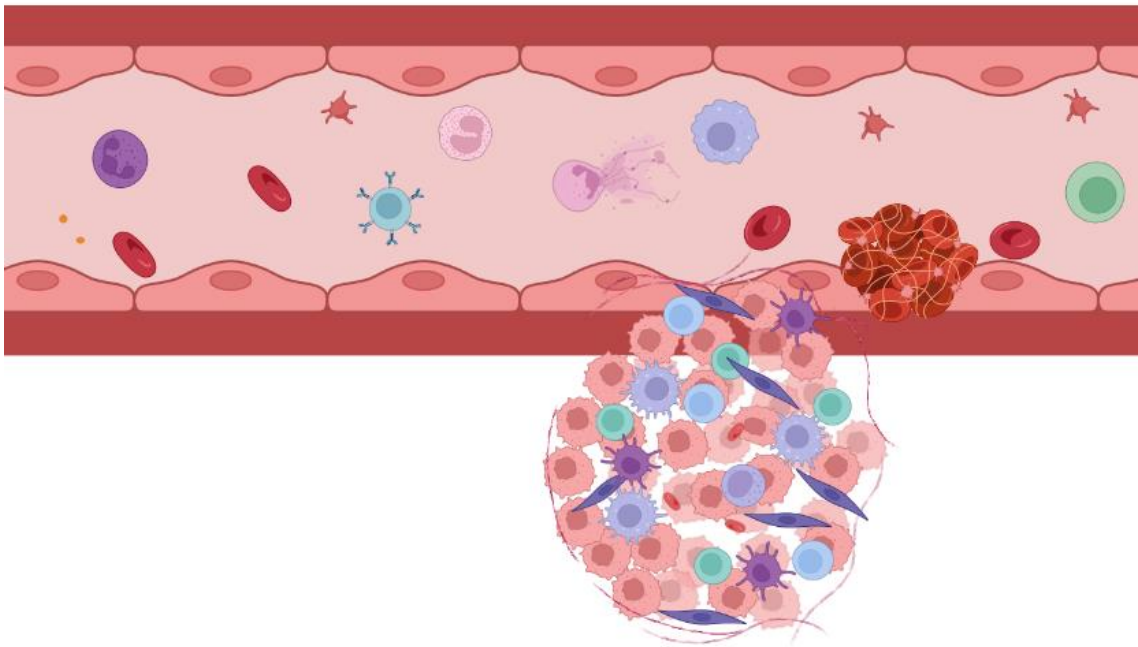
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Chapter V



CHAPTER V – Peripheral Blood Long Non-Coding RNAs: Shaping Venous Thrombogenesis and Ovarian Tumourigenesis

This chapter includes the following paper:

Inês Soares Marques *, Valéria Tavares *, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Inês Guerra de Melo, Joana Assis, Deolinda Pereira, Rui Medeiros. **Long Non-Coding RNAs: Bridging Cancer-Associated Thrombosis and Clinical Outcome of Ovarian Cancer Patients.** *International Journal of Molecular Sciences*. **2024**; 25 (1): 140. <https://doi.org/10.3390/ijms25010140>. (Impact factor and quartile reported in 2023: **4.9** and **Q1**).

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Long Non-Coding RNAs: Bridging Cancer Associated Thrombosis and Clinical Outcome of Ovarian Cancer Patients

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Abstract: Ovarian cancer (OC) and venous thromboembolism (VTE) have a close relationship, in which tumour cells surpass the haemostatic system to drive cancer progression. Long non-coding RNAs (lncRNAs) have been implicated in VTE pathogenesis, yet their roles in cancer-associated thrombosis (CAT) and their prognostic value are unexplored. Understanding how these lncRNAs influence venous thrombogenesis and ovarian tumourigenesis may lead to the identification of valuable biomarkers for VTE and OC management. Thus, this study evaluated the impact of five lncRNAs, namely MALAT1, TUG1, NEAT1, XIST and MEG8, on a cohort of 40 OC patients. Patients who developed VTE after OC diagnosis had worse overall survival compared to their counterparts (log-rank test, $p = 0.028$). Elevated pre-chemotherapy MEG8 levels in peripheral blood components (PBCs) predicted VTE after OC diagnosis (Mann–Whitney U test, $p = 0.037$; X^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; $p = 0.039$). Furthermore, low pre-chemotherapy NEAT1 levels in PBCs were associated with a higher risk of death (aHR = 6.25; $p = 0.008$). As for the remaining lncRNAs, no significant association with VTE incidence, OC progression or related mortality was observed. Future investigation with external validation in larger cohorts is needed to dissect the implications of the evaluated lncRNAs in OC patients.

Keywords: epithelial ovarian carcinoma; venous thromboembolism; RNA; long non-coding; prognosis; thromboprophylaxis

1. Introduction

Regarded as a silent killer, OC is often diagnosed at advanced stages and patients frequently develop therapy resistance [1–3]. These two factors contribute to a high rate of disease recurrence and a five-year survival rate lower than 50%, making OC the most lethal gynaecological tumour [1]. Therefore, more suitable prognostic biomarkers are required to personalise the disease management, which demands a better knowledge of ovarian tumourigenesis [4].

Although most studies on OC are focused on tumour cells, understanding the surrounding microenvironment and how tumour cells and stroma interact to promote disease aggressiveness is critical [5]. For instance, key players of the haemostatic system in the tumour microenvironment are known to establish a bidirectional relationship with cancer [6,7]. Specifically, tumour cells can activate the coagulation cascade and platelets and inhibit fibrinolysis through the secretion of several factors, alone or within

microparticles, namely tissue factor (TF), podoplanin and plasminogen activation inhibitor-1 (PAI-1), respectively [8]. In addition, cytokines derived from tumour cells can lead to the recruitment and activation of leucocytes, such as monocytes and neutrophils. Activated monocytes can release cancer procoagulant (CP) and TF-positive microparticles, while neutrophil extracellular traps (NETs) can capture and activate platelets, favouring the buildup of fibrin. In this process called immunothrombosis, platelets, leukocytes and endothelial cells interact, augmenting thrombogenesis [9–12]. On the other hand, haemostatic components hijacked by tumour cells can fuel their progression, for instance, by inducing tumour neoangiogenesis [8]. As a result, this complex network increases the susceptibility of oncological patients to thrombotic events while promoting tumour aggressiveness [13,14].

Venous thromboembolism (VTE) is a cardiovascular disease encompassing deep venous thrombosis (DVT) and pulmonary embolism (PE). This life-threatening condition, which results from a haemostatic imbalance, is common, particularly in regions with high Human Development Index (HDI) levels [4]. Cancer patients are four to seven times more likely to develop VTE than those without malignancy, with approximately 15% of the patients experiencing a VTE episode [15]. Conversely, the risk for cancer diagnosis is higher at least 2 years after the first episode of idiopathic VTE [16]. The disease is also associated with a poor prognosis, being the second-leading cause of death among oncological patients [8]. As such, there has been a growing effort to find predictive markers for cancer-associated thrombosis (CAT).

VTE occurrence is commonly observed among OC patients, with an incidence ranging from 5 to 20%, which increases their mortality rates [4]. Given the roles of the tumour coagulome in cancer growth and progression, it is important to identify biomarkers of OC-related VTE as they might improve the management of both VTE and OC [17]. Recently, multiple long non-coding RNAs (lncRNAs) were shown to influence venous thrombus formation and resolution; however, the data specifically among cancer patients are scarce [18]. Given the impact of platelets, endothelial cells and leukocytes on both thrombogenesis and tumour progression, as well as the high incidence and negative effect of VTE among OC patients, VTE-associated lncRNAs may influence the pathogenesis of OC-related VTE and constitute valuable OC prognostic biomarkers regardless of VTE. Thus, this study evaluated the expression of lncRNAs in peripheral blood components (PBCs) and its implications in CAT and the prognosis of OC patients.

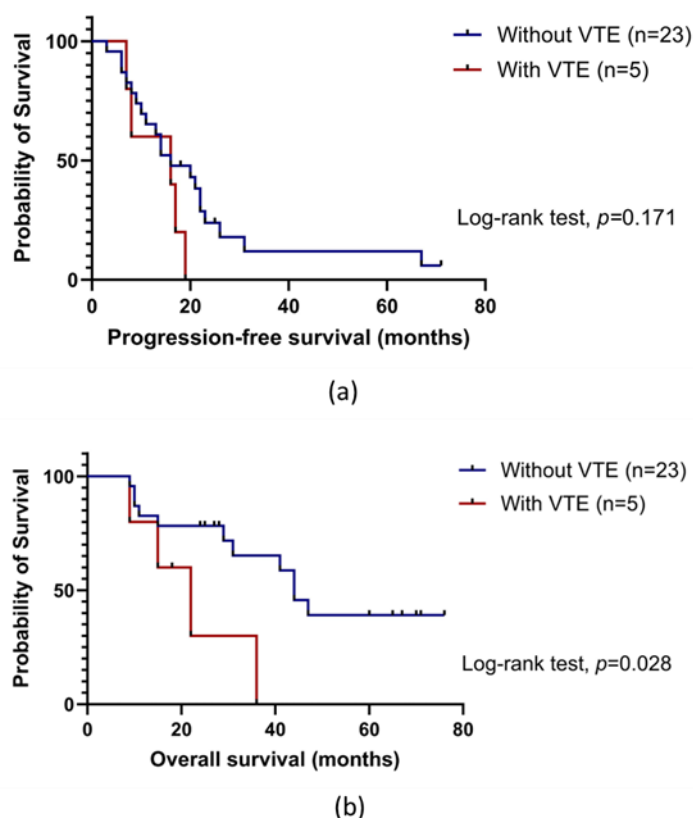
2. Results

2.1. Implications of VTE on Patients' Prognosis

Among 30 patients with sufficient follow-up time, seven (23.3%) developed OC-related VTE, including two patients with VTE before 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. Among the patients with VTE before cancer diagnosis, the mean 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.

The Khorana score (KS) did not present a significant predictive impact on VTE incidence among the OC patients (≥ 2 versus (vs.) < 2 ; X^2 , $p > 0.05$). Also, no significant association between VTE development and patients' demographical and clinicopathological features, baseline full blood count and coagulation tests was observed (X^2 , $p > 0.05$).

No association between VTE and patients' progression-free survival (PFS) was detected (log-rank test, $p = 0.171$, **Figure 1a**). Contrariwise, those with VTE had a worse overall survival (OS) than their counterparts (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 1b**).



Chapter V - Figure 1. Progression-free survival (a) and overall survival (b) by Kaplan–Meier and log-rank test for ovarian cancer (OC) patients (n = 28), according to venous thromboembolism (VTE) occurrence after cancer diagnosis. (a) No association between VTE and patients' progression-free survival (PFS) was observed among those with the condition after cancer diagnosis (log-rank test, $p = 0.171$). (b) Patients with VTE after OC diagnosis had lower overall survival (OS) compared to their counterparts (mean OS of 22.2 ± 5.6 months and 47.5 months ± 5.9 months, respectively; log-rank test, $p = 0.028$).

2.2. LncRNA Expression and Patients' Demographical and Clinicopathological Features, Baseline Full Blood Count and Coagulation Tests

Regarding patients' demographical and clinical features, no significant associations were found for MALAT1, TUG1 and NEAT1, regardless of the expression profile and the first treatment (X^2 , $p > 0.05$). In opposition, for MEG8, a significant association between its pre-chemotherapy levels in PBCs and KS (<2 vs. ≥ 2) was observed (profile A (see Section 4.7 for profile definition), X^2 , $p = 0.009$; profile B, X^2 , $p = 0.048$). When stratifying the analysis in terms of first treatment (surgery vs. chemotherapy), only those that would undergo chemotherapy as the first intervention (meaning they were treatment-naïve at the time of first sample collection) presented pre-chemotherapy MEG8 levels that were significantly associated with KS (profile A, X^2 , $p = 0.013$). In both analyses (entire cohort and subgroups),

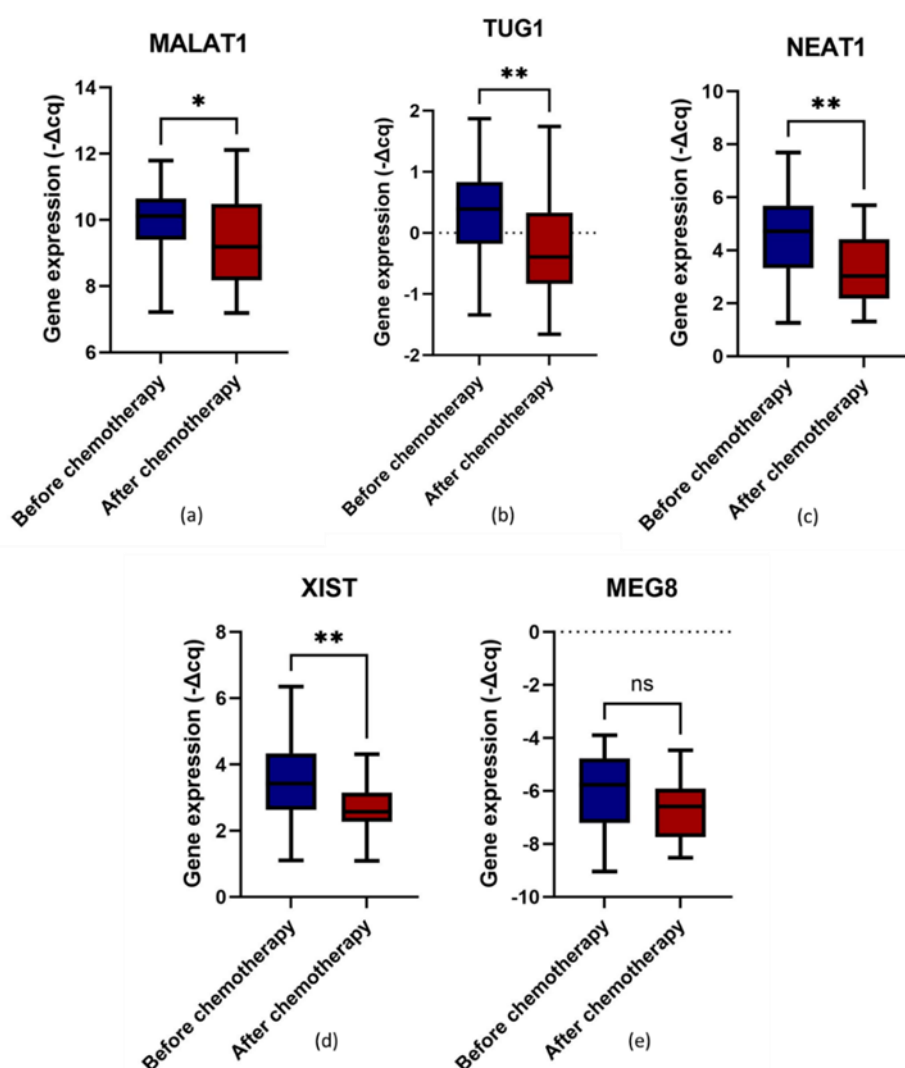
elevated pre-chemotherapy MEG8 levels were predominant in the $KS < 2$ group. Moreover, an association was found between XIST expression and cancer antigen 125 (CA 125) levels among those initially treated with chemotherapy. Specifically, intermediate and elevated pre-chemotherapy levels of the lncRNA were associated with high CA 125 levels (profile B, χ^2 , $p = 0.047$).

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

Concerning the baseline coagulation tests, namely prothrombin time (PT), activated partial thromboplastin time (aPTT) and international normalised ratio (INR), significant associations were detected only for MALAT1 and MEG8. Specifically, higher pre-chemotherapy MALAT1 levels were linked to lower PT times in the chemotherapy group (profile B, χ^2 , $p = 0.025$). For MEG8, irrespective of the first treatment, higher levels of this lncRNA were associated with a longer PT (profile A, χ^2 , $p = 0.014$; profile B and D, χ^2 , $p < 0.001$). When stratified by treatment, this association remained significant for the surgery group (profile A, χ^2 , $p = 0.038$) and in both chemotherapy and surgery (profile B, χ^2 , $p = 0.014$ and $p = 0.022$, respectively; profile D, χ^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 first treatment, with elevated pre-chemotherapy levels of MEG8 being more predominant among those with a higher INR (profile A and B, χ^2 , $p = 0.041$ and $p = 0.023$, respectively; profile D, χ^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, χ^2 , $p = 0.044$).

2.3. Chemotherapy Impact on lncRNA Expression

For patients whose samples prior to and after the first-line 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 (**Figure 2a** ($p = 0.047$), 2b ($p = 0.003$), 2c ($p = 0.005$) and 2d ($p = 0.009$), respectively). In opposition, only a marginally significant difference was observed for MEG8 (**Figure 2e**, $p = 0.058$). In the stratified analyses, TUG1 levels were significantly decreased after chemotherapy in the group that underwent chemotherapy as the first intervention ($n = 17$; $p = 0.029$). As for those first treated with surgery ($n = 20$), pre-chemotherapy TUG1 and XIST expression in PBCs significantly decreased after chemotherapy ($p = 0.035$ and $p = 0.006$, respectively). For the other lncRNAs, no statistically significant differences were detected in their expression levels in the stratified analyses (Wilcoxon matched-pairs signed rank test, $p > 0.05$).

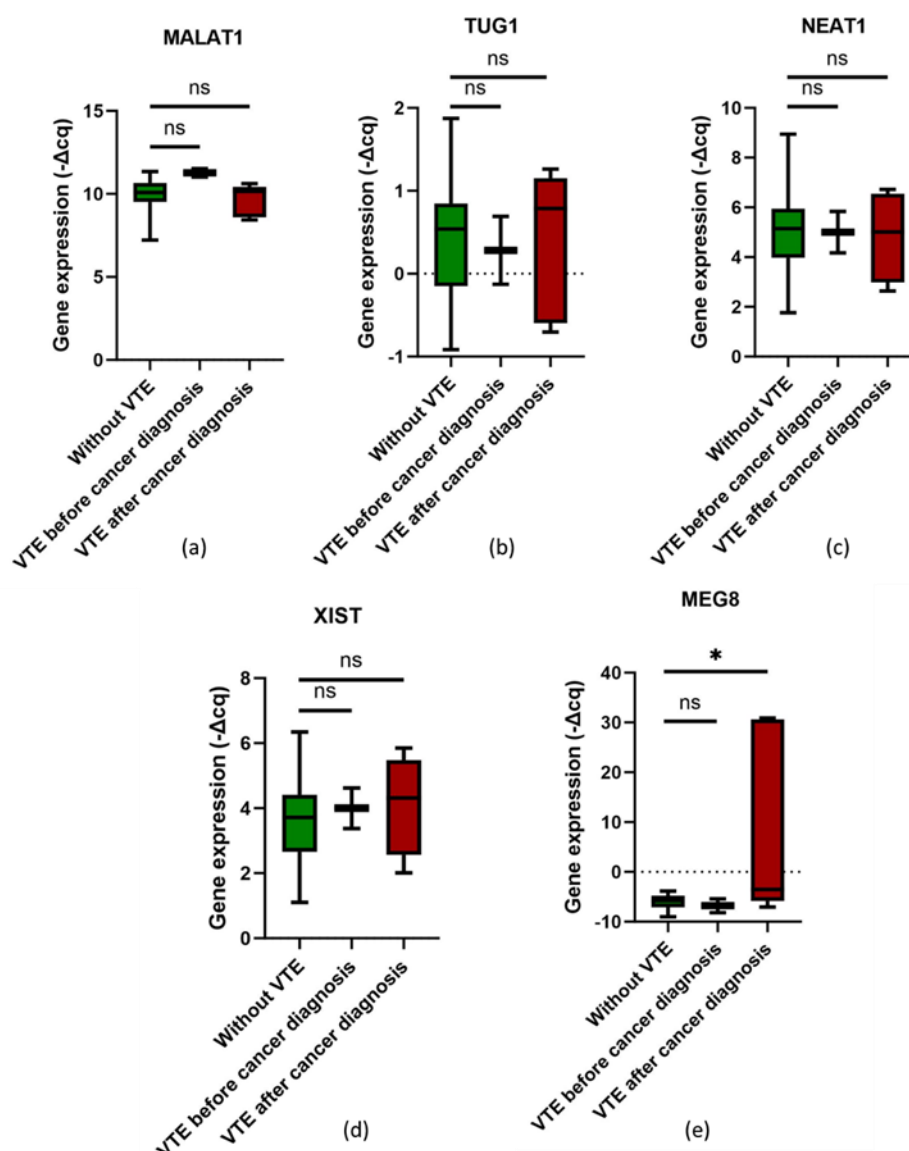


Chapter V - Figure 2. Normalised relative expression levels of lncRNAs ($-\Delta Cq$) in peripheral blood cells among ovarian cancer patients before and after the first-line chemotherapy: (a) MALAT1 expression; (b) TUG1 expression; (c) NEAT1 expression; (d) XIST expression; and (e) MEG8 expression; Wilcoxon matched-pairs signed rank test, * $p < 0.05$, ** $p < 0.01$; ns, non-significant.

2.4. LncRNA Expression and OC-Related VTE Incidence

The impact of the evaluated lncRNAs on OC-related VTE incidence was investigated by considering patients without VTE, those with the condition before 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 3a–d**), which was corroborated by the Chi-Square test (X^2) (profiles A, B, C and D; X^2 , $p > 0.05$). Concerning MEG8, its pre-chemotherapy expression levels were significantly elevated in OC patients who later developed a VTE event after cancer diagnosis compared to those without the condition (Mann–Whitney U test; $p = 0.037$;

Figure 3e). This result was further confirmed by the Chi-Square test (X^2) (profile C; X^2 test, $p = 0.033$).



Chapter V - Figure 3. Normalised relative expression levels of lncRNAs ($-\Delta Cq$) in peripheral blood 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.

2.5. lncRNA Expression and Patients' Prognosis

The influence of the lncRNAs on patients' clinical outcomes 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 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 OC progression and death was observed, both in univariable and multivariable Cox analyses, regardless of the expression profile ($p > 0.05$). 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 was observed. Specifically, its low pre-chemotherapy 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 1**). As for the impact of MEG8 on the risk of death, no significant association was identified (Cox regression analysis, $p > 0.05$). Concerning NEAT1, no significant association with the risk of disease progression was detected (Cox regression analysis, $p > 0.05$). As for its impact on the risk of death, a significant association was only observed in the multivariable Cox analysis adjusting for platinum sensitivity. Specifically, its low expression levels in PBCs 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 1**).

Chapter V - Table 1. Multivariable Cox regression analysis on the risk of disease progression ($n = 29$) and risk of death among OC patients ($n = 35$) according to pre-chemotherapy levels of MEG8 and NEAT1 in PBCs, respectively.

Variable	aHR	95% CI	<i>p</i> -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	
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	

¹ Reference group. Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; OC, ovarian cancer; PBCs, peripheral blood cells.

3. Discussion

Among other solid tumours, OC is one of the most associated with VTE [19–26]. Corroborating a two-way association between thrombosis and OC progression, increased D-dimer levels are linked to high tumour burden, chemoresistance and poor prognosis,

regardless of VTE status [4,27]. Likewise, high plasma thrombin levels have been shown to negatively impact OC patients' survival and prognosis, while elevated platelet count predicts disease recurrence [28,29]. Also, TF overexpression is linked to aggressive tumours, advanced stages and decreased survival among OC patients [30,31]. Ovary tumours are also known to stimulate platelets, endothelial and immune cells to present a procoagulant behaviour [2,32]. Together, all these players appear to act in a complex network to promote ovarian tumourigenesis 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 [33]. Subsequently, in recent years, multiple lncRNAs have been implicated in venous thrombus formation and resolution. However, the data on cancer patients are scarce [18]. Thus, this study evaluated the impact of VTE-associated lncRNAs on the incidence of OC-related VTE and their prognostic value among OC patients.

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 a high pro-thrombotic risk [19–26]. Furthermore, those with VTE had worse survival compared to their counterparts, particularly when focusing on patients with an event after OC diagnosis (log-rank test, $p = 0.028$), which corroborates the existing evidence [34–36]. As for PFS, no significant association was detected. Given the negative effect of VTE, it is imperative to delve into more effective thromboprophylaxis measures. Until now, the most studied score for predicting CAT is KS [37–40]. In this study, the score had a poor performance, unable to predict OC-related VTE onset, which was also previously described [25]. Although additional research in larger cohorts is needed, these preliminary findings reinforce the need for better and more tailored VTE predictive models.

Regarding the evaluated lncRNAs, starting with MALAT1, it has been pointed out as a major player in thrombogenesis due to its downregulation in atherosclerotic plaques and its association with endothelial function [41,42]. Inconsistent results, however, led to the hypothesis that this lncRNA may have a cell- and/or context-dependent role in VTE [18,42–44]. In the present study, higher pre-chemotherapy expression levels of MALAT1 in PBCs, irrespective of the first treatment (surgery vs. chemotherapy), were associated with higher leukocyte (profile A, X^2 , $p = 0.041$) and neutrophil (profile A, X^2 , $p = 0.041$) counts. This suggests that MALAT1 expression in PBCs is attributed, at least partially, to neutrophils. Indeed, as previously mentioned, neutrophils are implicated in immunothrombosis through the release of NETs (NETosis), which is promoted, for instance, by TF and platelets to increase the pro-thrombotic cascade [9]. Concordantly, exosomal MALAT1 from human umbilical vein endothelial cells (HUVECs) was previously revealed to stimulate NETosis, triggering a proinflammatory response [45]. The data hint that under pro-inflammatory and

pro-thrombotic settings (e.g., tumour microenvironment), endogenous or exosomal MALAT1 may promote neutrophil activation, favouring thrombus formation [42,43]. 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, $p = 0.047$). Indeed, by lessening the tumour burden, chemotherapy might have diminished the cancer stimulatory effect on the PBCs [46]. Moreover, the therapy itself is known to negatively impact leukocyte activity and counts, causing conditions such as neutropenia and leukopenia [47,48]. Also, chemotherapy can decrease platelet counts and cause endothelial dysfunction, altering the signal from these haemostatic key players to leukocytes (i.e., immunothrombosis) [9,49,50]. 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 and haemoglobin is, however, scarce and conflicting [51,52]. Furthermore, in the present study, pre-chemotherapy expression levels of MALAT1 in PBCs were also found to be associated with lower PT in the group that had chemotherapy as the first treatment (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 PT values), this finding suggests that, at OC diagnosis, MALAT1 expression in PBCs might be linked to haemostatic abnormalities, particularly in the extrinsic coagulation pathway [47,48,53,54]. However, no significant association with OC-related VTE was found, possibly due to the context-dependent role of this lncRNA and the limited cohort size. As for its implications in OC pathways, MALAT1 is usually upregulated, which is associated with advanced disease stages, high tumour burden and a poor prognosis [55–59]. However, no significant associations of MALAT1 with OS and PFS were detected in this study. Further investigation with larger cohorts is necessary to assess the implications of this lncRNA in PBCs among OC patients.

Regarding TUG1, by serving as a decoy, this lncRNA seems to exert a protective effect against DVT [60]. In this study, elevated pre-chemotherapy levels of TUG1 in PBCs, irrespective of the primary treatment, were linked to higher 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), a modulator of angiogenesis and inflammation, constitutively expressed by neutrophils. Interestingly, the little existing data indicate that LRG1 promotes the extravasation and activation of neutrophils, stimulating NETosis, which, as mentioned before, is implicated in immunothrombosis [61,62]. Furthermore, similar to MALAT1, TUG1 expression levels in PBCs decrease after the first-line chemotherapy (Wilcoxon matched-pairs signed rank test, $p = 0.003$), most likely due to the impact of the therapy on cancer burden, leukocytes (and neutrophils specifically), platelets and

endothelial cells, overall leading to a lower TUG1 expression. Regarding the baseline haemoglobin levels and the coagulation tests, no significant associations involving TUG1 were observed. The lncRNA also did not present a significant impact on VTE incidence among the OC patients. As for its implications in OC pathways regardless of VTE, the roles of this lncRNA also seem to be conflicting or context-dependent [63–66]. In the present study, TUG1 did not show any impact on the risk of disease progression or death. Given the reported roles of TUG1 in VTE and OC, further studies are warranted.

Serum levels of the lncRNA NEAT1 were previously found to be upregulated in DVT patients compared to healthy individuals. In HUVECs, this lncRNA is implicated in endothelial dysfunction and coagulation/fibrinolysis imbalances [67,68]. This study found no statistically significant associations between pre-chemotherapy NEAT1 expression levels in PBCs 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 [69,70]. Indeed, while cell counts can impact the expression levels of a gene within a sample, gene expression activity is often the primary contributor, which could explain the absent association between NEAT1 levels and leukocyte counts [71]. Furthermore, decreased levels of this lncRNA were observed after the first-line 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 endothelial cells. As for its roles in thrombogenesis, NEAT1 did not present a significant association with the baseline coagulation tests or OC-related VTE incidence. As previously mentioned, the study that linked NEAT1 to VTE was conducted by analysing the expression of the lncRNA in serum and HUVECs [67]. Additional investigation with a larger cohort size is needed to unravel the role of NEAT1 expression in PBCs in thrombogenesis among OC patients. Concerning ovarian tumourigenesis, a previous study demonstrated that NEAT1 was upregulated in OC compared to non-neoplastic tissues. Also, the lncRNA expression levels were positively associated with the International Federation of Gynecology and Obstetrics (FIGO) stage and tumour grade, being an independent prognostic factor of OC [68]. However, in the present study, diminished pre-chemotherapy NEAT1 expression levels in PBCs were significantly associated with an increased risk of mortality adjusting for platinum sensitivity (profile D, aHR = 6.25, 95% CI, 1.60–24.43, $p = 0.008$). Curiously, NEAT1 low levels were previously reported to lead to an excessive accumulation of promyelocytes, potentially leading to coagulation abnormalities [72–74]. Also, in OC, NEAT1-enriched extracellular vesicles derived from M2-polarised tumour-associated macrophages have been previously demonstrated to favour immune evasion [75]. Whether these two seemingly isolated mechanisms are interrelated and underlying the negative effect of low NEAT1 expression

in PBCs needs to be explored. No significant association between the lncRNA expression and PFS was detected. Overall, more studies with larger cohort sizes are required to establish the role of NEAT1 in VTE and OC.

The lncRNA XIST was previously 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), a pro-thrombotic protein that promotes the activation and aggregation of platelets [76,77]. In the present study, intermediate and higher pre-chemotherapy expression levels of XIST in PBCs 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 those that were first treated with chemotherapy (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 PBCs was attributed, at least partially, to neutrophil counts. Concordantly, this lncRNA has been previously implicated in neutrophil-associated biological processes, including NETosis [78,79]. Concerning chemotherapy's impact, XIST expression levels were significantly decreased after the first-line chemotherapy (Wilcoxon matched-pairs signed rank test, $p = 0.009$). Furthermore, a significant relationship with haemoglobin levels was found in the surgery group, with intermediate and elevated levels of XIST linked to higher haemoglobin levels (profile B, X^2 , $p = 0.033$). However, the existing data on this association are scarce. Regarding haemostatic abnormalities assessed by baseline coagulation tests and the incidence of OC-related VTE, no significant associations with XIST were detected. Nevertheless, the existing evidence on its implications in VTE pathways addresses the expression of the lncRNA in plasma [79]. Thus, additional investigation with a larger cohort size is needed to unravel the role of PBCs' expression of XIST in thrombogenesis. Concerning OC progression, there are contradictory findings surrounding this lncRNA. A study showed that XIST is highly expressed in epithelial OC (EOC) tissues and cell lines, being strongly associated with tumour grade, FIGO stage and distant metastasis, and representing an independent prognostic factor [79–81]. In opposition, its overexpression has been associated with longer survival in OC patients, being downregulated in advanced stages, compared to early disease stages [82]. In the present study, no impact of XIST on the risk of disease progression or death was found. However, within the chemotherapy group, intermediate and elevated levels of the lncRNA were associated with elevated levels of serum CA 125 (profile B, X^2 , $p = 0.047$). As this marker is known to be positively correlated with OC burden, XIST might also increase with tumour progression [83]. However, as mentioned before, the available literature has conflicting findings regarding the role of XIST in ovarian tumourigenesis, and the implications of PBCs' expression of this lncRNA in OC progression are yet unclear. Thus, further studies are needed.

The lncRNA MEG8 is dysregulated in several disorders, such as lung, ovarian and colorectal cancers, as well as gestational diabetes mellitus, diabetic nephropathy and ischemic heart disease [84,85]. In this study, MEG8 expression levels in PBCs were not associated with the baseline full blood count, nor impacted by the first-line chemotherapy. This suggests that the expression level of this lncRNA is mainly attributed to the gene expression activity in PBCs, which is sustained even with chemotherapy. Furthermore, elevated pre-chemotherapy MEG8 levels were significantly associated with lower 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 opposition, the lncRNA expression was not individually associated with either of the KS parameters (pre-chemotherapy body mass index (BMI), haemoglobin levels and platelet and leukocyte counts) [40]. Also, elevated MEG8 levels were associated with prolonged PT, aPTT and INR, which indicates its potential association with abnormalities of the haemostatic system, involving both the intrinsic and extrinsic pathways [53,54]. Concordantly, the lncRNA was significantly elevated among OC patients who later experienced a VTE event compared to those who remained VTE-free (Mann–Whitney U test, $p = 0.037$; X^2 test, $p = 0.033$). As mentioned, 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 with low VTE risk (KS < 3 or KS < 2 , depending on the cut-off) often end up suffering from the condition, according to different studies [39]. 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 tissue factor pathway inhibitor 2 (TFPI2) [18,85]. This haemostatic protein is expressed in cells such as monocytes or macrophages and endothelial cells, playing dual roles: as an anticoagulant by targeting the extrinsic coagulation pathway (also known as the TF coagulation pathway) and as an anti-fibrinolytic protein by inhibiting plasmin. Collectively, TFPI2 seems to play a context-dependent role in VTE pathways, influencing both thrombus formation and its resolution [86–89]. Therefore, as MEG8 downregulates the expression of TFPI2, which is also expressed among leukocytes, the present study results suggest that TFPI2 as an anticoagulant protein, rather than an anti-fibrinolytic protein, might be the primary mechanism underlying the promoting effect of MEG8 in blood clot formation. As such, due to the MEG8 inhibition of TFPI2 expression, the active TF coagulation pathway contributes to OC-related VTE occurrence. In accordance with its context-dependent role, elevated serum TFPI2 levels were reported to be a diagnostic marker of VTE among OC patients [90,91]. This hints that, in opposition to blood clot formation, MEG8 levels may be downregulated at thrombus resolution, leading to TFPI2 upregulation, inhibiting plasmin and

impairing thrombus resolution. In sum, elevated pre-chemotherapy MEG8 levels in PBCs 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 before 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, $p = 0.039$). In accordance with this, a study utilising integrated bioinformatics analysis demonstrated that diminished MEG8 levels in OC tissues were associated with a poorer prognosis [92]. Interestingly, in opposition to other tumours, TFPI2 is often overexpressed in ovarian clear cell carcinoma (the EOC subtype most associated with VTE), though its precise role and the underlying mechanisms remain unclear [93–96]. Furthermore, low MEG8 levels in endothelial cells are known to impair 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 tumourigenesis [85,97,98]. As for the implications of MEG8 expression in PBCs in ovarian tumourigenesis, the data are scarce. Nevertheless, MEG8 has been previously linked to immune infiltration in OC [92]. Additionally, its lower levels may lead to an overexpression of TFPI2, which, as previously mentioned, has an inhibitory activity towards plasmin [86]. In turn, this pro-fibrinolytic protein is also implicated in tumour growth and dissemination via extracellular matrix degradation, influencing tumour metastasis [99]. Of note, no significant association between MEG8 expression and OS was detected. Further studies with larger cohorts are needed for clarification.

Despite the promising results, the present study had limitations that need to be acknowledged. Firstly, the main constraint was the small cohort size, which may have lowered this study's statistical power, potentially impairing the generalisation of the findings to a broader population. Furthermore, the retrospective design of this study impeded the gathering of other relevant data. For instance, it would be important to assess the lncRNA expression close to VTE occurrence to evaluate the diagnostic capability of these non-coding RNAs. Also, 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. Moreover, there was an absence of control groups comprising healthy participants and VTE patients without cancer, which hindered the comparison of the lncRNA expression in health and disease contexts.

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, this study was focused on deregulated lncRNAs in PBCs, demonstrating the potential clinical relevance of liquid biopsies in OC prognosis. Overall, this study represents a pioneering effort in shedding light on the unexplored territory of VTE-related lncRNAs within the context

of CAT, since, up until now, no studies have been conducted on the role of these lncRNAs among cancer patients.

4. Materials and Methods

4.1. 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 Oncology Institute of Porto (IPO Porto) for first-line treatment. In this study, patients were excluded if they (1) had other malignant diseases prior to or after OC diagnosis; (2) were pregnant or breastfeeding at cancer diagnosis; (3) had autoimmune diseases and/or were under immunosuppressive treatments; (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. Therefore, 40 OC patients were enrolled, for whom biological material was available before the first line of platinum-based chemotherapy was available. Most patients had serous (33 (82.5%)), followed by clear-cell carcinoma (3 (7.5%)), carcinosarcoma (2 (5%)), endometrioid (1 (2.5%)) and mixed EOC (1 (2.5%)).

Disease staging was conducted according to the FIGO system updated in 2021 [100]. Data concerning the patients' demographical and clinicopathological features (including VTE history), baseline full blood count and coagulation tests were obtained from their medical data files. CAT was defined as an event occurring six months before to two years after cancer diagnosis [101]. It is worth noting that no active screening was conducted since this measure is not yet incorporated into the clinical routine procedures of IPO Porto. The median follow-up time was 24.5 months (minimum = 9.0 months; maximum = 76.0 months). A description of the study cohort characteristics is given in **Table 2**.

Chapter V - Table 2. Demographic and clinicopathological characterisation of OC patients (N = 40).

Variable	N (%)
Age (years) *	63.1 ± 12.5
<63	19 (47.5)
≥63	21 (52.5)
BMI (kg/m²)	
<26	19 (47.5)
≥26	20 (50.0)
Hormonal status	
Premenopausal	10 (25.0)
Postmenopausal	30 (75.0)
ECOG	
≤1	36 (90.0)
>1	4 (10.0)
Inherited mutations linked to ovarian cancer	
BRCA1/2	4 (10.0)
Other	1 (2.5)
None	35 (87.5)
Histological grade †	
Low	1 (2.5)
High	38 (95.0)
FIGO stage †	
I/II	10 (25.0)
III/IV	30 (75.0)
Serum CA 125 levels (U/mL)	
<1195	19 (47.5)
≥1195	20 (50.0)
First-line treatment	
Surgery plus adjuvant chemotherapy	21 (52.5)
Neoadjuvant chemotherapy plus surgery	1 (2.5)
Neoadjuvant chemotherapy 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 therapy §	2 (5.0)
Platelet anti-aggregation therapy	7 (17.5)
KS	
<2	21 (52.5)
≥2	15 (37.5)

* Presented as mean ± standard deviation. † Defined following the FIGO Cancer Report 2021 [100]. ‡ A platinum-sensitive patient was considered as having cancer progression after at least six months after the last dose of platinum-based chemotherapy. § Patients who had cancer-related venous thromboembolism prior to OC diagnosis. Except for first-line treatment, platinum sensitivity and maintenance therapy, all the factors were defined at cancer diagnosis. For patients' age, categories of the variable were defined based on the mean value given its normal distribution (Shapiro–Wilk test, $p = 0.738$), whereas for BMI and serum CA 125 levels, the median value was used as a cut-off since the variables were non-normally distributed (Shapiro–Wilk test, $p < 0.05$). For some patients, there was missing information, namely one for BMI, histological grade and serum CA 125 levels and four for KS. Abbreviations: BMI, body mass index; BRCA, Breast Cancer Gene; CA 125, cancer antigen 125; ECOG, Eastern Cooperative Oncology Group; FIGO, International Federation of Gynecology and Obstetrics; KS, Khorana score; OC, ovarian cancer; PARP, Poly (ADP-ribose) polymerase.

4.2. LncRNA Selection

Based on a literature review [18], lncRNAs were selected if they (1) had involvement in VTE; (2) had an association with haemostatic key players; (3) were expressed by PBCs, particularly monocytes, neutrophils and platelets; and (4) had available TaqMan assays for gene expression analysis. Additionally, those with known prognostic value in cancer were prioritised. Thus, the investigation was based around the subsequent lncRNAs: metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), taurine-upregulated gene 1 (TUG1), nuclear-enriched abundant transcript 1 (NEAT1; also known as nuclear paraspeckle assembly transcript 1), X-inactive-specific transcript (XIST) and maternally expressed 8 (MEG8).

4.3. Sample Collection and Processing

Peripheral blood samples were collected for each patient in ethylenediamine tetraacetic acid (EDTA)-coated tubes using a standard venipuncture technique. Samples were gathered before and, whenever possible, after the first-line chemotherapy. PBCs were isolated by first lysing the erythrocytes using an ammonium-chloride-potassium (ACK) solution (1×). The samples were then frozen at −20 °C for 20 min and then centrifugated for 10 min at 2500 rpm at room temperature. After discarding the supernatant, successive washes with ACK solution (1×) and phosphate-buffered saline (PBS) solution (1×) were conducted, followed by rounds of centrifugation for 10 min at 2500 rpm at room temperature to separate and discard the supernatant. The obtained pellet of PBCs (without erythrocytes) was further diluted and conserved in TriPure® Isolation Reagent (Roche Applied Science, Penzberg, Germany). Samples of PBCs were stored at −80 °C until further use.

4.4. Total RNA Extraction

Total RNA from PBC samples was extracted using the GRS RNA kit—Blood & cultured cells (#GK08.0100) (Grisp Research Resolutions®, Porto, Portugal) following the manufacturer's instructions with an adjustment (the duration of the DNase treatment (DNase I set (#GKC01.0100) was extended). RNA concentration and purity were analysed using the NanoDrop Lite spectrophotometer (Thermo Scientific®, Waltham, MA, USA). Following extraction, RNA samples were stored at −80 °C until use.

4.5. cDNA Synthesis

RNA samples (150 ng) were used as templates to generate complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Carlsbad, CA, USA) following the manufacturer's instructions. The conversion was conducted in a Mycycler™ Thermal cycler (Bio-Rad Laboratories, 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.

4.6. Relative Quantification of lncRNAs

The expression levels of the lncRNAs were quantified through quantitative real-time polymerase chain reaction (qRT-PCR) using a StepOnePlus™ qPCR system (Applied Biosystems®, Foster City, CA, USA). Data analysis was carried out using the StepOne software (version 2.3, Applied Biosystems®, Foster City, CA, USA). Each PCR reaction was performed using 5 µL of 2× TaqMan™ Gene Expression Master mix (Applied Biosystems®, Foster City, CA, USA); 3.5 µL of nuclease-free water; 0.5 µL of 20× TaqMan™ Gene Expression Assays (Applied Biosystems®, Foster City, CA, USA) 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 µL of cDNA sample, making a total volume of 10 µL. For all reactions, the thermal cycling conditions were: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The quantification was conducted in triplicate and negative controls (without cDNA) were included in all reactions. All targets and endogenous controls for each sample were amplified in the same plate. Standard deviations (SDs) 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 and endogenous controls in each sample. *GAPDH* and *HPRT1* were tested as endogenous controls.

4.7. Statistical Analysis

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

The lncRNAs' normalised relative expression was determined using the Livak method. Among the tested endogenous controls, *HPRT1* was used to normalise the lncRNAs' expression levels given its more stable expression (i.e., lowest SD values) compared to *GAPDH*. The interquartile range (IQR) method was applied to identify outliers in the normalised relative expression levels of the lncRNAs, which were further dismissed.

For each lncRNA, four profiles of expression were defined: profile A (low vs. high expression based on the median value of the normalised 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 a 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 lncRNAs' normalised relative expression levels as a categorical variable.

Associations of VTE incidence and the pre-chemotherapy levels of the lncRNAs with patient's demographic and clinicopathological factors (**Table 2**), baseline full blood count and coagulation tests (**Table 3**) were assessed using the Chi-Square test (χ^2), dismissing those with VTE before cancer diagnosis.

Chapter V - Table 3. Baseline full blood count and coagulation tests of OC patients (N = 40).

Baseline Full Blood Count		Baseline Coagulation Tests	
Variable *	N (%)	Variable *	N (%)
Haemoglobin levels (U/mL)		PT (s)	
<12.5	19 (47.5)	<14.3	17 (42.5)
≥12.5	18 (45.0)	≥14.3	18 (45.0)
Platelet count (×10⁹/L)		aPTT (s)	
<288	19 (47.5)	<26.9	17 (42.5)
≥288	18 (45.0)	≥26.9	17 (42.5)
Leukocyte count (×10⁹/L)		INR	
<8.2	19 (47.5)	<1.1	17 (42.5)
≥8.2	18 (45.0)	≥1.1	17 (42.5)
Neutrophil count (×10⁹/L)			
<5.1	19 (47.5)		
≥5.1	18 (45.0)		
Monocyte count (×10⁹/L)			
<0.6	17 (42.5)		
≥0.6	18 (45.0)		
Lymphocyte count (×10⁹/L)			
<1.6	17 (42.5)		
≥1.6	18 (45.0)		

* The categories were defined based on the median value since the variables were non-normally distributed (Shapiro–Wilk test, $p < 0.05$). For some patients, there was missing information, namely three for haemoglobin levels, platelet, leukocyte and neutrophil counts, five for monocyte and lymphocyte counts and PT, and six for aPTT and INR. Abbreviations: aPTT, activated partial thromboplastin time; INR, international normalised ratio; OC, ovarian cancer; PT, prothrombin time.

Depending on the data distribution according to the Shapiro–Wilk test, the Wilcoxon matched-pairs signed rank test or paired t-test was employed to evaluate the impact of first-line chemotherapy on the expression levels of the lncRNAs in PBCs. This analysis considered only patients with samples before and after the first-line chemotherapy.

The statistical differences in the pre-chemotherapy expression levels of the lncRNAs according to OC-related VTE occurrence were assessed using either Student's t-test or Mann–Whitney U test (for normal and non-normal distribution, respectively). For confirmation purposes, the Chi-Square test (χ^2) was also employed. Alive patients were characterised as VTE-free only if their follow-up time was at least 2 years without evidence

of thrombotic events. Thus, those with insufficient follow-up time were dismissed in analyses concerning VTE incidence.

To evaluate the impact of VTE and the lncRNAs on patients' prognosis, clinical outcome measures included PFS and 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 OC diagnosis until 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). The Kaplan–Meier method was employed to generate survival curves, while survival probabilities were analysed via the log-rank test. The impact of the evaluated lncRNAs on the risk of disease progression and death was assessed using the Cox proportional-hazards model, which was adjusted for the most relevant factors previously identified by applying the backward Wald method. In these analyses, those with VTE before OC diagnosis were dismissed.

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

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

5. Conclusions

OC stands out as the most lethal gynaecological malignancy. This condition has a close relationship with VTE, in which tumour cells hijack the haemostatic system to drive cancer progression [102]. Recently, lncRNAs have been implicated in VTE pathogenesis but their roles among cancer patients are yet to be explored [18]. As they might constitute valuable biomarkers for both CAT prediction and OC management, 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, these patients have a high pro-thrombotic risk and the VTE negatively impacts their survival. Among the evaluated lncRNAs, MEG8 in PBCs was a predictor of OC-related VTE, with its association with the baseline coagulation tests reflecting a possible role of this lncRNA in haemostatic abnormalities. Thus, MEG8 might hold 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, MEG8 was also demonstrated to impact the risk of OC progression regardless of VTE. Moreover, NEAT1 in PBCs was associated with the risk of death, emerging as a potential OC prognostic biomarker. While these preliminary findings are promising, they should be addressed carefully given the small cohort size. Further validation studies in larger cohorts are needed, preferably using a multicentric approach for more robust data. To our current understanding, this investigation is the first to propose lncRNAs in PBCs as potential predictors of OC-related VTE. These RNAs might enable a more focused screening and justifiable thromboprophylaxis for those at high risk, thereby safely reducing the occurrence of VTE events without adversities (e.g., bleeding disorders). Likewise, they could assess the prognosis among these patients in a non-invasive manner, helping tailor treatment strategies to improve their clinical outcomes. Further, performing single-cell RNA sequencing would be important to better understand which individual PBCs are responsible for expressing these lncRNAs. Additionally, it would be imperative to include control groups comprising healthy individuals and VTE subjects without cancer. Beyond PBCs, the expression of these lncRNAs in OC and normal ovarian tissues is important to offer deeper insights into their roles in different pathophysiological settings. Furthermore, functional studies should be conducted to dissect the biological processes underlying the role of these lncRNAs in OC-related VTE and OC progression, including their target proteins. In sum, more studies in this field and with more 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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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available, due to the privacy of participating patients and the hospital.

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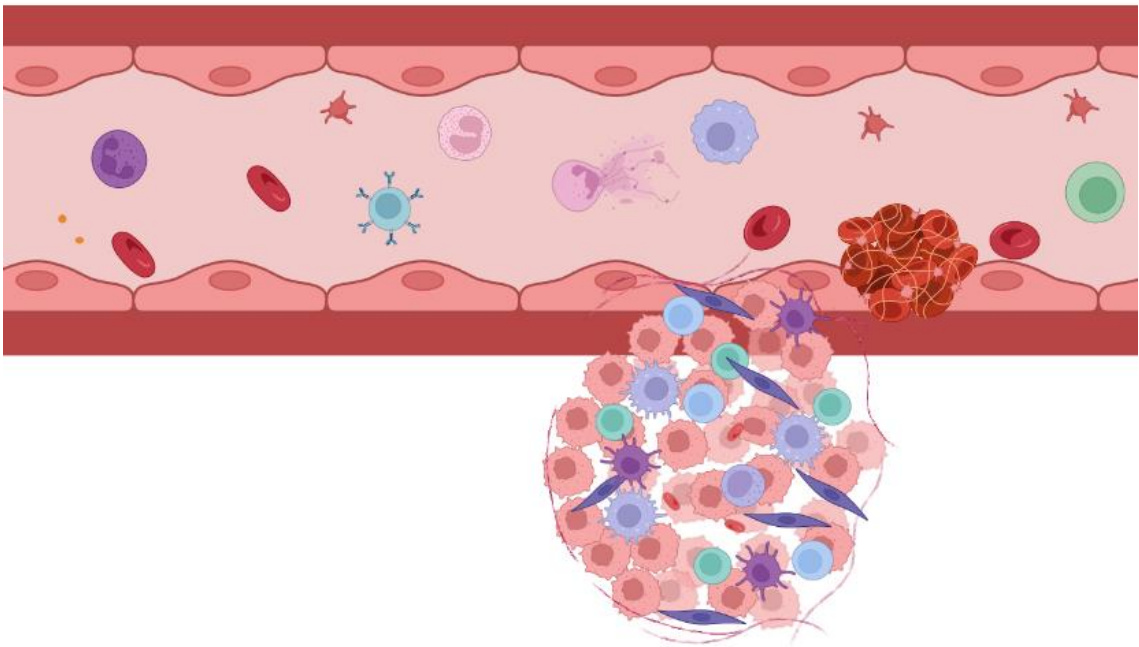
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Chapter VI



CHAPTER VI – Peripheral Blood MicroRNAs: Their Impact on Cancer-Associated Thrombosis and Ovarian Tumour Behaviour

This chapter includes the following paper:

Valéria Tavares, Joana Savva-Bordalo, Mariana Rei, Joana Liz-Pimenta, Joana Assis, Deolinda Pereira, Rui Medeiros. **Plasma microRNA environment linked to tissue factor pathway and cancer-associated thrombosis: prognostic significance in ovarian cancer.** *Biomolecules*. **2024**; 14 (8): 928. <https://doi.org/10.3390/biom14080928>. (Impact factor and quartile reported in 2023: **4.8** and **Q1**).

Plasma microRNA Environment Linked to Tissue Factor Pathway and Cancer Associated Thrombosis: Prognostic Significance in Ovarian Cancer

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Abstract: Ovarian cancer (OC) is a leading cause of death among gynaecological malignancies. The haemostatic system, which controls blood flow and prevents clotting disorders, paradoxically drives OC progression while increasing the risk of venous thromboembolism (VTE). MicroRNAs (miRNAs) have emerged as crucial in understanding VTE pathogenesis. Exploring the connection between cancer and thrombosis through these RNAs could lead to novel biomarkers of cancer-associated thrombosis (CAT) and OC, as well as potential therapeutic targets for tumour management. Thus, this study examined the impact of eight plasma miRNAs targeting the tissue factor (TF) coagulation pathway—miR-18a-5p, -19a-3p, -20a-5p, -23a-3p, -27a-3p, -103a-3p, -126-5p and -616-3p—in 55 OC patients. Briefly, VTE occurrence post-OC diagnosis was linked to shorter disease progression time (log-rank test, $p = 0.024$) and poorer overall survival (OS) (log-rank test, $p < 0.001$). High pre-chemotherapy levels of miR-20a-5p (targeting *coagulation factor 3 (F3)* and *tissue factor pathway inhibitor 2 (TFPI2)*) and miR-616-3p (targeting *TFPI2*) predicted VTE after OC diagnosis (χ^2 , $p < 0.05$). Regarding patients' prognosis regardless of VTE, miR-20a-5p independently predicted OC progression (adjusted hazard ratio (aHR) = 6.13, $p = 0.005$), while miR-616-3p significantly impacted patients' survival (aHR = 3.72, $p = 0.020$). Further investigation is warranted for their translation into clinical practice.

Keywords: ovarian neoplasms; thrombosis; prognosis; microRNAs; liquid biopsy

1. Introduction

In 2022, there were nearly 324,000 newly diagnosed cases of ovarian cancer (OC) worldwide, with approximately 206,000 related deaths. This malignancy stands as the eighth deadliest cancer among women, being the most lethal gynaecological tumour [1,2]. Furthermore, the prognosis of OC patients is worsened by the occurrence of cancer-associated thrombosis (CAT), particularly venous thromboembolism (VTE) [3,4].

In the tumour microenvironment, cancer cells interact with key haemostatic players, including endothelial cells, platelets and plasma proteins. In physiological conditions, these components prevent haemorrhage and thrombosis while ensuring endothelium integrity [5]. However, their complex interplay with cancer cells and leukocytes can disrupt the haemostatic balance, augmenting the thrombogenic and bleeding potential. In parallel, haemostatic abnormalities can fuel tumour neo-angiogenesis, cancer-promoted inflammation, immune escape and metastasis [5,6]. Indeed, a two-way association between cancer and VTE, known as the Trousseau syndrome, is well-characterised [4]. Regarding OC patients, they have an incidence of VTE events, primarily deep venous thrombosis

(DVT) and pulmonary embolism (PE), of approximately 20%, placing them among those most affected by CAT [3].

MicroRNAs (miRNAs) are single-stranded and highly conserved non-coding RNAs with 18 to 25 nucleotides that control gene expression at a post-transcriptional level. They primarily bind to the 3' UTR of target messenger RNAs (mRNAs), leading to translation inhibition and potential mRNA degradation [5]. A single miRNA can target the expression of several genes, with a wide range of biological implications. Conversely, a single gene can be the target of multiple miRNAs [7,8]. Given their roles and characteristics, miRNAs have emerged as attractive disease biomarkers. Their high sensitivity and elevated stability in biofluids, even with pH and temperature variations, freeze-thaw cycles and RNase treatment, make them particularly attractive for liquid biopsy applications [5]. In addition, their ability to target several biological pathways puts them at the forefront of non-coding RNA-based therapeutics [7].

Like other biological processes, haemostasis is controlled by miRNAs, which regulate the expression of plasma proteins and the activity of platelets, endothelial cells and leucocytes. Consistently, serum/plasma miRNAs have been implicated in VTE pathogenesis in both the general population and cancer patients. Several of them target essential members of the tissue factor (TF) pathway (also known as the extrinsic coagulation pathway): TF, tissue factor pathway inhibitor 1 (TFPI1 or simply TFPI) and tissue factor pathway inhibitor 2 (TFPI2) [5]. Overexpression of TF (coagulation factor III) is documented in various solid tumours, including OC, promoting both venous thrombogenesis and ovarian tumourigenesis [9–14]. The anticoagulant TFPI1 is the main inhibitor of the TF pathway, while TFPI2 can additionally act as a plasmin inhibitor, regulating fibrinolysis and the remodelling of endothelial cell matrix (ECM) [5]. TFPI1 and TFPI2 are deemed tumour suppressors, yet their role can vary depending on the cancer type and its specific molecular environment [5,15,16]. Considering the involvement of miRNAs in the Trousseau syndrome, this study explored the impact of peripheral blood-based miRNAs (i.e., circulating miRNAs) on CAT occurrence and patients' prognosis (regardless of VTE).

2. Materials and Methods

2.1. Patients

This retrospective cohort study is part of a broader research protocol exploring CAT biomarkers and their implications in OC patients [17,18]. The study included adult Caucasian patients with confirmed epithelial ovarian carcinoma (EOC) admitted at the Clinic of Gynaecology of the Portuguese Oncology Institute of Porto (IPO Porto) between March

2017 and September 2023 for initial treatment. The treatment protocol included cytoreductive surgery and platinum-based chemotherapy. The number of cycles varied based on the treatment approach (neoadjuvant, adjuvant or as a standalone therapy for patients who were ineligible for surgery). Exclusion criteria encompassed: (1) a history of previous and concurrent tumours, (2) immunosuppression and/or autoimmune disorders, (3) acute infections upon OC diagnosis, (4) pregnancy or postpartum status (up to six weeks after childbirth) at diagnosis, (5) anticoagulation therapy unrelated to VTE, (6) presence of relevant VTE-related genetic polymorphisms, specifically prothrombin G20210A or factor V Leiden and (7) refusal to provide informed consent. A total of 55 OC patients, whose biological samples were stored in the institutional biobank, were included in the study. Their demographical and clinical factors were revised using electronic medical records (**Table 1**). Each patient signed a written informed consent following the principles of the Helsinki Declaration. The ethics committee at the research centre of IPO Porto (CI-IPO Porto) approved the study (CES. 69/021).

Chapter VI - Table 1. Demographic and clinicopathological data of OC patients (N = 55).

Variable	N (%)
Age at OC diagnosis (years) †	63.8 ± 11.9
≥64	31 (56.4)
Hormonal status at OC diagnosis	
Post-menopausal	43 (78.2)
Baseline BMI (kg/m²) †	26.6 ± 4.7
≥27.0	22 (40.0)
ECOG PS at OC diagnosis	
>1	7 (12.7)
OC-related germline mutations	6 (10.9)
Histology	
Serous	46 (83.6)
Clear cell	3 (5.5)
Endometrioid	2 (3.6)
Mixed	2 (3.6)
Unusual	2 (3.6)
Histological grade	
High	51 (92.7)
FIGO stage §	
I/II	11 (20.0)
III/IV	44 (80.0)
Baseline CA-125 levels (U/mL) ‡	
≥913.0	27 (49.1)
Baseline aPTT (s) ‡	
≥27.2	24 (43.6)
Baseline PT (s) ‡	
≥14.2	22 (40.0)
Baseline INR ‡	
≥1.06	23 (41.8)
KS ‡‡	
≥2	21 (38.2)
Anticoagulation therapy ††	3 (5.5)
Platelet anti-aggregation therapy	7 (12.7)
First-line treatment	
Surgery and adjuvant chemotherapy	24 (43.6)
Neoadjuvant chemotherapy, surgery and adjuvant chemotherapy	15 (27.3)
Chemotherapy only	12 (21.8)
Neoadjuvant chemotherapy and surgery	4 (7.3)
Platinum sensitivity §§	39 (70.9)
Maintenance therapy	
PARP inhibitors	17 (30.9)
bevacizumab	9 (16.4)

† Given the normal distribution, the categories were defined based on the mean value. Age at OC diagnosis and baseline BMI were also presented as mean ± standard deviation. § Based on FIGO Cancer Report 2021 [19]. ‡ Since the variables were non-normally distributed, the categories were defined based on the median value. §§ Defined as OC progression after six months following the completion of first-line platinum-based chemotherapy [3]. ‡‡ Determined for patients with available data using these parameters: the cancer site, baseline haemoglobin levels, BMI and platelet and leukocyte count. The cut-off of two points was considered [20]. †† Patients with cancer-associated thrombosis before OC diagnosis. Certain patients lacked specific information: eleven had missing data for aPTT, ten for PT and INR, five for platinum sensitivity, two for OC-related inherited mutations and histological grade, and one for BMI and CA-125 levels. Abbreviations: aPTT, activated partial thromboplastin time; BMI, body mass index; CA-125, cancer antigen 125; ECOG PS; Eastern

Cooperative Oncology Group Performance Status; FIGO, International Federation of Gynecology and Obstetrics; INR, international normalised ratio; KS, Khorana score; N, number of patients; OC, ovarian cancer; PT, prothrombin time.

CAT was defined as an event occurring within the period of six months before to two years after the cancer diagnosis [18]. Active screening for OC-related VTE was not performed as it is not part of the standard clinical procedures at IPO Porto. The median follow-up time was 27.0 months (minimum = 1.0 months; maximum = 85.0 months).

2.2. miRNA Selection

A comprehensive review of all miRNAs associated with VTE in the general population and among cancer patients was conducted to identify the most suitable miRNAs to be evaluated [5]. From the initial list, only those targeting *coagulation factor 3 (F3)*, *TFPI1* and *TFPI2* were considered, given their pivotal role in CAT. Furthermore, miRNAs evaluated in plasma samples and with more evidence (i.e., reported in several studies) were prioritised. Data from studies on other pathophysiological conditions were gathered from the literature to explore and confirm the specific miRNA–mRNA interactions. Additionally, several miRNA databases were utilised, including the latest versions of TargetScanHuman (v8), miRDB (v6), miRmap (v1.1), miRWalk (v3), DIANA-TarBase (v9) and miRTarBase (v9.0), with all resources last accessed on 6 March 2024. Based on these criteria, hsa-miR-18a-5p, hsa-miR-19a-3p, hsa-miR-20a-5p, hsa-miR-23a-3p, hsa-miR-27a-3p, hsa-miR-103a-3p, hsa-miR-126-5p and hsa-miR-616-3p were selected (**Table 2**).

Chapter VI - Table 2. Selected miRNAs and their targets.

miRNAs	Target Gene	miRNA–mRNA Interaction on Physio-Pathological Settings	Predicted miRNA–mRNA Interactions				Experimentally Validated miRNA–mRNA Interactions *		VTE **
			TargetScanHuman	MiRDB	miRmap	miRwalk	DIANA-TarBase	miRTarBase	
			Site Type (Context++ Score Percentile)	Target Score	miRmap Score	Score (Region)	MicroT Score	Level of Evidence	
miR-18a-5p	<i>F3</i>	-	7mer-m8 (98)	51	98.28	0.92 (3' UTR)	0.71	Less strong	-
miR-19a-3p	<i>F3</i>	[21–27]	8mer (98)	92	85.15	-	0.97	-	B [28]
miR-20a-5p	<i>F3</i>	[24,29,30]	8mer (99)	95	84.88	-	0.25	Less strong	-
	<i>TFPI2</i>	-	7mer-A1 (88)	-	87.88	-	0.74	-	
miR-23a-3p	<i>TFPI2</i>	[31]	7mer-m8 (92)	-	52.23	0.92 (CDS)	0.75	-	B [32]
miR-27a-3p	<i>F3</i>	-	7mer-m8 (92)	57	79.20	0.85 (3' UTR)	0.83	-	A [33]
	<i>TFPI1</i>	[34,35]	8mer (97)	72	84.00	0.92 (3' UTR)	0.96	-	
miR-103a-3p	<i>TFPI2</i>	-	-	-	52.23	1.00 (CDS)	0.72	-	C [28,36,37]
miR-126-5p	<i>F3</i>	[22,25]	-	-	-	-	-	-	C [32,38,39]
miR-616-3p	<i>TFPI2</i>	[40,41]	7mer-m8 (96)	-	48.40	-	-	Strong	-

* When available, the MicroT Score from DIANA-TarBase and the level of evidence from miRTarBase were provided for *F3*, *TFPI1* and *TFPI2*. ** A: VTE in the general population; B: VTE in cancer patients; C: VTE in the general population and among cancer patients. Abbreviations: CDS, coding sequence; miRNA, microRNA; mRNA, messenger RNA; UTR, untranslated region; VTE, venous thromboembolism.

2.3. Blood Sample Collection and Processing

Peripheral blood samples were obtained from each patient before initiating the first-line chemotherapy (baseline) using a standard venipuncture method. The collected samples, stored in EDTA-coated tubes, underwent centrifugation at room temperature for 5 min at 3000 rpm to isolate the plasma fraction. The prepared plasma samples were kept at –80 °C until needed.

2.4. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from plasma samples using the MagMAX™ mirVana™ Total RNA Isolation Kit (CAT A27828, Thermo Fisher Scientific, Waltham, MA, USA) in a KingFisher™ Duo Prime Magnetic Particle Processor (Thermo Fisher Scientific, Waltham,

MA, USA). RNA purity and concentration were evaluated using the NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Following extraction, RNA samples were stored at -80°C until use.

RNA samples (50 ng) were reversely transcribed into complementary DNA (cDNA) using the TaqMan™ MicroRNA Reverse Transcription Kit (CAT 4366596, Thermo Fisher Scientific, Carlsbad, CA, USA) and the sequence-specific stem-loop reverse transcription primers for hsa-miR-18a-5p (assay ID 002422), hsa-miR-19a-3p (assay ID 000395), hsa-miR-20a-5p (assay ID 000580), hsa-miR-23a-3p (assay ID 000399), hsa-miR-27a-3p (assay ID 000408), hsa-miR-103a-3p (assay ID 000439), hsa-miR-126-5p (assay ID 000451), hsa-miR-616-3p (assay ID 002414), U6 snRNA (assay ID 001973), hsa-miR-1228-3p (assay ID 002919) and hsa-miR-451a (assay ID 001141), following the manufacturer's instructions. The conversion was performed in a Mycycler™ Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycle conditions: 30 min at 16°C , 60 min at 42°C and 10 min at 85°C . To evaluate potential contaminations, controls devoid of RNA were included in all conversion reactions.

2.5. Relative Quantification of miRNAs

The miRNA expression was quantified via real-time polymerase chain reaction (RT-PCR) in a StepOnePlus™ qPCR system (Applied Biosystems®, Foster City, CA, USA). Each PCR reaction was conducted using 5.0 μL of 2× TaqMan™ Fast Advanced Master Mix (Applied Biosystems®, Foster City, CA, USA), 2.5 μL of nuclease-free water, 0.5 μL of 20× specific TaqMan™ MicroRNA Assays for hsa-miR-18a-5p (assay ID 002422), hsa-miR-19a-3p (assay ID 000395), hsa-miR-20a-5p (assay ID 000580), hsa-miR-23a-3p (assay ID 000399), hsa-miR-27a-3p (assay ID 000408), hsa-miR-103a-3p (assay ID 000439), hsa-miR-126-5p (assay ID 000451), hsa-miR-616-3p (assay ID 002414), U6 snRNA (assay ID 001973), hsa-miR-1228-3p (assay ID 002919) and hsa-miR-451a (assay ID 001141) and 2.0 μL of cDNA sample, in total volume of 10 μL . According to previous studies assessing miRNA expression in plasma samples, U6 snRNA and miR-1228-3p were tested as housekeeping genes (endogenous controls) [42,43]. In addition to being evaluated as a VTE-related miRNA, miR-23a-3p (recognised for its stability in the presence of haemolysis) together with miR-451a (highly expressed in erythrocytes) were employed to assess potential haemolysis. A ΔCq (hsa-miR-23a-3p–hsa-miR-451a) value ≥ 8 indicates a high likelihood of haemolysis [44–46]. Since none of the samples exhibited a ratio exceeding 7, all of them were included in the analysis. The PCR conditions were as follows: 2 min at 50°C , 10 min at 95°C , 45 cycles of 15 s at 95°C and 60 s at 60°C . Measures of quality control and the methods for reliable generation of cycle threshold (Ct) values were applied as previously described [17,18].

2.6. Statistical Analysis

Statistical analysis and graphing were conducted using IBM SPSS Statistics for Windows (version 29, IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 9.0.0, GraphPad Software Inc., La Jolla, CA, USA), respectively.

Among the tested endogenous controls, miR-1228-3p exhibited a more consistent expression, as indicated by the lowest standard deviation values. The Livak method was employed to normalise the miRNAs' expression levels using this miRNA as an endogenous control.

Severe outliers in miRNA normalised relative expression levels were identified with the interquartile range (IQR) method and subsequently excluded. Four expression profiles were established to assess miRNA expression as a nominal variable, as previously defined [17,18].

Data normality was evaluated using the Shapiro–Wilk or Kolmogorov–Smirnov tests depending on the cohort size ($N \leq 50$ and $N > 50$, respectively). Based on the distribution, either Spearman's rank correlation coefficient test or Pearson's correlation coefficient test was computed to study the relationship between the miRNAs' expression. Results were considered relevant if $p < 0.05$ and the coefficient was ≥ 0.500 .

VTE-free patients included those who died without experiencing CAT or remained without an event during a two-year follow-up period. Thus, live patients with less than two years of follow-up were excluded in analyses involving VTE implications. Associations of VTE occurrence and miRNAs' expression levels with patients' characteristics (**Table 1**) were evaluated using the Chi-squared test (χ^2), excluding those who had CAT before OC diagnosis. In the analysis concerning the miRNAs, stratified evaluations according to the first treatment (surgery vs. chemotherapy) were also performed. Statistical differences in the miRNAs' expression levels according to CAT status were analysed employing the Kruskal–Wallis test or one-way ANOVA followed by Dunnett's test, depending on data normality. Patients who experienced a VTE event before and after being diagnosed with OC were compared, respectively, with their VTE-free counterparts. χ^2 was employed for confirmation.

Two clinical outcome measures, namely progression-free survival (PFS) and overall survival (OS), were utilised in the study. These measures were previously defined [17,18]. The impact of VTE occurrence and miRNA expression on PFS and OS was assessed using the Kaplan–Meier method and log-rank test. Regarding the implications of miRNA expression, the most appropriate profile was appointed for each miRNA after an initial assessment of the survival curves. Additionally, the influence of miRNA expression on the risk of OC progression and mortality was evaluated using the Cox model. Multivariate Cox analyses were conducted for all the miRNAs, adjusting for the most relevant patients'

characteristics. These were identified by applying the backward Wald method. Only factors with prognostic value according to univariate Cox analyses were included in this analysis. Patients with VTE before their OC diagnosis were excluded from these analyses. Harrell's concordance (C)-index was used to compare the predictive ability of the proposed models, with a value greater than 0.5 indicating a good predictive ability.

In the study, a significance level of 5% was set. Additionally, *p*-values falling within the range of 0.05 to 0.06 were considered marginally significant.

2.7. *In Silico* Analysis

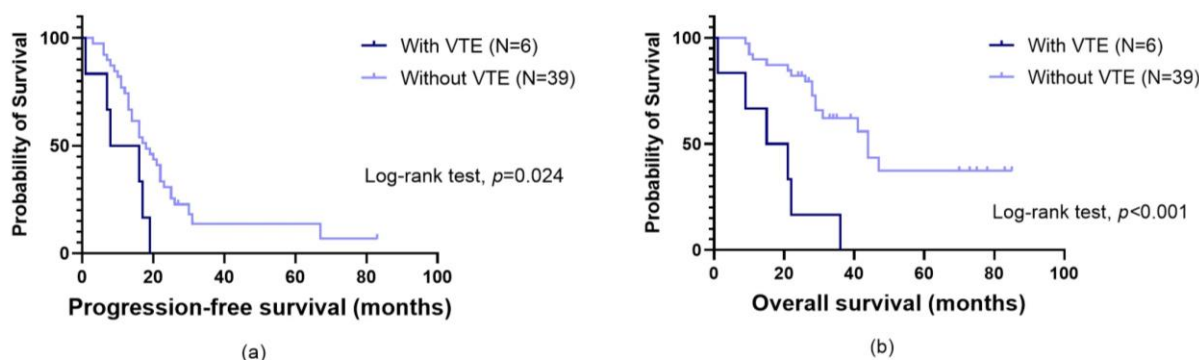
In silico analyses were conducted to explore the biological implications of the relevant miRNAs. The miRTargetLink 2.0 database and the STRINGapp Protein Query from Cytoscape 3.10.2 were employed to identify miRNA targets and generate a protein-protein interaction (PPI) network for each miRNA, respectively [42]. Markov clustering (MCL) was applied to cluster proteins based on their STRING interaction scores. Subsequently, a functional enrichment analysis was performed, eliminating redundant terms with a cut-off of 0.5. The top 20 enriched terms with a false discovery rate (FDR) < 0.05 for Gene Ontology (GO) categories, Diseases, KEGG and Reactome pathways were provided for each relevant miRNA.

3. Results

3.1. Impact of VTE on Patients' Prognosis

Among those with a two-year follow-up (N = 48), nine (18.8%) had OC-related VTE, including seven DVT and two PE, with the majority being symptomatic (N = 6 (66.7%)). Six VTE patients (66.7%) were treated with low-molecular-weight heparin (LMWH), two (22.2%) with direct oral anticoagulants (DOACs) and one (11.1%) with vitamin K antagonists (VKAs). Those with thrombotic events previous to cancer diagnosis (N = 3) exhibited a median period between VTE and OC diagnosis of 2.0 months (minimum = 0; maximum = 5 months). Regarding patients with VTE after OC detection (N = 6), the median time to thrombogenesis was 4.5 months (minimum = 1; maximum = 24 months). Notably, except for one patient treated with DOAC, those in this latter subgroup were treated with LMWH. Thrombotic events did not impact patients' PFS (log-rank test, *p* = 0.113), although they significantly influenced patients' survival (log-rank test, *p* < 0.001). Specifically, those with VTE had lower OS compared to those unaffected (mean OS of 22.3 ± 4.4 months and 50.8 ± 5.6 months, respectively). Upon excluding patients with VTE before OC diagnosis, a notable association

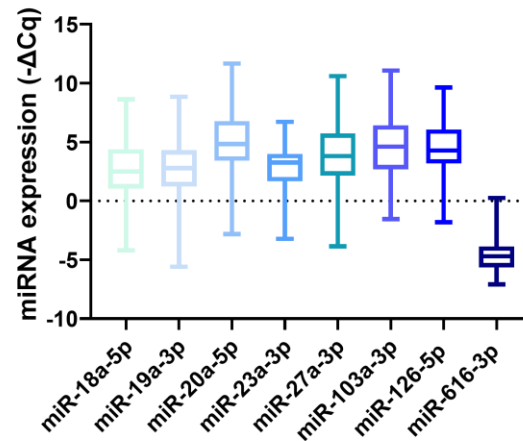
between thrombotic events and PFS emerged (log-rank test, $p = 0.024$; **Figure 1a**). In the same subgroup, the negative influence of VTE on OS was sustained (log-rank test, $p < 0.001$; **Figure 1b**). No patients' characteristics (**Table 1**) were found to be predictors of OC-related VTE (χ^2 , $p > 0.05$).



Chapter VI - Figure 1. Progression-free survival (PFS) (a) and overall survival (OS) (b) by Kaplan–Meier and log-rank test among ovarian cancer (OC) patients, according to venous thromboembolism (VTE) occurrence after tumour diagnosis. (a) Patients with VTE had a lower PFS than their counterparts (mean PFS of 11.3 ± 2.9 months and 24.9 ± 3.8 months, respectively; log-rank test, $p = 0.024$). **(b)** Likewise, they also exhibited a lower OS (mean OS of 17.3 ± 4.9 months and 50.8 ± 5.6 months, respectively; log-rank test, $p < 0.001$). The VTE patients were treated with low-molecular-weight heparin (LMWH), except for one patient who received direct oral anticoagulants (DOACs).

3.2. Correlation between Baseline miRNA Expression

Except for miR-616-3p, miRNAs' expression levels were positively correlated (Spearman's $\rho \geq 0.50$ and $p < 0.05$; **Figure 2**).



(a)

		miR-18a-5p	miR-19a-3p	miR-20a-5p	miR-23a-3p	miR-27a-3p	miR-103a-3p	miR-126-5p	miR-616-3p
miR-18a-5p	Spearman's ρ	-	0.893	0.960	0.672	0.844	0.911	0.815	0.314
	<i>p</i> -Value	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.021
miR-19a-3p	Spearman's ρ	0.893	-	0.871	0.512	0.742	0.806	0.825	0.443
	<i>p</i> -Value	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
miR-20a-5p	Spearman's ρ	0.960	0.871	-	0.695	0.851	0.911	0.792	0.301
	<i>p</i> -Value	<0.001	<0.001	-	<0.001	<0.001	<0.001	<0.001	0.027
miR-23a-3p	Spearman's ρ	0.672	0.512	0.695	-	0.829	0.800	0.661	0.025
	<i>p</i> -Value	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	0.859
miR-27a-3p	Spearman's ρ	0.844	0.742	0.851	0.829	-	0.906	0.808	0.315
	<i>p</i> -Value	<0.01	<0.001	<0.001	<0.001	-	<0.001	<0.001	0.021
miR-103a-3p	Spearman's ρ	0.911	0.806	0.911	0.800	0.906	-	0.826	0.307
	<i>p</i> -Value	<0.001	<0.001	<0.001	<0.001	<0.001	-	<0.001	0.024
miR-126-5p	Spearman's ρ	0.815	0.825	0.792	0.661	0.808	0.826	-	0.336
	<i>p</i> -Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-	0.013
miR-616-3p	Spearman's ρ	0.314	0.443	0.301	0.025	0.315	0.307	0.336	-
	<i>p</i> -Value	0.021	<0.001	0.027	0.859	0.021	0.024	0.013	-

Moderate correlation
 Strong correlation
 Very strong correlation

(b)

Chapter VI - Figure 2. Normalised relative expression of plasma miRNAs ($-\Delta Cq$) in ovarian cancer (OC) patients preceding first-line chemotherapy (a) and the correlation between the baseline expression levels of these miRNAs according to Spearman's rank correlation coefficient test (b). The strength of the correlations (Spearman's $\rho \geq 0.500$ and $p < 0.05$) was classified as follows: moderate (Spearman's $\rho < 0.700$), strong ($0.700 \leq \text{Spearman's } \rho < 0.900$), and very strong (Spearman's $\rho \geq 0.900$).

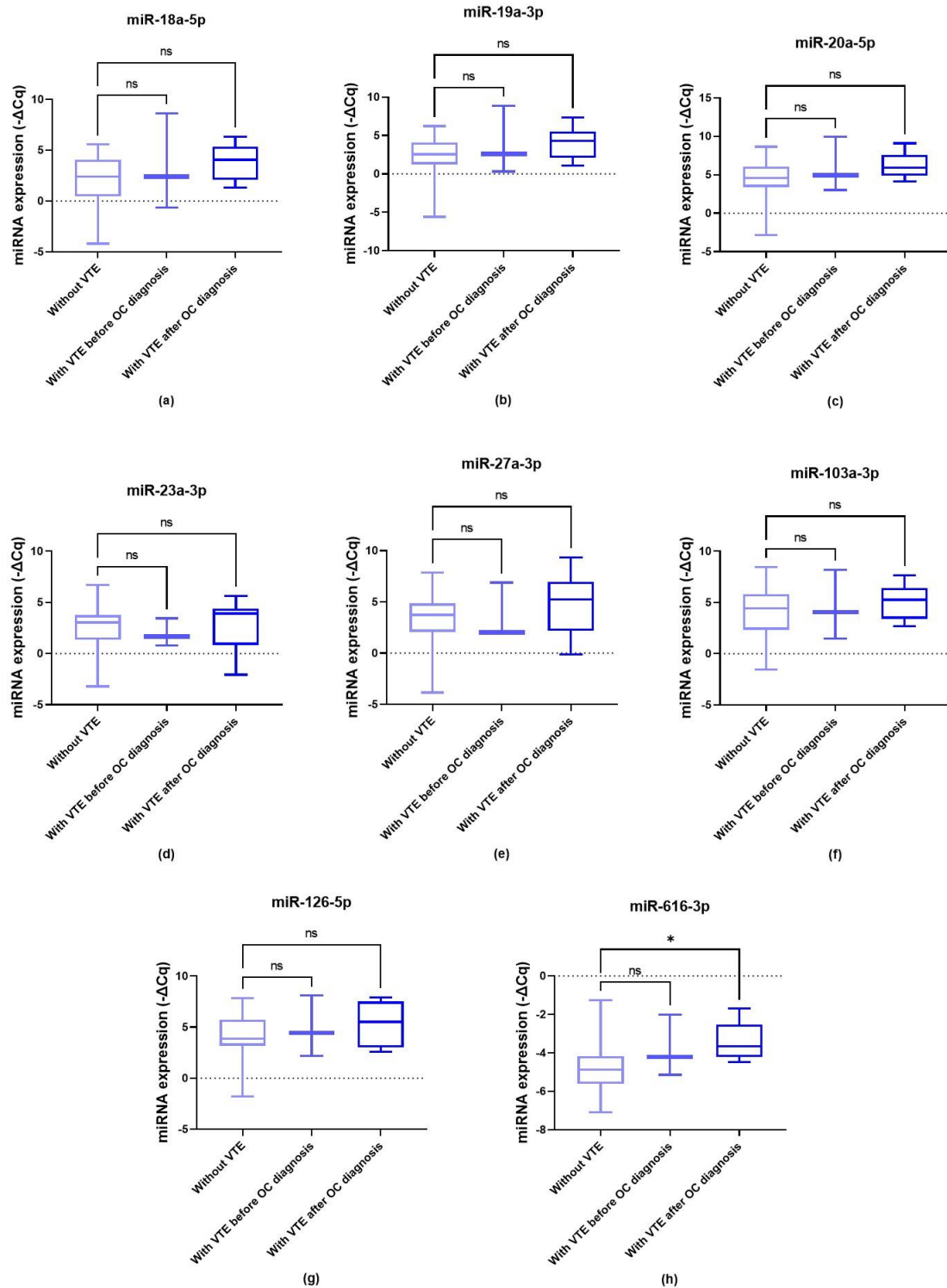
3.3. Baseline miRNA Levels and Patients' Characteristics

Concerning miR-18a-5p, patients with an Eastern Cooperative Oncology Group Performance Status (ECOG PS) ≤ 1 tended to exhibit high miRNA levels more frequently (profile 2, χ^2 , $p = 0.047$; profile 3, χ^2 , $p = 0.031$). Patients with lower cancer antigen 125 (CA-125) baseline levels (<913.0 U/mL) often showed intermediate-to-high miRNA levels

(profile 2, χ^2 , $p = 0.055$). Regarding miR-20a-5p, those with an ECOG PS ≤ 1 commonly displayed high miRNA levels (profile 2, χ^2 , $p = 0.047$; profile 3, χ^2 , $p = 0.031$). Patients at the International Federation of Gynecology and Obstetrics (FIGO) III/IV stages more frequently exhibited elevated miR-23a-3p levels (profile 2, χ^2 , $p = 0.024$; profile 4, χ^2 , $p = 0.022$). The same trend was noted for individuals with lower CA-125 baseline levels (profile 1, χ^2 , $p = 0.035$; profile 2, χ^2 , $p = 0.042$; profile 3, χ^2 , $p = 0.031$). Concerning miR-27a-3p, patients at III/IV stages also commonly showed high levels of this miRNA (profile 2, χ^2 , $p = 0.028$; profile 4, χ^2 , $p = 0.022$). Elevated miR-103a-3p levels were predominantly observed among patients with lower CA-125 baseline levels (profile 2, χ^2 , $p = 0.034$; profile 4, χ^2 , $p = 0.023$). High miR-616-3p levels were more frequently found among those with Khorana score (KS) ≥ 2 (profile 3, χ^2 , $p = 0.044$). As for miR-19a-3p and miR-126-5p, no significant association with patients' characteristics was detected. It is worth noting that the first treatment (surgery vs. chemotherapy) had no significant impact on the baseline miRNA levels.

3.4. Baseline miRNA Levels and OC-Related VTE Susceptibility

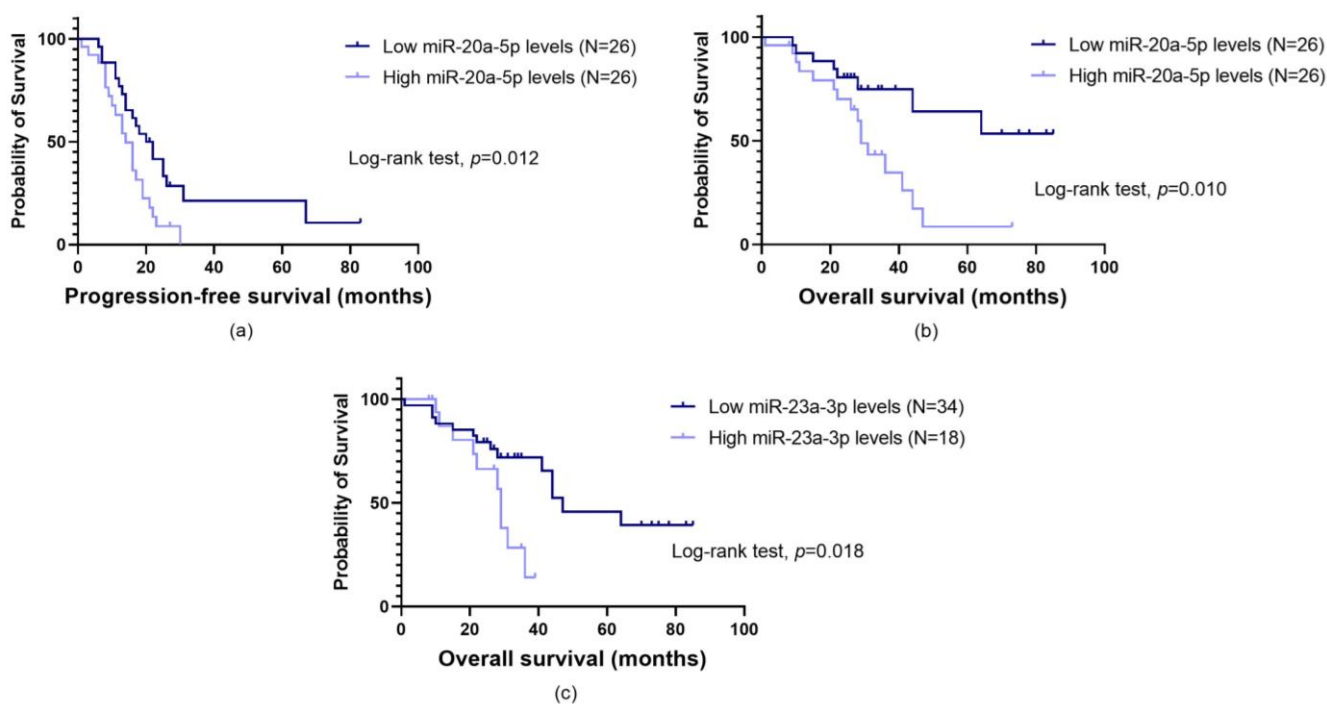
Except for miR-616-3p, there were no associations between the miRNAs and VTE development according to the Kruskal–Wallis test (**Figure 3a–g**, $p > 0.05$). As for miR-616-3p, significantly elevated levels were observed among those who would later develop VTE compared to VTE-free patients (Kruskal–Wallis test, $p = 0.022$; **Figure 3h**). Regarding the analysis with χ^2 , the predictive value of miR-616-3p was confirmed (profile 1, $p = 0.048$; profile 2, $p = 0.017$; profile 3, $p = 0.029$). Furthermore, miR-20a-5p levels were significantly elevated in patients with VTE after OC diagnosis compared to VTE-free subjects (profile 2, χ^2 , $p = 0.034$). Considering the expression profile 4 of this miRNA, the likelihood test indicated a p -value of 0.021, reflecting a potentially significant finding (χ^2 , $p < 0.05$) if the cohort size was larger.



Chapter VI - Figure 3. Normalised relative expression of plasma miRNAs (–ΔCq) in ovarian cancer (OC) patients preceding first-line chemotherapy, according to venous thromboembolism (VTE) status. (a) miR-18a-5p; (b) miR-19a-3p; (c) miR-20a-5p; (d) miR-23a-3p; (e) miR-27a-3p; (f) miR-103a-3p; (g) miR-126-5p; and (h) miR-616-3p; Kruskal–Wallis test, * $p < 0.05$; ns, non-significant.

3.5. Impact of Baseline miRNA Levels on Patients' Prognosis

A significant association between miR-20a-5p expression and PFS was observed. Namely, patients with high miRNA levels had a lower time to disease progression than their counterparts (profile 1, log-rank test, $p = 0.012$; **Figure 4a**). This result was confirmed with univariate Cox regression analysis (profile 1, hazard ratio (HR) = 2.17, 95% confidence interval (CI), 1.15–4.12, $p = 0.017$). As for OS, significant associations were detected for miR-20a-5p and miR-23a-3p. Those with high miR-20a-5p levels had a lower survival time (profile 1, log-rank test, $p = 0.010$; **Figure 4b**). These patients presented almost a three-fold increase in the risk of death (profile 1, HR = 2.92, 95%CI, 1.23–6.93, $p = 0.015$). Likewise, elevated miR-23a-3p levels were associated with a decreased OS (profile 3, log-rank test, $p = 0.018$; **Figure 4c**), which was confirmed by Cox regression analysis (profile 3, HR = 2.83, 95%CI, 1.14–7.00, $p = 0.025$).



Chapter VI - Figure 4. Progression-free survival (PFS) (a) and overall survival (OS) (b, c) by Kaplan–Meier and log-rank test in a cohort of ovarian cancer (OC) patients, according to the baseline expression levels of plasma miRNAs. (a) Patients with high miR-20a-5p levels had a lower PFS than their counterparts (mean PFS of 14.8 ± 1.5 months and 29.8 ± 5.4 months, respectively, log-rank test, $p = 0.012$). (b) These patients also had a lower OS (mean OS of 32.5 ± 4.1 months and 61.6 ± 6.7 months, respectively, log-rank test, $p = 0.010$). (c) Likewise, those with elevated miR-23a-3p levels had a lower survival time (mean OS of 27.1 ± 2.4 months and 53.7 ± 5.9 months, respectively, log-rank test, $p = 0.018$).

Stratified analysis based on the patient's baseline characteristics—age (≥ 64 vs. < 64 years), ECOG PS (> 1 vs. ≤ 1), FIGO stage (III/IV vs. I/II), CA-125 levels (≥ 913.0 vs. < 913.0 U/mL) and KS (≥ 2 vs. < 2)—were conducted. All the miRNAs had a detrimental impact on PFS and/or OS when accessed at a specific subgroup (only the subgroups with significant results are shown). Among patients at early stages, a lower PFS was observed for those with high levels of miR-18a-5p (profile 3), miR-20a-5p (profile 1), miR-23a-3p (profile 3) and miR-27a-3p (profile 3) (log-rank test, $p = 0.010$, $p = 0.010$, $p < 0.001$ and $p < 0.001$, respectively). In the subgroup with lower CA-125 levels, a shorter PFS was experienced by those with high levels of miR-20a-5p (profile 1), miR-23a-3p (profile 4) and miR-27a-3p (profile 4) (log-rank test, $p = 0.034$, $p = 0.017$ and $p = 0.028$, respectively). The same was observed for those with high miR-23a-3p levels in the KS ≥ 2 subgroup (profile 4, log-rank test, $p = 0.012$).

Among the younger patients, a lower OS was observed for those with elevated levels of miR-18a-5p (profile 3), miR-19a-3p (profile 3), miR-20a-5p (profile 1), miR-23a-3p (profile 3), miR-27a-3p (profile 3), miR-103a-3p (profile 3), miR-126-5p (profile 3) and miR-616-3p (profile 3) (log-rank test, $p = 0.001$, $p < 0.001$, $p = 0.020$, $p = 0.002$, $p = 0.019$, $p < 0.001$, $p = 0.001$ and $p = 0.011$, respectively). In the subgroup with ECOG PS ≤ 1 , OS was lower in patients with high levels of miR-20a-5p (profile 1) and miR-23a-3p (profile 3) (log-rank test, $p = 0.031$ and $p = 0.036$, respectively). Among patients at early cancer stages, those with high levels of miR-18a-5p (profile 3), miR-19a-3p (profile 3), miR-20a-5p (profile 1), miR-23a-3p (profile 4) and miR-27a-3p (profile 4) exhibited a lower survival time (log-rank test, $p = 0.011$, $p = 0.045$, $p = 0.011$, $p = 0.045$ and $p = 0.045$, respectively). In the subgroup with lower CA-125 levels, patients with high levels of miR-20a-5p had a lower OS (profile 1, log-rank test, $p = 0.031$). The same was seen in the KS < 2 subgroup for those with elevated levels of miR-20a-5p (profile 1) and miR-616-3p (profile 3) (log-rank test, $p = 0.030$ and $p = 0.008$, respectively).

Multivariate Cox analyses were performed for all the miRNAs, adjusting for relevant demographic and clinicopathological factors. Significant predictors of disease progression included patients' age (≥ 64 years, HR = 1.98, 95%CI, 1.05–3.76, $p = 0.036$), FIGO stage (III/IV, HR = 2.71, 95%CI, 1.12–6.60, $p = 0.028$), first treatment (surgery, HR = 0.33, 95%CI, 0.17–0.64, $p = 0.001$) and surgery (yes, HR = 0.13, 95%CI, 0.06–0.30, $p < 0.001$). In a multivariate Cox analysis applying the backward Wald method, surgery (yes vs. no) was the most relevant predictor of disease progression (clinic model A1). This variable was defined as having or not having the intervention during the patient's first-line treatment. Regarding the risk of death, patients' age (≥ 64 years, HR = 6.12, 95%CI, 2.20–16.98, $p < 0.001$), hormonal status (post-menopause, HR = 5.66, 95%CI, 1.31–24.47, $p = 0.020$), surgery (yes, HR = 0.14, 95%CI, 0.05–0.37, $p < 0.001$) and platinum sensitivity (no, HR = 5.49,

95%CI, 2.68–11.24, $p < 0.001$) had a significant predictive value. Among these factors, patients' age, surgery and platinum sensitivity were the most relevant predictors of mortality (clinic model B1). Furthermore, by focusing on factors with available data at the start of the patient's treatment (i.e., dismissing surgery and platinum sensitivity), primary treatment (clinic model A2) and patients' age (clinic model B2) emerged as the most informative factors concerning the risk of disease progression and death, respectively. Considering these models, multivariate Cox analyses, including the miRNAs (integrative models), were conducted (**Table 3**). The C-index was computed to compare the predictive ability of the models for disease progression and death. The integrative models (including data on the baseline miRNA levels) outperformed their clinical counterparts in predictive capacity. Integrative model A1, the top predictor for disease progression, exhibited a 5% improvement in predictive ability over its clinical counterpart (c-index, 0.952 vs. 0.902; **Table 3**). Notably, integrative model A2 demonstrated a 34% increase in predicting disease progression risk compared to its clinical counterpart (c-index, 0.898 vs. 0.671; **Table 3**). For predicting the risk of death, the best model—integrative model B1—showed a 3% enhancement (c-index, 0.848 vs. 0.821; **Table 3**).

Chapter VI - Table 3. Multivariable Cox regression analysis on the risk of disease progression and mortality.

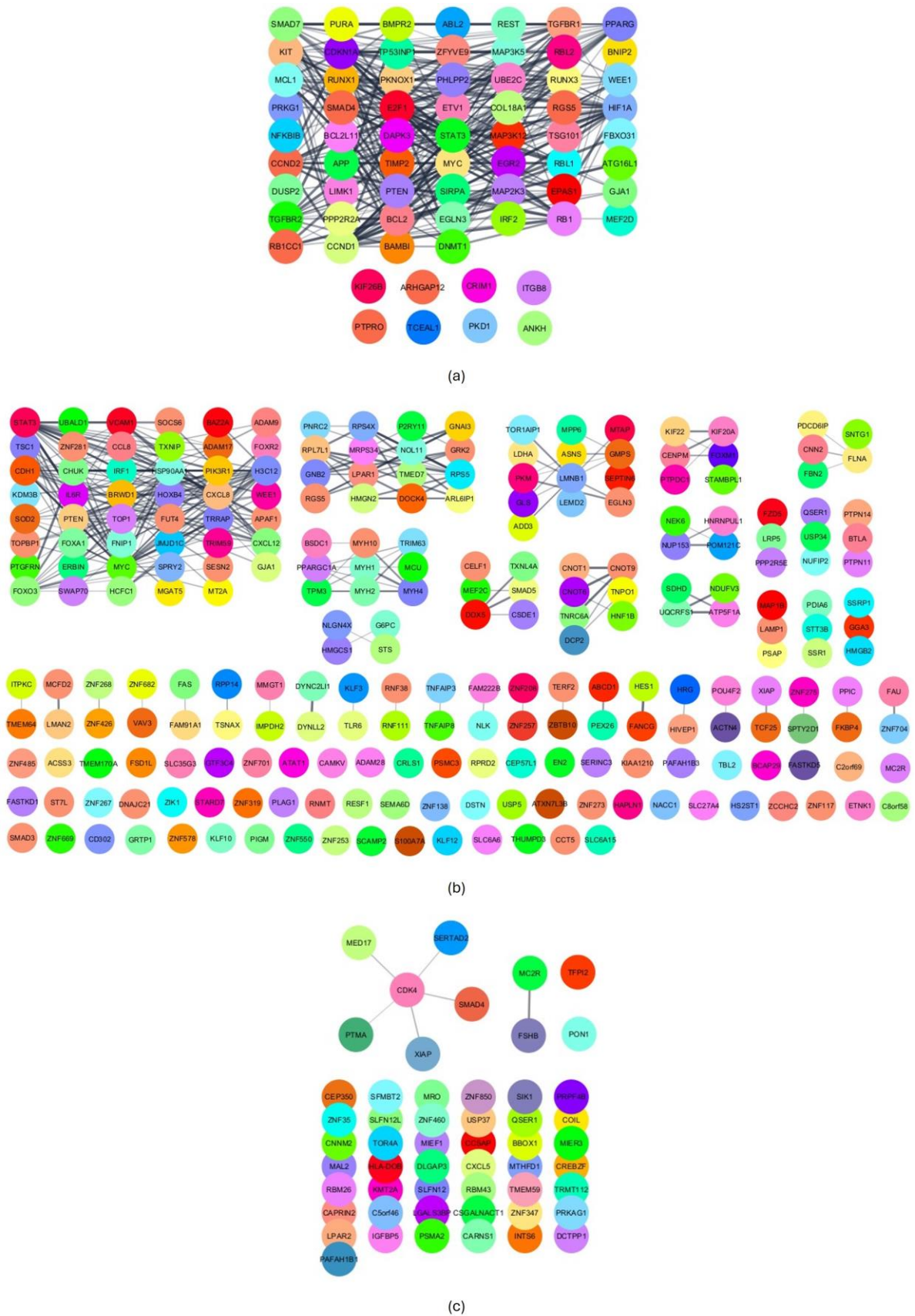
Model (N)	Variable	aHR	95%CI	p-Value	C-Index		Event
					Clinic Model	Integrative Model	
Integrative model A1 (47)	Surgery (Yes vs. no ¹)	0.16	0.07–0.37	<0.001	0.902	0.952	Risk of disease progression
	miR-19a-3p *** (Low vs. high levels ¹)	4.85	1.37–17.16	0.014			
	miR-20a-5p * (High vs. low levels ¹)	6.13	1.72–21.83	0.005			
Integrative model A2 (52)	Primary treatment (Surgery vs. chemotherapy ¹)	0.33	0.17–0.64	0.001	0.671	0.898	
	miR-20a-5p * (High vs. low levels ¹)	2.17	1.14–4.15	0.019			
Integrative model B1 (46)	Patients' age at OC diagnosis (≥64 vs. <64 years ¹)	7.43	2.24–24.65	0.001	0.821	0.848	Risk of death
	Surgery (Yes vs. no ¹)	0.16	0.05–0.47	<0.001			
	Platinum sensitivity (No vs. yes ¹)	4.90	1.96–12.24	<0.001			
	miR-616-3p *** (High vs. low levels ¹)	3.72	1.23–11.21	0.020			
Integrative model B2 (52)	Patients' age at OC diagnosis (≥64 vs. <64 years ¹)	6.03	2.15–16.88	<0.001	0.699	0.713	
	miR-23a-3p ** (High vs. low levels ¹)	2.68	1.06–6.77	0.037			

¹ Reference group. * Profile 1; ** Profile 3; *** Profile 4. Clinic models incorporated only demographic and clinicopathological factors, while integrative models also encompassed the baseline miRNAs' expression. Bold

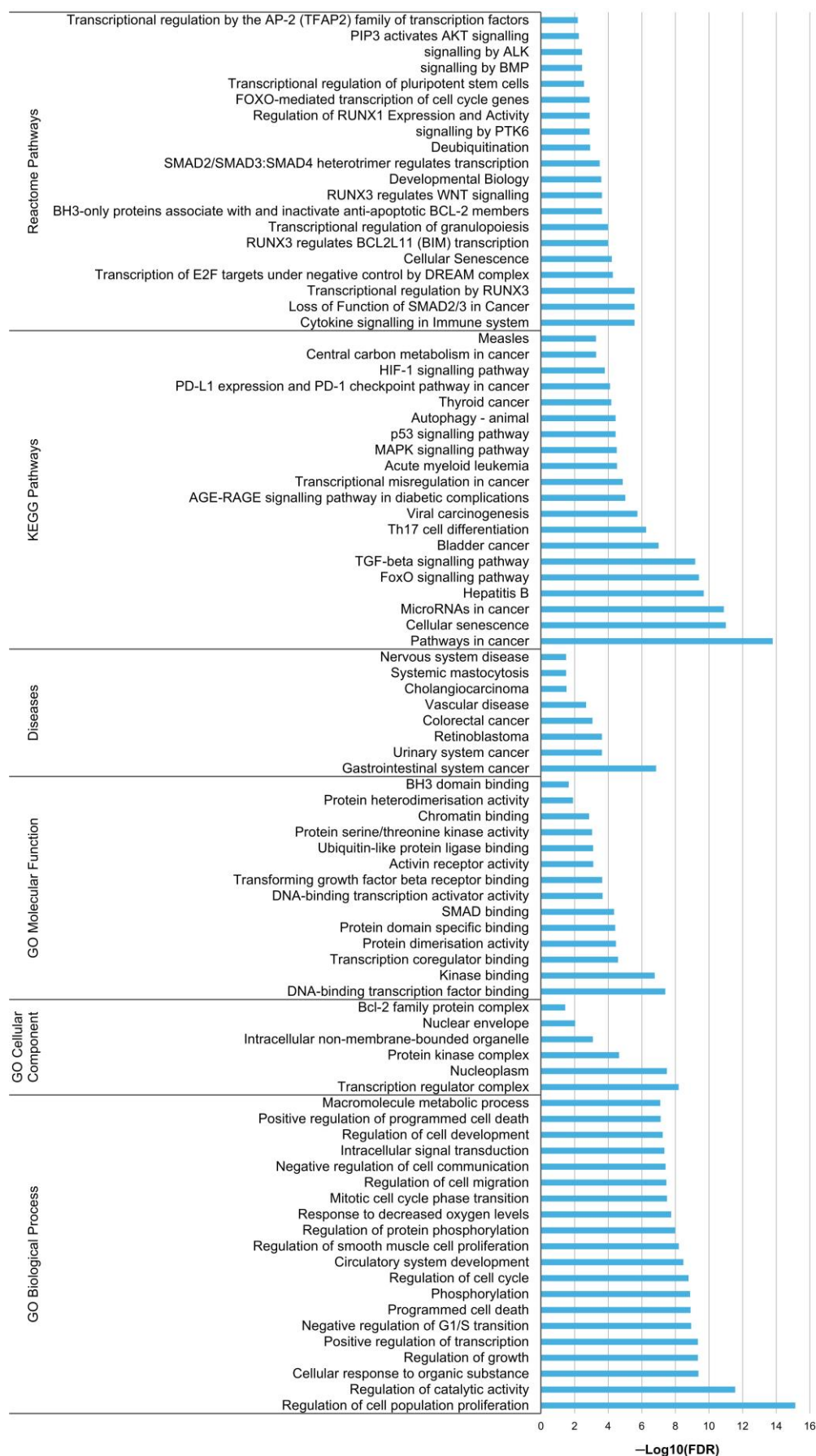
models were deemed the most suitable. Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; N, number of patients; OC, ovarian cancer.

3.6. *In Silico* Analysis

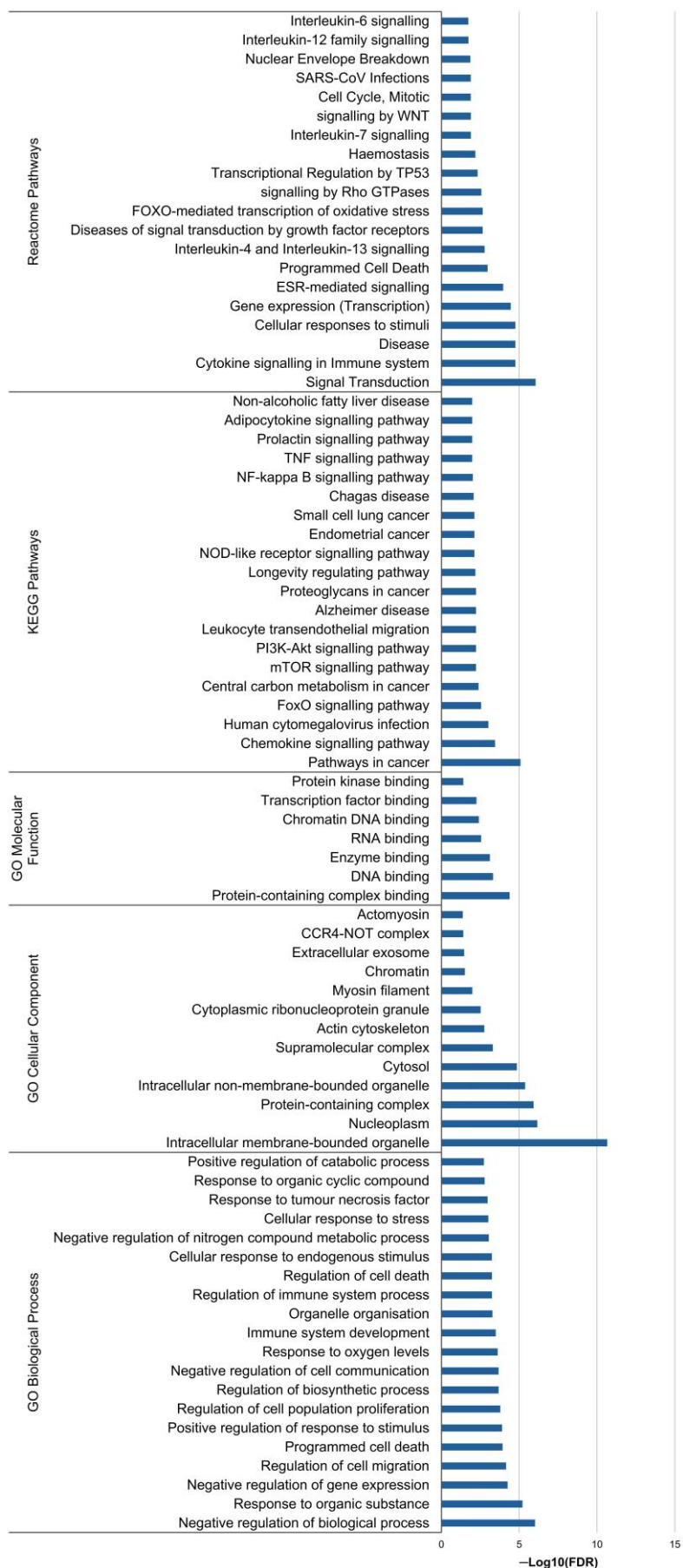
In this study, miR-20a-5p, miR-23a-3p and miR-616-3p exhibited a more predominant role. Thus, *in silico* analyses were conducted to explore their potential biological implications. For miR-20a-5p, since it had more than 300 validated targets (cut-off defined to ensure clear visualisation of the targets), only the strongly validated ones were considered (N = 68). For miR-23a-3p and miR-616-3p, 248 and 53 targets (including TFPI2 as a strongly validated target of miR-616-3p) were reported, respectively. The PPI networks generated for the targets of miR-20a-5p (68 nodes and 317 edges, $p = 1.0 \times 10^{-16}$; **Figure 5a**) and miR-23a-3p (246 nodes and 312 edges, $p = 7.9 \times 10^{-7}$; **Figure 5b**) had significant enrichment. The functional enrichment analysis focused on the clusters (60 nodes and 317 edges for miR-20a-5p and 183 nodes and 312 edges for miR-23a-3p) identified 250 and 154 enriched terms for miR-20a-5p and miR-23a-3p, respectively. The top 20 enriched terms for each miRNA concerning GO categories, Diseases, KEGG and Reactome pathways are presented in **Figures 6** and **7**. Notably, no enriched term regarding Diseases was found for miR-23a-3p (**Figure 7**). Moreover, no significant enrichment was found for miR-616-3p due to the limited interaction among its targets (52 nodes and six edges, $p = 0.74$; **Figure 5c**), which prevented further functional enrichment analysis.



Chapter VI - Figure 5. Protein–protein interaction (PPI) networks of miR-20a-5p (a), miR-23a-3p (b) and miR-616-3p (c) validated targets.



Chapter VI - Figure 6. Functional enrichment analysis for miR-20a-5p.



Chapter VI - Figure 7. Functional enrichment analysis for miR-23a-3p.

4. Discussion

Cancer is linked to a broad range of haemostatic issues, with the tumour coagulome fuelling tumourigenesis. Recent investigation delves into understanding how tumour cells “educate” the haemostatic system to facilitate cancer progression [47]. This study examined the implications of VTE and the role of miRNAs targeting the TF pathway in OC patients. A CAT incidence of nearly 19% was observed, which is consistent with the literature reporting an incidence range of 10% to 30% [3]. Particularly among those with VTE after OC diagnosis, VTE negatively affected their PFS and OS (long-rank test, $p = 0.024$ and $p < 0.001$, respectively). Unlike survival time, thrombotic events did not significantly impact patients’ PFS when considering patients with VTE before OC diagnosis (log-rank test, $p = 0.113$). The use of anticoagulants prior to treatment may have potential effects, yet further research is needed to elucidate the underlying biological mechanisms. Notably, among those with VTE after OC diagnosis, LMWH was the commonly chosen anticoagulant therapy, aligning with the existing literature. Although with some data inconsistency, LMWH is thought to be less effective than DOACs among cancer patients, while the latter is seemingly linked to a heightened risk of bleeding and additional complications [48,49]. Regarding CAT prediction, neither demographic and clinicopathological factors nor KS were reliable predictors of OC-related VTE in this study, an observation that warrants investigation in larger cohorts.

Except for miR-616-3p, some correlation was observed among the evaluated plasma miRNAs. MiR-18a-5p, miR-19a-3p and miR-20a-5p exhibited strong correlations. Consistently, they belong to the miR-17-92 cluster (chromosome 13q31.3) and are experimentally validated to target *F3* [50]. In addition, miR-19a-3p has been associated with CAT development [28]. Regarding miR-27a-3p, this *F3*- and *TFPI1*-targeting miRNA is a potential diagnostic biomarker of VTE in the general population [33–35]. Concerning miR-103a-3p, its circulating levels have been implicated in first and recurrent VTE events, as well as CAT [28,36,37]. This miRNA potentially modulates the risk of thrombosis by influencing endothelial cell function and targeting genes regulating TF expression [51,52]. Also, according to miRmap, miRwalk and DIANA-TarBase, miR-103a-3p targets *TFPI2* expression. As for miR-126-5p, increased circulating levels of this *F3*-targeting miRNA have been found to be a diagnostic and prognostic biomarker of VTE in the general population, as well as a predictive biomarker of CAT [22,25,32,38,39]. Overall, miR-18a-5p, miR-19a-3p, miR-20a-5p, miR-27a-3p, miR-103a-3p and miR-126-5p seem to all impact *F3* expression, which may partially explain the correlation between these miRNAs. MiR-23a-3p and miR-616-3p are validated *TFPI2*-targeting miRNAs [31,40,41]. The former showed a moderate correlation with miR-18a-5p, miR-19a-3p, miR-20a-5p and miR-126-5p and a

strong correlation with miR-27a-3p (both are members of the miR-23a~27a~24-2 cluster, chromosome 9q22) and miR-103a-3p, whereas the latter did not correlate with any of the miRNAs [53].

In the study, miR-20a-5p, miR-23a-3p and miR-616-3p were the most relevant miRNAs when considering the entire cohort. Starting with miR-20a-5p, this validated *F3*-targeting miRNA is reported to inhibit and promote OC cell proliferation, migration, invasion and cisplatin resistance, depending on the context [54–56]. Xie et al. (2014) [57] have found that elevated circulating miR-20a levels were linked to poor OS. In the present study, high plasmatic levels of miR-20a-5p were observed among patients with an ECOG PS ≤ 1 (profile 2, χ^2 , $p = 0.047$; profile 3, χ^2 , $p = 0.031$). This upregulation was significantly associated with VTE development (profile 2, χ^2 , $p = 0.034$), which could be partially explained by *F3* and *TFPI2* targeting, the latter with less evidence. In addition to TF, recent studies have highlighted TFPI2 as a predictive and diagnostic biomarker of OC-related VTE, particularly when combined with D-dimer levels, as well as a diagnostic and prognostic marker of OC [58–60]. Notably, most data link this haemostatic protein to the pathogenesis of ovarian clear cell carcinoma—the OC subtype most strongly associated with VTE. In this subtype, TFPI2 is overexpressed [61–63].

In this study, miR-20a-5p upregulation was also associated with a faster disease progression (profile 1, log-rank test, $p = 0.012$) and a decreased survival time (profile 1, log-rank test, $p = 0.010$), which is in line with the findings of Xie et al. (2014) [57]. Patients with heightened levels faced a two-fold increase in the risk of OC progression (profile 1, $p = 0.017$). Based on the stratified analysis, the miRNA appears to have a more preponderant influence on disease progression among individuals at early cancer stages and with lower CA-125 levels. Those with elevated levels of miR-20a-5p also experienced nearly a three-fold rise in the risk of mortality (profile 1, $p = 0.015$). This adverse effect was more pronounced in younger patients, those at early cancer stages, with an ECOG PS ≤ 1 , lower CA-125 levels and a KS < 2 . Interestingly, Xie et al. (2014) [57] found that elevated serum miR-20a expression was significant only in OC patients with CA-125 levels ≤ 500 U/mL. Overall, this miRNA appears to be more significant in the pre-metastatic phase and/or when the tumour burden is low. This supports our previous findings that certain VTE-related biomarkers may have a greater impact before metastasis, potentially aiding in the spread of tumour cells [64,65]. As for patients' age and KS, the former is a significant factor influencing the risk of thrombogenesis, whereas KS stands out as the most extensively studied risk assessment model (RAM) for CAT, which corroborates the influence of haemostatic mechanisms in miR-20a-5p function [4]. This study's best predictive model for disease progression—integrative model A1—combined information on surgery (yes vs. no) and baseline levels of miR-19a-3p and miR-20a-5p. Likewise, the integrative model A2,

designed for use at the beginning of first-line treatment, showed a 34% improvement in predictive accuracy over its clinical counterpart. This model encompasses data on primary treatment options (surgery vs. chemotherapy) and baseline miR-20a-5p levels, highlighting the potential clinical significance of this miRNA for an early prognosis evaluation of OC patients.

According to the *in silico* analysis, miR-20a-5p seems to be implicated in cell proliferation, differentiation, migration and senescence/apoptosis, also participating in host immune response. Importantly, this miRNA appears to regulate cell cycle and transcription activity and play an important role in circulatory system development. Overall, miR-20a-5p seems to be involved in several signalling pathways that foster both tumour progression and cancer-associated hypercoagulation, particularly HIF- α , PI3K/Akt and TGF- β /Smad pathways [66–69]. All in all, focusing on the TF pathway, the deregulation of TF and TFPI2, both targeted by miR-20a-5p, might contribute to the tumour coagulome and OC progression. According to the literature, cells actively or passively release miRNAs into the extracellular space through four main mechanisms: miRNAs encapsulated within extracellular vesicles, complexed with the Argonaute2 protein (Ago2), bound to high-density lipoprotein (HDL) or associated with the RNA-binding protein nucleophosmin (NPM1). Passive release occurs due to tissue injury, chronic inflammation, apoptosis or necrosis, which are common in cancer [70]. The requirement to maintain TF expression, combined with the upregulated plasma levels of miR-20a-5p linked to a poor prognosis, may suggest an active release of this miRNA from the OC cells, key haemostatic and/or immune players into the bloodstream, a hypothesis that requires further investigation. Currently, the expression pattern of *TFPI2* in the more aggressive ovarian tumours is unclear when considering all histological subtypes. A better understanding of the specific molecular and cellular environment in which miR-20a-5p operates, along with its expression dynamics, is crucial to effectively target it for prognostic and therapeutic purposes.

Like miR-20a-5p, miR-23a-3p is reported to have contradictory roles in OC. Overexpression of this validated *TFPI2*-targeting miRNA in OC tissue has been associated with tumour cell proliferation, invasion, migration and chemoresistance, as well as a poor PFS [71–75]. However, pro-apoptotic and chemosensitive effects have also been observed in certain contexts within OC cells [76]. Regarding the impact of circulating miR-23a-3p in OC patients, data are limited. This study found that elevated miRNA levels were more common among patients at advanced cancer stages and those with lower CA-125 levels, an intriguing and inconclusive finding. A previous study reported that miR-23a downregulates *TFPI2* expression in pancreatic cancer cells and exacerbates their malignant characteristics, thus acting as an oncogene [31]. Furthermore, elevated plasma levels of miR-23a-3p have been shown to predict the occurrence of CAT [32]. In this study,

no impact on venous thrombogenesis was observed. Nevertheless, upregulated miR-23a-3p levels were associated with a decreased survival time (profile 3, log-rank test, $p = 0.018$). Patients with high levels face nearly a three-fold increase in the risk of death (profile 3, $p = 0.025$). According to stratified analysis, the miRNA appears to influence disease progression among younger patients, those with lower CA-125 levels and with $KS \geq 2$. Furthermore, elevated levels of the miRNA seem to negatively impact the survival time of younger patients with an ECOG PS ≤ 1 and at early cancer stages. Overall, like miR-20a-5p, miR-23a-5p may have an important role in a pre-metastatic setting. Lastly, the multivariate Cox analysis indicated that miR-23a-3p is an independent predictor of risk of death.

According to the *in silico* analysis, miR-23a-3p is mainly involved in regulating immune response, cell proliferation, migration and apoptosis, which aligns with the existing evidence [71–76]. Interestingly, miR-23a-3p's involvement in haemostasis was corroborated. Also, this miRNA participates in PI3K/Akt and FOXO signalling pathways, which are implicated in cancer and thrombogenesis [68,77]. All in all, miR-23a-3p appears to have a context-dependent role in OC pathways, echoing the functions of its target, TFPI2. However, further studies are necessary to elucidate miRNA expression dynamics and its mechanism of release in peripheral blood.

Recently, miR-616 has been addressed as a cancer-associated miRNA, playing an oncogenic role in gastric, ovarian and breast cancers [78–80]. In OC, elevated miR-616 levels correlated with poor differentiation, invasion, migration and metastasis in experimental settings [79]. However, data on circulating miR-616-3p in OC patients are scarce. In this study, high levels of this miRNA were more common among those with $KS \geq 2$ (profile 3, χ^2 , $p = 0.044$), suggesting a potential role in CAT pathogenesis. Concordantly, its upregulated levels were found among patients who later developed VTE after OC diagnosis compared to VTE-free patients (Kruskal–Wallis test, $p = 0.022$; profile 1, $p = 0.048$; profile 2, $p = 0.017$; profile 3, $p = 0.029$). This prothrombotic effect could be due to *TFPI2*, one of the few strongly validated targets of miR-616-3p [41]. Similarly, a prior study conducted by our research group found that elevated pre-chemotherapy levels of MEG8 (a long non-coding RNA targeting *TFPI2* expression) in peripheral blood components (PBCs) were predictive of VTE after an OC diagnosis [17,81].

While no significant association was found in the overall cohort regarding its influence on patients' prognosis, increased levels of miR-616-3p were associated with a lower OS in younger patients and those with $KS < 2$ (long-rank test, $p < 0.05$). Consistently, the best predictive model for the risk of death integrated information on patients' age, surgery, platinum sensitivity and baseline miR-616-3p levels. Although it only yielded a marginal 3% enhancement in predictive accuracy compared to the clinical model alone, the integrated

model B1 demonstrated the independent predictive capability of this miRNA. Unfortunately, *in silico* analysis was unfeasible due to the limited interactions among the miRNA targets. Overall, miR-616-3p could be a valuable tool for predicting OC-related VTE and improving prognostic accuracy. More investigation is needed to elucidate the miRNA expression dynamics and its role in OC.

According to the stratified analyses, miR-18a-5p, miR-19a-3p, miR-27a-3p, miR-103a-3p and miR-126-5p appear to be particularly relevant in the pre-metastatic phase and among younger patients, consistent with findings for miR-20a-5p, miR-23a-3p and miR-616-3p. Furthermore, miR-19a-3p was a predictor of the risk of OC progression in the multivariate Cox analysis adjusting for surgery (yes vs. no) and baseline miR-20a-5p (integrative model A1). Whether these results indicate a context-dependent role or are influenced by the correlations between the miRNAs or the cohort size remains to be determined.

This study presented some limitations. Due to the relatively low incidence of OC and the need to control for major confounders associated with CAT development, the cohort size was small, impacting the statistical power. This study's retrospective nature prevented the evaluation of miRNA expression levels near the occurrence of VTE, which would offer valuable insights into their diagnostic and prognostic potential concerning thrombosis. Also, no active VTE screening was conducted, potentially underestimating asymptomatic events. Moreover, *in silico* analyses have intrinsic limitations. Given the complexity of OC and CAT pathogenesis, other genes and pathways with an impacting role may have been overlooked. As previously mentioned, a single miRNA can target several mRNAs, influencing multiple biological pathways. More real-world data are needed for an integrative view of the related mechanisms. Despite these constraints, the study has notable strengths in accounting for major risk factors associated with VTE and CAT.

5. Conclusions

Among gynaecological tumours, OC is the most lethal. Investigating haemostasis deregulation in cancer progression is promising for identifying OC biomarkers and therapeutic targets. Meanwhile, circulating miRNAs have emerged as valuable tools for understanding not only CAT pathogenesis but also ovarian tumourigenesis. These RNAs are accessible, resistant to environmental conditions and allow for minimally invasive sample collection, making them suitable for routine clinical use and longitudinal monitoring. This study evaluated plasma expression levels of miRNAs targeting the TF coagulation pathway in OC patients. The findings confirmed that VTE negatively impacts their

prognosis, affecting both disease progression and survival. Baseline levels of miR-20a-5p and miR-616-3p were shown to predict OC-related VTE, with miR-20a-5p being a relevant indicator of OC progression and miR-616-3p significantly impacting survival, which underscores the bidirectional relationship between thrombosis and ovarian tumourigenesis. Additionally, miR-23a-3p influenced patients' prognosis, though it did not affect thrombogenesis. Although more studies with larger cohorts are required to validate their clinical applicability, these miRNAs appear to be valuable for tailoring thromboprophylaxis and could potentially aid in the selection of the most effective anticoagulation therapy to prevent recurrent events. Importantly, they could also enhance prognosis accuracy and potentially serve as therapeutic targets for OC management. Overall, this could pave the way for more personalised medicine. To our knowledge, this is the first study to address the implications of plasma miRNAs targeting the TF coagulation pathway in OC patients, with the findings suggesting a pivotal role of TF and TFPI2 in OC pathogenesis.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Portuguese Institute of Oncology of Porto (CES. 69/021, approved on 11 March 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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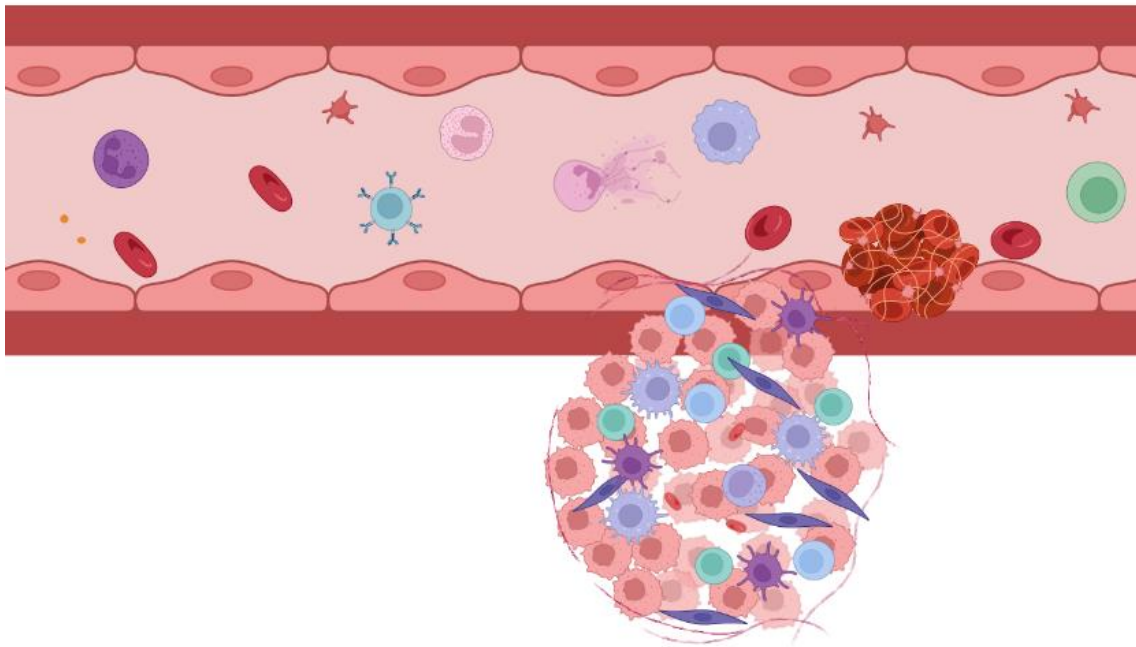
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Chapter VII



CHAPTER VII – General Discussion

A paradigm shift in ovarian cancer (OC) management underscores the need for more reliable prognostic biomarkers to personalise treatment. Accumulating data highlights the role of haemostatic components in cancer progression and dissemination, prompting the question of whether biomarkers related to cancer-associated thrombosis (CAT), serving as surrogate markers of coagulation activation, could aid in prognostication, monitoring treatment response, and early detection of OC recurrence [1]. Thus, the **primary aim** of this thesis was to identify clinically relevant prognostic biomarkers for OC by examining the tumour coagulome and CAT pathogenesis using liquid biopsies. **Specific aims** were established to achieve this goal (**Chapter II**). This chapter summarises the main findings related to each specific aim and the associated main limitations.

1. Evaluate the incidence of CAT occurrence among OC patients and study its implications on patients' clinical outcomes.

The findings of this project reveal that OC patients face a significant pro-thrombotic risk, with incidence rates of CAT between 19 and 23%, aligning with the existing literature [2]. CAT negatively impacted tumour progression and survival in OC patients, particularly when dismissing those with a history of other tumours (specifically in **Chapter III**) and those under anticoagulation therapy at OC diagnosis (including those with CAT before tumour diagnosis). The lack of a significant impact of thrombogenesis on tumour progression observed in **Chapter V** is mostly likely attributed to the small cohort size compared with the other Chapters.

Besides leucocyte count, patient characteristics failed to predict CAT development (**Chapters III-VI**). A high leucocyte count was associated with an increased risk for CAT development (**Chapter IV**). This finding, consistent with the scarce literature, highlights the role of the immune system in venous thrombogenesis among cancer patients [3]. The most studied and validated CAT risk assessment model, the Khorana score (KS), also showed poor predictive performance, consistent with previous research [4,5]. Inclusively, those classified as low CAT risk often end up suffering from the disease, which aligns with the existing data [4]. Furthermore, baseline (before cancer treatment) coagulation assays commonly used in our clinical practice – prothrombin time (PT), international normalised ratio (INR) and activated partial thromboplastin time (aPTT) - proved unsuitable as biomarkers for assessing the pro-thrombotic potential of OC patients. However, INR had

prognostic value. This coagulation assay might be an important prognostic tool in OC, especially when combined with D-dimer (DD) levels, as previously suggested [6].

Associations of KS and the routine baseline coagulation assays with the evaluated biomarkers were also evaluated. KS was found to be associated with the distribution of the genotypes of *coagulation factor 5 (F5)* rs6025 and *zinc finger protein, FOG family member 2 (ZFPM2)* rs4734879 in **Chapter III**. The score was also associated with different pre-chemotherapy expression levels of *coagulation factor 8 (F8)* (CAT predictor), *tissue factor pathway inhibitor 1 (TFPI1)* and *thrombomodulin (THBD)* in **Chapter IV**. Furthermore, pre-chemotherapy levels of maternally expressed 8 (MEG8) (**Chapter V**) and miR-616-3p levels (**Chapter VI**), both predictive biomarkers of CAT, varied significantly according to KS. Baseline PT was associated with pre-chemotherapy levels of *coagulation factor 3 (F3)* (CAT predictor) and *TFPI1* in **Chapter IV**. Likewise, the coagulation assay was linked to pre-chemotherapy levels of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and MEG8 (CAT predictor) in **Chapter V**. Baseline INR was linked to the distribution of *integrin subunit beta 3 (ITGB3)* rs5918 genotypes in **Chapter III**. This coagulation assay was also associated with pre-chemotherapy levels of *F8* and MEG8 (both predictive biomarkers of CAT, as previously mentioned) in **Chapter IV** and **Chapter V**, respectively. As for aPTT, pre-chemotherapy levels of MEG8 were found to vary significantly with this coagulation assay in **Chapter V**. Overall, these associations corroborate the influence of the evaluated biomarkers in blood coagulopathies. However, the generalisation of findings is hindered by the small cohort size in each study and the lack of an evaluation considering other physiopathological conditions.

2. *Since VTE is a highly heritable disease, investigate the role of haemostatic gene variations in the tumour coagulome and their impact on the clinical outcomes of OC patients.*

In **Chapter III**, haemostatic gene variations were evaluated as predictors of CAT development and prognostic biomarkers of OC patients (regardless of venous thrombogenesis). Impactful haemostatic gene variations were found to fall into three roles: sole predictors of CAT development, dual-purpose biomarkers for CAT and OC prognosis (independent of thrombogenesis), and exclusive prognostic biomarkers for OC. The SNP *protein C receptor (PROCR)* rs10747514, a sole predictor of CAT in our cohort of OC patients, may help stratify patients by CAT risk and enhance thromboprophylaxis. *Serpin family E member 1 (SERPINE1)* rs2070682 was identified as both a predictor of CAT and a prognostic factor for OC, supporting the two-way relationship between thrombosis and ovarian tumourigenesis. The SNPs *regulator of G protein signalling 7 (RGS7)* rs2502448, *F3* rs1361600 and *fibrinogen gamma chain (FGG)* rs2066865 were shown to be solely

prognostic biomarkers of OC. Among them, *F3* rs1361600 stand out as a predictor of both OC progression and mortality, aligning with the pivotal role of tissue factor (TF) in malignant diseases, including OC. Since these biomarkers are heritable and thus stable, they could facilitate timely intervention in OC. However, the small cohort size requires a cautious interpretation of these results. Furthermore, the functional consequence of these genetic variants is not completely understood.

3. Considering the inflammatory processes in CAT pathogenesis and the pivotal role of coagulation in venous thrombogenesis, decode the expression of haemostasis-related genes involved in immunothrombosis within peripheral blood components (PBCs) and their clinical significance for OC patients.

Chapter IV delves into the expression of genes encoding coagulation factors and anticoagulants within PBCs of OC patients, exploring the concept of cancer thrombo-inflammation or cancer immunothrombosis. Pre-chemotherapy expression levels of *F3* and *F8* in platelets and leukocytes were found to be predictive of CAT development. However, only *F8* expression was linked to patients' time to disease progression and survival. These results imply that TF in PBCs might not be a key driver of OC tumourigenesis. Nonetheless, the cohort size in the study was small. Also, identifying the specific cell populations expressing *F3* would be crucial to understanding its expression dynamics and underscore a potential cell-specific role of TF. These limitations also apply to *F8* expression.

4. Examine the biomarker potential of emerging VTE predictors, specifically long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), and their contribution to CAT pathogenesis and the clinical outcomes of OC patients.

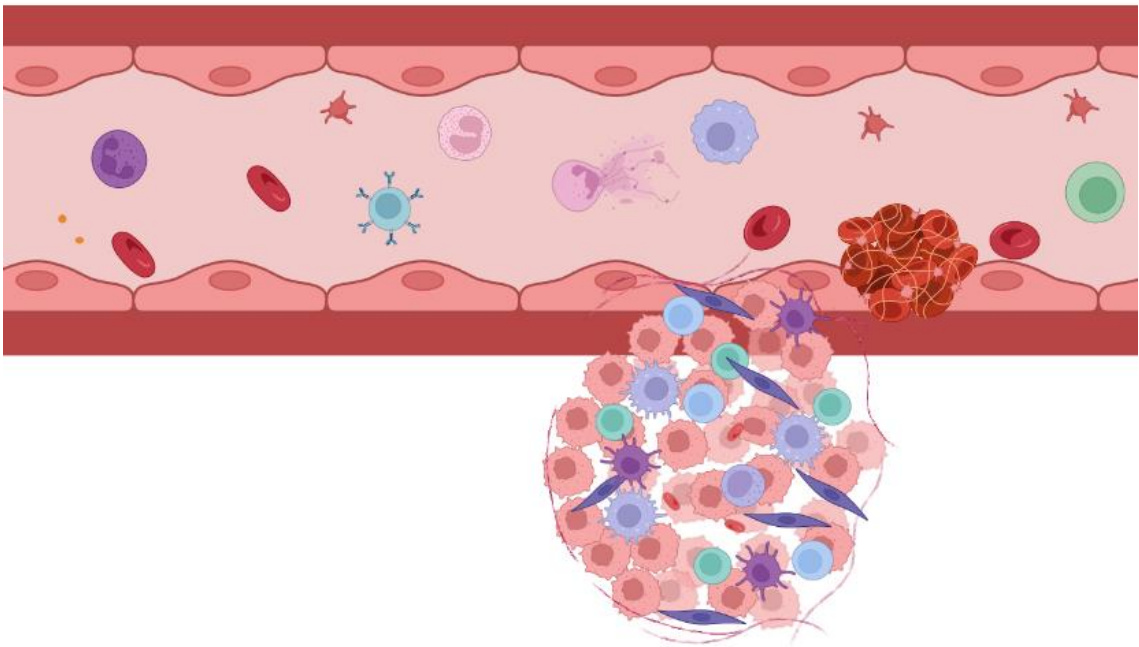
Chapters V and **VI** examined the impact of VTE-related lncRNAs and miRNAs on CAT development and the clinical outcomes of OC patients. In **Chapter V**, MEG8 [targeting tissue factor pathway inhibitor 2 (*TFPI2*) expression] in PBCs was a predictor of OC-related VTE, with its association with the baseline coagulation tests reflecting a possible role of this lncRNA in haemostatic abnormalities. Thus, MEG8 might hold the potential to be an attractive tool for predicting CAT among OC patients. This lncRNA was also demonstrated to impact the risk of OC progression regardless of VTE. Moreover, NEAT1 in PBCs was associated with the risk of death, emerging as a potential OC prognostic biomarker. Like in **Chapter IV**, expanding the cohort size and identifying the specific cell populations expressing these lncRNAs would be essential to gain deeper insights into the underlying biological mechanisms.

In **Chapter VI**, baseline levels of plasma miR-20a-5p (targeting *TF* and *TFPI2*, the latter with less evidence) and miR-616-3p (targeting *TFPI2*) were shown to predict OC-related VTE, with miR-20a-5p being a relevant indicator of OC progression and miR-616-3p significantly impacting survival. Furthermore, miR-23a-3p (also targeting *TFPI2*) influenced patients' prognosis but not CAT susceptibility. When performing subgroup analysis, all the evaluated miRNAs were shown to have a detrimental impact on OC progression and/or patients' survival. Whether these results indicate a context-dependent role or are influenced by the correlations between the miRNAs, or the cohort size remains to be determined. Also, the mechanisms underlying the release of miRNAs in peripheral blood are currently poorly explored.

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Chapter VIII



CHAPTER VIII – Main Conclusions & Future Perspectives

This thesis project was designed to identify clinically relevant prognostic biomarkers for ovarian cancer (OC) by examining the tumour coagulome and pathogenesis of cancer-associated thrombosis (CAT) using liquid biopsies. The impact of venous thromboembolism (VTE) on patients with this malignant disease was assessed through several tasks. Considering the existing body of evidence, the clinical implications of haemostatic gene variations, the expression of haemostasis-related genes in peripheral blood components (PBCs) and the expression of VTE-related long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) were evaluated aiming to uncover CAT predictors and prognostic tools of OC to improve tumour management. The **integrative view of the main findings** and **future perspectives** are present in this chapter.

The results of this project reveal that OC patients have a high risk for CAT development, with the disease imposing a negative impact on their clinical outcomes. Neither the Khorana score (KS) nor coagulation assays routinely used in clinical practice were able to predict CAT occurrence. Focusing on the evaluated haemostasis and VTE-related biomarkers, from SNPs to miRNAs, the findings highlight the significance of tissue factor (TF) and tissue factor pathway inhibitor 2 (TFPI2) in ovarian tumourigenesis. TF is a pivotal player in CAT pathogenesis and exerts tumourigenic roles through coagulation-dependent and independent mechanisms [1,2]. Consistently, numerous studies underscore TF's central role in ovarian tumourigenesis, with recent data suggesting a potential diagnostic value [3]. In this thesis project, the SNP in *coagulation factor 3* (*F3*, gene encoding for TF) rs1361600, and miR-20a-5p (targeting *F3*) emerged as relevant prognostic biomarkers for OC. Although *F3* expression in PBCs was not significantly linked to OC clinical outcomes, it was a predictive biomarker for CAT development. Regarding TFPI2, this anticoagulant and anti-fibrinolytic protein has gained attention as a serodiagnostic and prognostic biomarker for OC, also showing potential diagnostic value for CAT in patients with this tumour [4]. While TFPI2 generally exhibits an anti-invasion effect in most human cancers, it paradoxically promotes disease progression in certain OC cases, particularly in the clear cell carcinoma (CCC) subtype, which has the highest reported CAT risk [5,6]. In this thesis project, the lncRNA maternally expressed 8 (MEG8) and the miRNAs miR-20a-5p and miR-616-3p, all targeting *TFPI2*, were able to predict CAT development. These biomarkers, along with miR-23a-3p, also targeting *TFPI2*, held prognostic value in OC patients, independent of venous thrombogenesis.

Other findings include the associations of *protein C receptor* (*PROCR*, gene encoding for the endothelial receptor of the anticoagulant protein C) rs10747514, *serpin family E member 1* (*SERPINE1*, gene encoding for the anti-fibrinolytic protein, PAI-1) rs2070682 and the expression of *coagulation factor 8* (*F8*) in PBCs with CAT development, with the latter two demonstrating prognostic value. The SNPs *regulator of G protein signalling 7* (*RGS7*) rs2502448 (regulating platelet activity) and *fibrinogen gamma chain* (*FGG*) rs2066865 (modulating the composition of fibrinogen, the precursor of fibrin) were also shown to impact OC patients' prognosis, regardless of CAT development.

The potential biomarkers revealed in this project may have several potential applications. Namely, they can serve as CAT predictors, aiding in disease prediction, allowing a more personalised thromboprophylaxis based on individual risk profiles. This is important given that VTE incidence has shown a consistent rise among various types of tumours, driven by factors related to the patient, tumour and treatment [7]. Furthermore, the identified biomarkers could also facilitate VTE treatment monitoring, ensuring the effectiveness of therapeutic interventions and enabling timely adjustments. In-depth studies on these biomarkers may also facilitate the identification of novel therapeutic targets for VTE prevention and management. This could be valuable given the occurrence of thrombotic events even among patients undergoing thromboprophylaxis, demonstrating a lack of efficacy. Additionally, thromboprophylaxis is associated with bleeding adverse events, underscoring the importance of accurate CAT risk stratification and appropriate therapeutic interventions to improve patient prognosis [8]. In terms of OC management, the prognostic value of these biomarkers is significant, as demonstrated by their independent prognostic value in multivariable Cox analyses adjusted for relevant clinical factors. Furthermore, by reflecting the dynamic changes in the OC microenvironment, these biomarkers could potentially predict treatment response, allowing for more tailored and effective therapeutic strategies. More recently, haemostasis-related factors have been suggested as diagnostic tools for OC to improve early diagnosis and intervention. Lastly, understanding the role of these biomarkers in ovarian tumourigenesis may help identify new therapeutic targets for OC management, potentially leading to more effective treatments. Inclusively, as previously mentioned, case-control studies and randomised placebo-controlled trials have long suggested that low-molecular-weight heparin (LMWH) could enhance survival rates in cancer patients. Nevertheless, recent phase III trials have not substantiated these benefits, highlighting the pressing need for further research to unravel the mechanisms by which coagulation drives tumour progression [9]. This could aid in patient selection for anticoagulation therapy with additional anti-neoplastic effects.

Despite the promising findings, in-depth and more statistically powered studies will be required to validate the clinical applications of the evaluated haemostasis and VTE-related

biomarkers among OC patients. The REMARK criteria, widely recognised in the literature, outline essential guidelines for biomarker research [10]. Briefly, cancer biomarkers must be validated through large, prospective studies involving diverse patient cohorts, demonstrating a correlation with disease activity and known predictive and prognostic molecular factors. A biomarker must effectively differentiate between pathological and physiological conditions and have a well-defined molecular mechanism of action. Its validity should be supported by rigorous specimen collection and assay results showing adequate specificity, sensitivity, reproducibility, and robustness along with stringent patient follow-up. Moreover, cancer biomarkers can be identified through molecular, cellular, or imaging techniques and should be detectable in easily accessible biological samples, such as liquid biopsies. These biomarkers might include normal endogenous products overexpressed or hyperactivated in tumour cells but not in normal cells. They can be intracellular molecules within tissues or released into the bloodstream and other bodily fluids. Finally, clinical assays for biomarker detection and evaluation should be straightforward, cost-effective, and compatible with high-throughput technologies. Hence, the clinical implications of haemostasis and VTE-related biomarkers should be confirmed in prospective well-designed studies with larger cohort sizes, preferably using a multicentric approach. Enabled by the prospective nature of the studies, active VTE screening should be integrated into the protocols to account for symptomatic and non-symptomatic (subclinical) cases. Further research should also pursue validation in other populations to dissect the reproducibility and generalisation of the findings. Particularly concerning the transcripts (mRNAs, lncRNAs and miRNAs), their expression should also be evaluated in OC tissues to gain more insights into their complex regulatory networks in different compartments. Also, their expression dynamics should be explored in other physiopathological conditions, including healthy individuals and cancer-free patients with VTE. In these studies, matching VTE risk factors across groups is essential. Focusing on the biomarkers evaluated in PBCs, conducting single-cell RNA sequencing would be important to identify the specific cell populations responsible for their expression and thrombotic role. More functional studies are also crucial to better understanding the mechanisms through which the SNPs, lncRNAs and miRNAs are implicated in CAT and/or OC pathogenesis. Comparing the predictive and prognostic value of these biomarkers with those already used in clinical practice, such as D-dimer (DD), is also warranted [11]. Lastly, the implications of these biomarkers should be examined in patients with other tumours to explore their spectrum of applications.

Overall, this thesis corroborates the pivotal role of haemostatic mechanisms in OC pathogenesis and underscores attractive biomarkers that could aid in disease management. Given the complex nature of CAT and OC pathogenesis, it is improbable that a single biomarker will meet all criteria for acceptance as a cancer biomarker. Therefore,

the objective should be to integrate multiple haemostasis-related biomarkers with clinical factors to enhance prognostic accuracy, refine OC management strategies and ultimately improve clinical outcomes. In this context, artificial intelligence algorithms and big data analytics emerge as invaluable tools. They can help synthesise and interpret complex datasets, enabling the creation of a comprehensive prognostic panel based on specific patterns that may not be obvious [12]. Likewise, the applications of multi-omics, including genomics (DNA), transcriptomics (RNA), proteomics (proteins) and metabolomics (metabolites), may aid in the identification of new relevant haemostatic mechanisms in OC pathogenesis. These integrative approaches hold the potential to revolutionise OC management by providing personalised, precise and effective treatment plans [13].

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DOUTORAMENTO

PROGRAMA DOUTORAL EM PATOLOGIA E GENÉTICA MOLECULAR

Tumour Coagulome and Venous Thromboembolism-related Biomarkers for Prognosis Assessment of Ovarian Cancer Patients

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D

2024

