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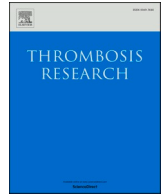
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# Tumor gene expression is associated with venous thromboembolism in patients with ductal pancreatic adenocarcinoma

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## ABSTRACT

**Introduction:** In patients with pancreatic cancer, the risk of venous thromboembolism (VTE) is high compared to other cancer types, suggesting that tumor-intrinsic features drive hypercoagulability. Tumor gene expression analysis may help unravel the pathogenesis of VTE in these patients and help to identify high-risk patients.

**Aim:** To evaluate the association between tumor gene expression patterns and VTE in patients with pancreatic cancer.

**Methods:** In this retrospective cohort study RNA-sequence data from surgically resected tumor material from patients with pancreatic ductal adenocarcinoma (PDAC) was used to identify genes associated with the presence of venous thromboembolism (i.e., pulmonary embolism or deep-vein thrombosis) within one year follow-up after surgery. Additionally, VTE risk and expression of coagulation related genes in two molecular subtypes of pancreatic cancer was assessed.

**Results:** Out of 151 patients, 10 (6.6 %) developed deep-vein thrombosis or pulmonary embolism within one year follow-up. Differential expression analysis yielded 89 genes significantly differentially expressed in patients with VTE compared to those without VTE, including *ATP6V0A4*, *SYT14* and *ZNF114*. The incidence of VTE in classical subtype was higher ( $n = 9$ ; 7.6 %) than in basal-like subtype ( $n = 1$ ; 4 %), but this difference was not statistically significant (SHR 1.79; 95 % CI 0.22–14.3). Forty-two coagulation-associated genes were identified that were differentially expressed between these molecular subtypes, including *F5*, *PLAU*, *SERPINE1*, and *C4BPB*.

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**Conclusions:** Patients with pancreatic cancer and VTE show a different tumor gene expression profile than those without VTE. Multiple coagulation-related genes were differentially expressed in classical versus basal-like molecular subtype, suggesting that there is a difference in pro-thrombotic phenotype.

## 1. Introduction

Venous thromboembolism (VTE), which encompasses pulmonary embolism and deep-vein thrombosis, occurs in approximately 7 % of cancer patients during the first 6 months after their cancer diagnosis [1,2]. VTE frequently leads to interruption or discontinuation of cancer treatment, decreased quality of life, morbidity, and death [3,4]. The incidence of VTE is heavily dependent on the type of cancer. Patients with breast or prostate cancer have a significantly lower VTE incidence (~1 % per year) than those with high-risk tumors, including gynecological, biliary, and pancreatic cancer [5]. Patients with ductal pancreatic adenocarcinoma (PDAC) have the highest risk of VTE, with an estimated incidence of up to 20 % in the first 6 months after diagnosis in those with metastatic disease and up to 11 % in the first year after surgery with curative intent [5,6]. The reasons for this disparity in VTE risk across tumor types is still largely unclear, but is hypothesized to be highly related to tumor intrinsic properties. PDAC cells may induce hypercoagulability by releasing thrombogenic factors, such as tissue factor-positive extracellular vesicles [7–9], or inhibitors of fibrinolysis, such as plasminogen activator inhibitor 1 (PAI-1) [10]. Molecular information holds promise to improve risk assessment, which can be used to identify patients for thromboprophylaxis. Additionally, it will provide insights in the pathophysiology of cancer-associated thrombosis.

Other studies have demonstrated an association between tumor gene expression and the risk of VTE in patients with lung cancer and colorectal cancer [11,12]. To the best of our knowledge, this association has not been studied in PDAC, which is a tumor type known to carry the highest risk of VTE. We hypothesize that tumor gene expression is associated with VTE risk in PDAC patients. Therefore, we sought to evaluate the association between tumor gene expression assessed by RNA-sequencing and development of VTE in patients with PDAC after resection.

## 2. Methods

### 2.1. Cohorts, outcome data and analysis

RNA sequence data were analyzed retrospectively in two independent cohorts, across three academic hospitals in the Netherlands, in which tumor specimens were collected after surgery. In the first cohort, patients underwent surgery in the Amsterdam UMC between 1993 and 2015. The second cohort included patients who underwent pancreatic surgery between 2015 and 2018 in the Amsterdam UMC, or between 1993 and 2018 in the University Medical Center Utrecht or Leiden University Medical Center. Patients were included in these cohorts if they had histologically proven PDAC; underwent a pancreateoduodenectomy; were 18 years or older and provided written informed consent for tumor sample collection. Differences in tumor gene expression and their association with VTE were assessed.

The main outcome was radiologically confirmed proximal deep vein thrombosis (DVT) of the leg or symptomatic or incidental pulmonary embolism (PE) within one year after surgery, since VTE risk remains high in the first year after PDAC surgery [6]. Abdominal vein thrombosis and catheter-related thrombosis were not included in this homogeneous outcome because they are often also caused by local factors (e.g. vein compression by the tumor and foreign material, postoperative complications) rather than hypercoagulability. Patients with arterial thromboembolism were also excluded since mechanisms of thrombosis are likely different which could interfere with differential expression results. Outcomes were adjudicated by a vascular medicine specialist who was

blinded for the RNA-sequencing outcomes.

RNA-sequencing data was used to assess differences in gene expression in patients who developed VTE compared to those who did not develop a VTE event during the first year after surgery for PDAC. Gene set enrichment analysis was performed to find significantly enriched pathways associated with VTE. Additionally, we compared genes significantly associated with VTE in our analysis with two previous studies on VTE and differentially expressed genes in patients with colorectal cancer [11] and lung cancer [12]. To assess a possible difference in thrombogenic phenotype, we compared VTE outcomes and expression of coagulation-related genes in two molecular subtypes of PDAC, classical versus basal-like [15]. In this analysis, samples were classified as classical or basal-like molecular subtype according to the PurIST classifier, introduced by Rashid et al., which classifies samples as classical or basal-like based on the expression of 16 different genes [16,17]. Basal-like and classical subtype were further categorized as strong, likely, or leaning basal-like and strong, likely, or leaning classical molecular subtype. Finally, differential expression of 324 coagulation-related genes and gene ontology for coagulation pathways was assessed in classical vs. basal-like molecular subtype to find possible differences in expression which could be related to VTE risk. Three external PDAC datasets were used to confirm coagulation-related gene expression in these two subtypes.

### 2.2. Data collection, validation cohorts and gene sets

Data on patient characteristics and outcomes were retrospectively collected from the patients' medical charts using Castor EDC, an electronic case record form. Patients without complete one year follow-up were excluded.

Methodology on tissue collection and RNA-sequencing is discussed in detail elsewhere [18]. In short, tumor specimens were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . RNA was isolated from 30 sections of 20  $\mu\text{m}$  using RNABee (Bio-Connect, Huissen, the Netherlands) and the RNeasy Mini kit (Qiagen, Hilden, Germany). Samples were DNase-treated. RNA was amplified using the Total Prep RNA Amplification kit (Illumina, San Diego, CA). Poly-A enriched libraries were synthesized using TruSeq RNA Library Prep kit and sequenced in three batches (Illumina HiSeq2500). All sequencing data were quality-controlled using FastQC and found to be of high quality. RNA-Seq reads were aligned to the human reference genome (GRCh38). rRNAs, tRNAs and chromosome M were masked.

Retrospective collection for RNA sequencing was conducted in accordance with ethical guidelines 'Code for Proper Secondary Use of Human Tissue in The Netherlands' (Dutch Federation of Medical Scientific Societies), approved by the Amsterdam UMC institutional review board (METC\_A1 15.0122) [18]. For prospectively collected material, written informed consent was obtained from all patients in the BioPAN biobank from 2011 (METC 2018-181) and PancreasParel biobank from 2015 (BTC 2014\_180). Clinicopathological data were obtained through the departments of Surgery and Pathology. Normalized gene expression data from these cohorts are available upon request at R2: Genomics Analysis and Visualization Platform (<https://r2.amc.nl>) under identifier 'Tumor PDAC Spacious 1+2 - Vijver - 221 - custom - ensh38e99'. Raw read data can be downloaded at EMBL-EBI ArrayExpress (E-MTAB-6830).

For the analysis of differences in expression of coagulation related genes in classical versus basal-like molecular subtype, we used genes from gene ontology pathway 'coagulation' (GO:0050817; 178 genes), KEGG pathways 'Complement and coagulation cascades' (hsa04610; 86 genes), and 'Platelet activation' (hsa04611; 124 genes), resulting in a list

of 324 unique coagulation and platelet related genes. For external validation of gene expression of coagulation related genes in the two molecular subtypes, three publicly available PDAC datasets were used. These included the Moffitt dataset (GSE71729), the Canadian pancreatic cancer dataset by the International Cancer Genome Consortium (ICGC PACA-CA, available at <https://dcc.icgc.org/projects/PACA-CA>), and The Cancer Genome Atlas PDAC dataset (TCGA-PAAC, available at <http://portal.gdc.cancer.gov/projects/TCGA-PAAD>).

### 2.3. Statistical analysis

Analyses were performed in R (statistical software, version 3.2.0). Differential gene expression analysis was performed using the DESeq2 package and LFC shrinkage was performed using the 'ashr' method [20,21]. A P-value <0.05 after multiple testing correction, using the Benjamini-Hochberg method, was considered statistically significant. Analyses were restricted to protein coding genes only ( $n = 17,311$ ). The 'ComplexHeatmap' package was used to construct heatmaps. Gene Set Enrichment Analysis (GSEA) was performed on genes significant differentially expressed (false discovery rate [FDR] <0.05) using the gseGO function in the clusterProfiler package. Gene expression in external datasets were assessed using the 'pdacR' package, in which gene expression analysis can be performed using publicly available expression data of previously reported PDAC datasets [22].

VTE in both molecular subgroups was visualized using cumulative incidence curves. Subdistributional hazard ratios were calculated to assess risk differences of VTE in both groups with death not related to VTE as a competing risk according to Fine & Gray [23]. Survival curves were constructed to assess a difference in mortality in classical vs. basal-like tumors using the log-rank test.

Coagulation-related genes that were significantly differentially expressed with a  $\log_2\text{FC} \geq 0.5$  or  $\leq -0.5$  were assessed in the three external PDAC dataset mentioned above (GSE71729, ICGC PACA-CA, and TCGA-PAAC).

## 3. Results

### 3.1. Description of the cohort

RNA-sequencing data were available for 221 patients from the two cohorts, including expression data of 17,311 protein coding genes. Eight patients (3.6 %) were excluded because they did not have a certain diagnosis of pancreatic ductal adenocarcinoma after pathology revision and one (0.4 %) was excluded because of therapeutic-dose anticoagulation for atrial fibrillation during follow-up. Of the remaining 212 patients, 162 (76.4 %) had complete one-year follow-up available. Eleven patients were excluded from the analysis due to other thromboembolic events, including abdominal vein thrombosis ( $n = 5$ ), catheter-related upper extremity thrombosis ( $n = 3$ ), and arterial thromboembolism ( $n = 3$ ). Of the remaining 151 patients, 10 (6.7 %) patients developed lower-extremity DVT or PE, 65 (43.0 %) had recurrent disease, and 48 (31.8 %) died. Of 10 patients with VTE, 7 (70 %) had disease recurrence within one year. Of those, 2 had recurrent disease prior to VTE. The other 5 had recurrent disease within six months after VTE. Baseline and tumor characteristics are listed in Table 1.

### 3.2. Gene expression in PDAC patients with VTE versus no VTE

Differential gene expression analysis between patients with VTE and no VTE yielded 20 genes significantly associated with VTE and 69 genes significantly associated with no VTE after multiple testing correction (Fig. 1). Genes with the strongest association with VTE, in terms of  $\log_2\text{fold change}$  ( $\log_2\text{FC}$ ), were *ATP6V0A4* ( $\log_2\text{FC}$  3.77, FDR  $1.22 \times 10^{-6}$ ), *SYT14* ( $\log_2\text{FC}$  2.91, FDR  $4.31 \times 10^{-6}$ ), and *ZNF114* ( $\log_2\text{FC}$  2.55, FDR 0.0008). Genes associated with no VTE were *KRT20* ( $\log_2\text{FC}$  -5.75, FDR  $1.22 \times 10^{-6}$ ), *TMPRSS15* ( $\log_2\text{FC}$  -4.78, FDR 0.006), and

**Table 1**

Baseline characteristics of patients with and without thromboembolic events year follow-up.

|                               | Patients with<br>VTE<br>$n = 10$ | Patients without<br>VTE<br>$n = 141$ | P-<br>value |
|-------------------------------|----------------------------------|--------------------------------------|-------------|
| Sex (male) (%)                | 6 (60)                           | 84 (59.6)                            | 1.00        |
| Age (median, IQR)             | 67.0 (60.0–73.0)                 | 65.5 (64.0–69.3)                     | 0.89        |
| Tumor diameter (%)            |                                  |                                      | 0.86        |
| 0–2 cm                        | 0                                | 14 (9.9)                             |             |
| 2–4 cm                        | 7 (70)                           | 90 (63.8)                            |             |
| 4–6 cm                        | 2 (20)                           | 27 (19.1)                            |             |
| >6 cm                         | 1 (10)                           | 9 (6.4)                              |             |
| Tumor location (%)            |                                  |                                      | 0.15        |
| Head                          | 8 (80)                           | 119 (84.4)                           |             |
| Corpus                        | 2 (20)                           | 6 (4.2)                              |             |
| Tail                          | 0                                | 11 (7.8)                             |             |
| Differentiation (%)           |                                  |                                      | 0.87        |
| Well                          | 1 (10)                           | 12 (8.5)                             |             |
| Moderate                      | 4 (40)                           | 50 (35.5)                            |             |
| Poor                          | 5 (50)                           | 70 (49.6)                            |             |
| Unknown                       | 0                                | 5 (3.5)                              |             |
| Lymph node metastasis (%)     | 9 (90)                           | 105 (74.5)                           | 0.47        |
| Irradical resection* (%)      | 5 (50)                           | 90 (53.9)                            | 0.93        |
| Adjuvant chemotherapy† (%)    | 9 (90)                           | 110 (78.0)                           | 0.80        |
| Neo-adjuvant chemotherapy (%) | 0 (0)                            | 4 (2.9)                              | 0.59        |

Differences between groups are assessed using a chi-squared test for categorical variables and an ANOVA tests for continuous variables. \*Margin <1 mm or tumor in resection margin. †adjuvant treatment data missing for 3 patients without VTE. Abbreviations: VTE, venous thromboembolism; SD, standard deviation.

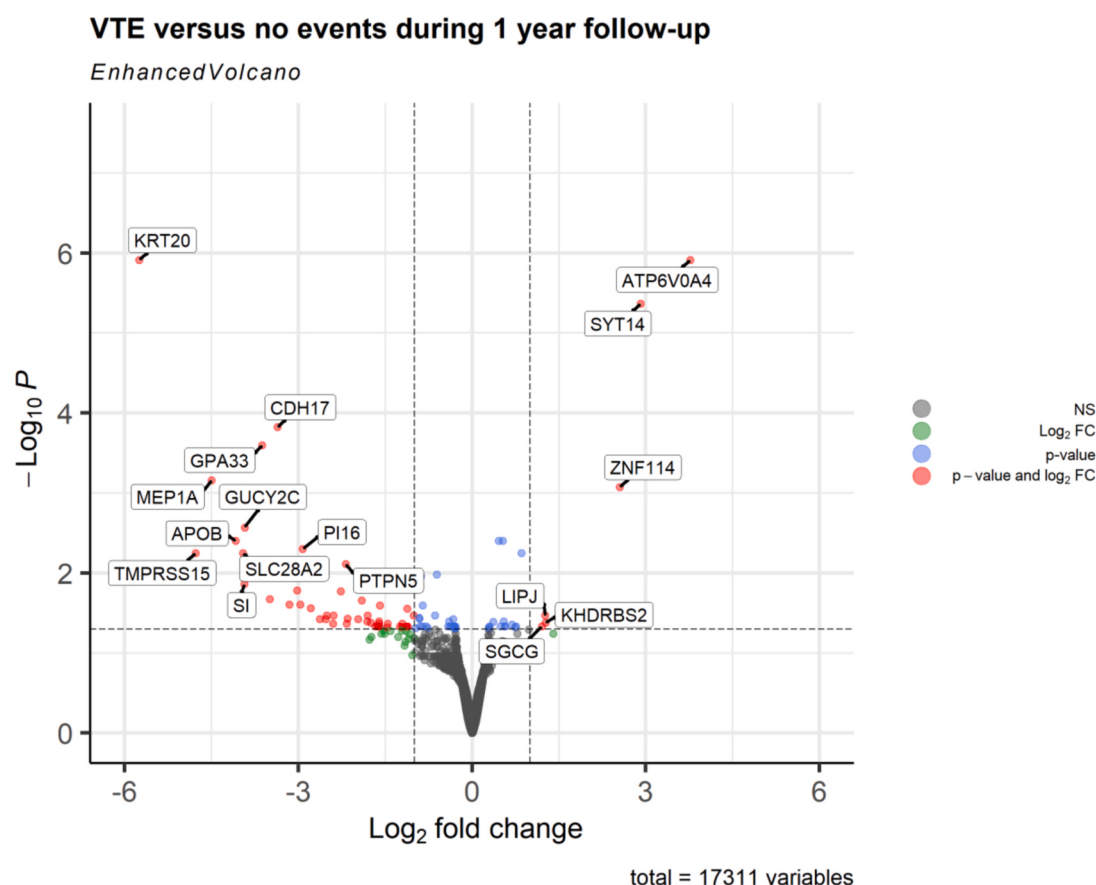
*MEP1A* ( $\log_2\text{FC}$  -4.50, FDR 0.004). Genes differentially expressed with a  $\log_2$  fold change higher than 2 or lower than -2 are summarized in Table 2. Remarkably, no genes known to be associated with thrombosis, coagulation pathways, or platelet function were differentially expressed. Gene set enrichment analysis resulted in four pathways significantly enriched in patients with VTE: keratinization (Enrichment score [ES] 0.82, Q-value 0.001), keratinocyte differentiation (ES 0.63, Q-value 0.003), epidermis development (ES 0.44, Q-value 0.04), and protein-containing complex remodeling (ES -0.90, Q-value 0.04).

Validation of signature genes in external datasets of patients with colorectal and lung cancer revealed that *SYT14* was significantly differentially expressed between patients with and without VTE in the current cohort ( $\log_2\text{FC}$  2.91; FDR  $4.31 \times 10^{-6}$ ) as well as in those with lung cancer. Several other genes previously described as associated with VTE were differentially expressed with high  $\log_2$  fold change in the current cohort, including *DEFA5* ( $\log_2\text{FC}$  -5.87), *SPINK4* ( $\log_2\text{FC}$  -5.82) and *REG4* ( $\log_2\text{FC}$  -3.37) in colorectal cancer and *DSG1* ( $\log_2\text{FC}$  3.29) in lung cancer, although these association did not reach statistical significance after adjusting for multiple testing. (sup Table 1).

Finally, we noted that 3 genes significantly associated with no VTE are amongst the 25 signature genes for classical molecular subtype as proposed by Moffitt et al., including *KRT20*, *CDH17* ( $\log_2\text{FC}$  -3.37, FDR 0.0002) and *MYO1A* ( $\log_2\text{FC}$  -2.59, FDR 0.01) [15]. Therefore, VTE incidence was also assessed in classical versus basal-like molecular subtype.

### 3.3. VTE in patients with classical vs. basal-like molecular subtype

Since several signature genes for classical subtype PDAC were significantly downregulated in patients with VTE, we assessed whether classical or basal-like molecular subtype was associated with VTE. For this analysis we classified samples as classical or basal-like according to the PuriST classifier [16]. Classical and basal-like molecular subtype can further be classified as strong, likely, or leaning classical or basal-like. 126 samples (83.4 %) were classified as classical molecular subtype, of which 117 were classified as strong classical (77.5 %). Twenty-five



**Fig. 1.** Volcano plot of differentially expressed genes in patients VTE versus no VTE during one year of follow-up. In red genes with a false discovery rate < 0.05 and Log2 fold change > 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(16.6 %) samples were basal-like, of which 11 (7.3 %) were strong basal-like. 2805 (16.2 %) genes were differentially expressed in patients with classical versus basal-like subtype. Differential expression analysis in strong classical versus strong basal-like resulted in 3996 (23 %) significantly differentially expressed genes. 33 (26.2 %) patients with classical subtype died during follow-up, compared with 15 (60 %) in the basal-like subtype group ( $p = 0.0001$ ; Fig. 2A). One-year VTE incidence was 7.2 % ( $n = 9$ ) in patients with classical subtype tumors compared to 4.0 % ( $n = 1$ ) in patients with basal-like tumor (SHR 1.79; 95 % CI 0.22–14.3) (Fig. 2B).

To formally ascertain genes associated with VTE regardless of molecular subtype, we assessed gene expression in patients with VTE versus no VTE in patients with classical subtype only, in which 90 % of VTE events occurred. Differential expression of genes in patients with classical subtype and VTE ( $n = 9$ ) versus classical subtype and no VTE ( $n = 116$ ) showed that 4 genes significantly associated with VTE and 23 genes associated with no VTE. Genes associated with no VTE were comparable to differentially expressed genes in VTE versus controls in the whole cohort, including the three signature genes for classical subtype *KRT20* (log2FC -5.83, FDR  $8.74 \times 10^{-6}$ ), *CDH17* (log2FC -3.57, FDR 0.0002), and *MYO1A* (log2FC -2.64, FDR 0.03). *EREG* (log2FC 1.85, FDR 0.05) and *LIPJ* (log2FC 1.64, FDR 0.03) were significantly associated with VTE (sup. Table 2).

### 3.4. Expression of coagulation related genes in classical vs. basal-like molecular subtype

To assess a potential difference in thrombogenic phenotype between subtypes, we assessed differential expression of coagulation related genes in basal-like and classical molecular subtype. A total of 324 genes

from the Gene ontology and KEGG coagulation, complement and platelet pathways (GO:0050817, hsa04610, hsa04611) were assessed. Out of 324 genes, 42 (13.0 %) were significantly differentially expressed (Fig. 3). Notable genes associated with the classical subtype were coagulation factor V (*F5*, log2FC 1.42, FDR  $1.7 \times 10^{-6}$ ), factor XI (*F11*, log2FC 1.31, FDR 0.002), and complement Component 4 Binding Protein Beta (*C4BPB*, log2FC 1.18, FDR  $7.1 \times 10^{-5}$ ), which is responsible for inhibition of the natural anticoagulant protein S [26,27]. Transcription factors *HNF4α* (log2FC 2.0, FDR  $4.4 \times 10^{-15}$ ) and *FOXA2* (log2FC 1.23, FDR  $2.19 \times 10^{-6}$ ) both involved in regulation of coagulation related gene expression, were significantly associated with classical subtype [28–30]. Amongst genes significantly associated with basal-like subtype were proteins associated with thrombosis, including S100 calcium-binding protein A9 (*S100A9*, log2FC -1.52, FDR  $2.1 \times 10^{-6}$ ) [31], Plasminogen activator inhibitor-1 (*SERPINE1*, log2FC -0.85, FDR 0.002) [10], and interleukin 6 (*IL6*, log2FC -1.14, FDR 0.002) [32] and proteins associated with fibrinolysis including plasminogen activator, urokinase (*PLAU*, log2FC -0.85, FDR 0.0004) [33] and the thrombin inhibitor protease nexin-1 (*SERPINE2*, log2FC -1.18, FDR  $6.7 \times 10^{-7}$ ) [34]. Caveolin 1 (*CAV1*, log2FC -1.25, FDR  $2.05 \times 10^{-6}$ ) a signal transduction protein, reported to be involved in exposure and function of tissue factor pathway inhibitor, was significantly associated with basal-like subtype [35,36].

Next, we assessed differentially expressed genes in patients with ‘strong classical’ ( $n = 115$ ) versus ‘strong basal-like’ ( $n = 12$ ). This analysis revealed 74 (22.5 %) differentially expressed coagulation-related genes. Similar to the previous analysis, *F5*, *F11*, *C4BPB*, *HNF4α* and *FOXA2* were significantly associated with classical subtype, while *PLAU*, *PAI-1*, *CAV-1* and *S100A9* were significantly associated with basal-like subtype. Amongst other differentially expressed genes were

Table 2

Genes significantly differentially expressed and log2 fold change >2 in patients with deep vein thrombosis or pulmonary embolism compared to patients with no thromboembolic events.

| Downregulated |                                                         |                                                                                       |                  |                       |
|---------------|---------------------------------------------------------|---------------------------------------------------------------------------------------|------------------|-----------------------|
| Gene symbol   | Name                                                    | Function                                                                              | log2 Fold Change | FDR                   |
| KRT20         | Keratin 20                                              | Maintaining keratin filament organization                                             | −5.75            | 1.22*10 <sup>−6</sup> |
| TMPRSS15      | Transmembrane protease, serine 15                       | Activation of pancreatic proteolytic proenzymes                                       | −4.78            | 0.005652              |
| MEP1A         | Meprin A, alpha                                         | Hydrolysis of protein and peptide substrates                                          | −4.50            | 0.000689              |
| APOB          | Apolipoprotein B                                        | Recognition signal for the cellular binding and internalization of LDL                | −4.08            | 0.003973              |
| SLC28A2       | Solute carrier family 28, member 2                      | Sodium-dependent and purine-selective transporter                                     | −3.96            | 0.005652              |
| SI            | Sucrase-isomaltase                                      | Carbohydrate digestion.                                                               | −3.94            | 0.013764              |
| GUCY2C        | Guanylate cyclase 2C                                    | Catalyzes synthesis of cyclic GMP                                                     | −3.93            | 0.00269               |
| GPA33         | Glycoprotein A33                                        | Cell-cell recognition and signalling                                                  | −3.63            | 0.000253              |
| MTRNR2L8      | MT-RNR2-like 8                                          | Neuroprotective and antiapoptotic factor                                              | −3.50            | 0.021237              |
| CDH17         | Cadherin 17, LI cadherin                                | Calcium-dependent cell adhesion protein                                               | −3.37            | 0.00015               |
| CHST5         | Carbohydrate Sulfotransferase 5                         | Sulfotransferase                                                                      | −3.16            | 0.024646              |
| NEU4          | Sialidase 4                                             | Catalyzes the hydrolytic cleavage of sialic acids                                     | −3.03            | 0.01638               |
| NPPC          | Natriuretic peptide C                                   | Endochondral ossification                                                             | −2.97            | 0.024646              |
| PI16          | Peptidase inhibitor 16                                  | May inhibit cardiomyocyte growth                                                      | −2.93            | 0.004975              |
| MOGAT2        | Monoacylglycerol O-acyltransferase 2                    | Catalyzes the formation of diacylglycerol from 2-monoacylglycerol and fatty acyl-CoA. | −2.79            | 0.02751               |
| SHD           | Src homology 2 domain containing transforming protein D | May function as an adapter protein                                                    | −2.63            | 0.03757               |
| MYO1A         | Myosin IA                                               | Movement of organelles along actin filaments                                          | −2.59            | 0.011156              |
| ADH4          | Alcohol dehydrogenase 4                                 | Oxidizes long chain omega-hydroxy fatty acids                                         | −2.53            | 0.03757               |
| APC2          | Adenomatosis polyposis coli 2                           | Stabilizes microtubules                                                               | −2.52            | 0.034081              |
| CYP3A4        | Cytochrome P450, family 3, subfamily A, polypeptide 4   | Involved in the metabolism of sterols, steroid hormones, Retinoids and fatty acids    | −2.40            | 0.042837              |
| TUBAL3        | Tubulin, alpha-like 3                                   | Constituent of microtubules                                                           | −2.39            | 0.034081              |

Table 2 (continued)

| Downregulated |                                                   |                                                                                                   |                  |                       |
|---------------|---------------------------------------------------|---------------------------------------------------------------------------------------------------|------------------|-----------------------|
| Gene symbol   | Name                                              | Function                                                                                          | log2 Fold Change | FDR                   |
| SOX2          | SRY (sex determining region Y)-box 2              | Controls expression of genes involved in embryonic development                                    | −2.27            | 0.016974              |
| PTPN5         | Protein tyrosine phosphatase, non-receptor type 5 | activity of several effector molecules involved in synaptic plasticity and neuronal cell survival | −2.19            | 0.007677              |
| MOGAT3        | Monoacylglycerol O-acyltransferase 3              | Catalyzes the formation of diacylglycerol from 2-monoacylglycerol and fatty acyl-CoA              | −2.17            | 0.042837              |
| CNTFR         | Ciliary neurotrophic factor receptor              | Receptor for heterodimeric neurotrophic cytokine                                                  | −2.15            | 0.0369                |
| Upregulated   |                                                   |                                                                                                   |                  |                       |
| Gene Symbol   | Name                                              | Function                                                                                          | log2 Fold Change | FDR                   |
| ZNF114        | Zinc finger protein 114                           | Involved in transcriptional regulation                                                            | 2.55             | 0.000841              |
| SYT14         | synaptotagmin XIV                                 | trafficking and exocytosis of secretory vesicles in non-neuronal tissues                          | 2.91             | 4.31*10 <sup>−6</sup> |
| ATP6V0A4      | ATPase, H+ transporting, lysosomal V0 subunit a4  | Hydrolyzes ATP                                                                                    | 3.77             | 1.22*10 <sup>−6</sup> |

Abbreviations: FDR, false discovery rat.

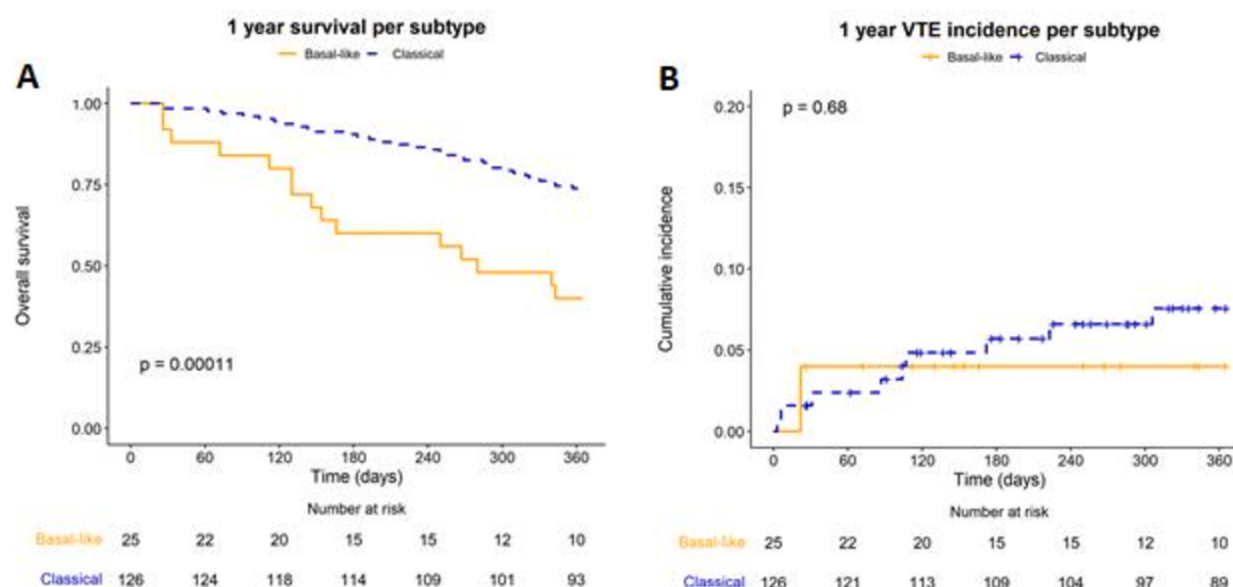
coagulation factor X (*F10*, Log2FC 1.48, FDR 1.0\*10<sup>−5</sup>), Protein C inhibitor (*SERPINA5*, Log2FC 1.05, FDR 0.007), both associated with classical subtype (sup. Fig. 1).

GSEA showed no significantly enriched coagulation related pathways in classical versus basal-like subtype. GSEA in strong classical versus strong basal-like subtype yielded 209 pathways significantly enriched, including the pathways ‘coagulation’ (GO:0050817, ES 0.49, Q-value 0.04) and ‘blood coagulation’ (GO:0007596, ES 0.49, Q-value 0.04) (sup. Fig. 2).

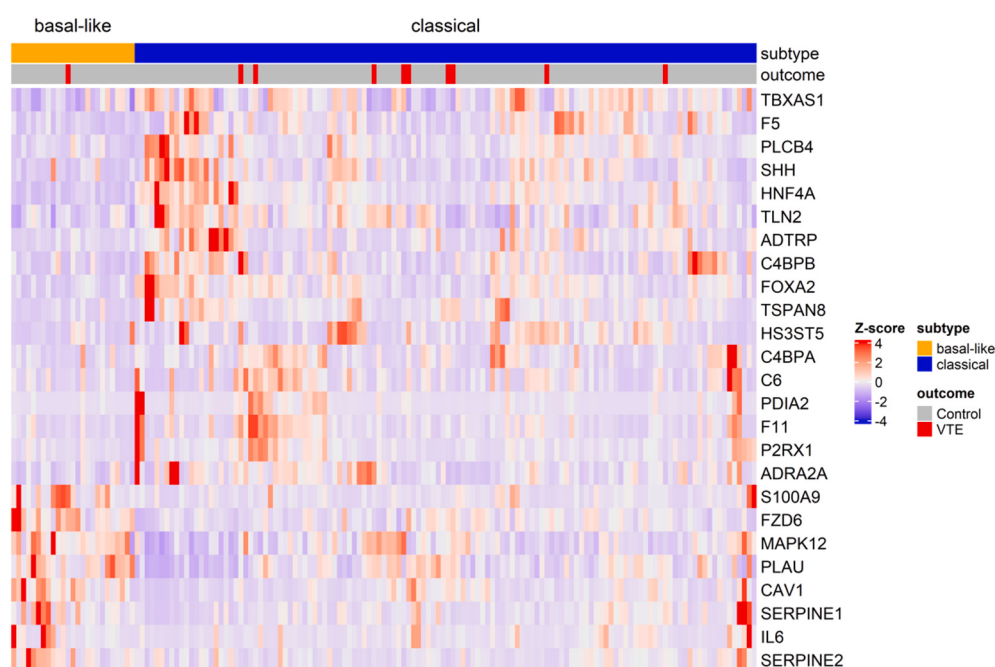
Expression of coagulation related genes in classical versus basal-like subtype with a log2 fold change ≥0.5 or ≤ −0.5 were evaluated in three external dataset of PDAC in which classical vs. basal-like subtype per sample was available. Clustered heatmaps showed clustering of basal-like and classical subtype in all three cohorts with similar over- and under expression in both subtypes as in our study cohort (Fig. 4).

4. Discussion

Tumor gene expression analysis of patients with pancreatic cancer showed that expression of 89 genes were significantly associated with VTE, of which 20 genes showed significantly higher expression in patients with VTE and 69 genes showed significantly lower expression in patients with VTE. In an analysis stratified into two different commonly used molecular subtypes of PDAC [15,16], the incidence of VTE was numerically higher in patients with classical compared to basal-like molecular subtype, but this difference was not statistically significant. In an analysis of classical subtype only, we found that expression of *KRT20*, *MYO1A* and *CDH17*, three genes previously classified as signature genes for classical subtype, were amongst genes associated with no



**Fig. 2.** One year overall survival (A) and cumulative incidence of VTE (B) in patients with classical (blue) versus basal-like (yellow) molecular subtype of pancreatic ductal adenocarcinoma. Significance assessed using the log-rank test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

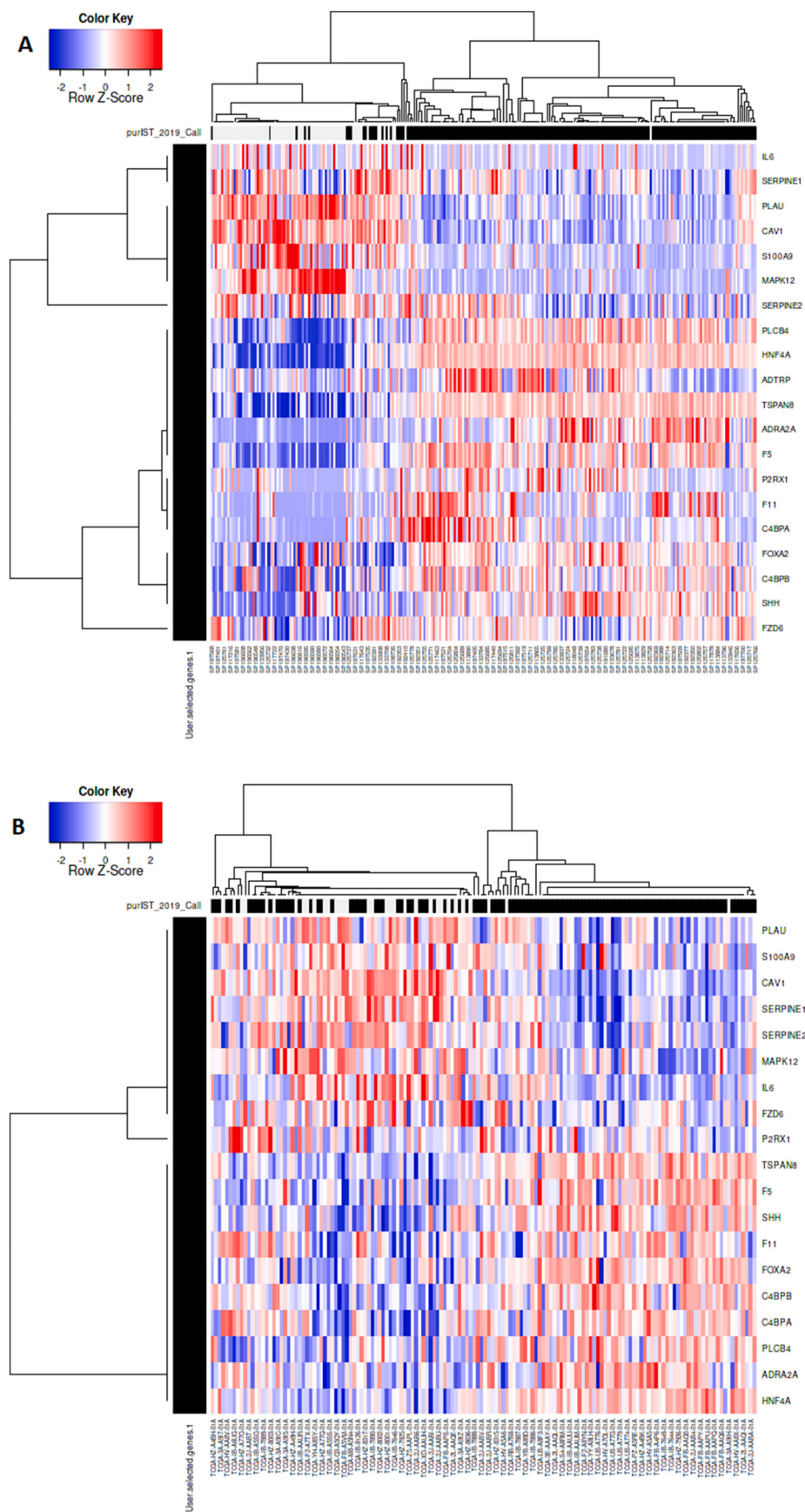


**Fig. 3.** Heatmap of significantly differentially expressed coagulation related genes with a log2 fold change  $>0.5$  or  $<-0.5$  in patients with classical vs basal-like subtype.

VTE. Finally, we found an abundance of coagulation-related genes differentially expressed across the two molecular subtypes, including prothrombotic coagulation factors upregulated in classical subtype while proteins involved in fibrinolysis were more upregulated in basal-like subtype, suggesting that VTE risk might be different in these subtypes.

While several genes were significantly differentially expressed in patients with VTE versus controls, none of these have a known association with thrombosis, coagulation, or platelet activation. Several differentially expressed genes are known to be associated with poor prognosis or tumor progression. *ATP6V0A4* is a gene which encodes a

component of vacuolar-type ATPase, a proton pump responsible for controlling the intracellular and extracellular pH of cells, which has been reported to predict survival in patients with pancreatic cancer [37,38]. A high level of *ZNF114*, a transcription regulator found to be upregulated in several tumor types, has been reported to be associated with poor outcome in renal cell carcinoma [39,40]. *SYT14* is a gene known to be responsible for cell proliferation in glioma tumors, which was also found to be significantly upregulated in lung cancer patients with VTE compared to no VTE [12,41]. The expression of all these three genes was significantly associated with VTE. Expression of *EREG*, the gene encoding epiregulin, an epidermal growth factor, was significantly



**Fig. 4.** Heatmaps of coagulation related genes expressed in ICGC-PACA-CA (A) and TCGA PAAD (B). molecular subtype call per sample according to PuriST classifier is noted at the top of the map in grey (basal-like) and black (classical). Heatmaps of coagulation related genes expressed in data set from Moffitt et al. (GSE71729) (C). molecular subtype call per sample according to PuriST classifier is noted at the top of the map in grey (basal-like) and black (classical).

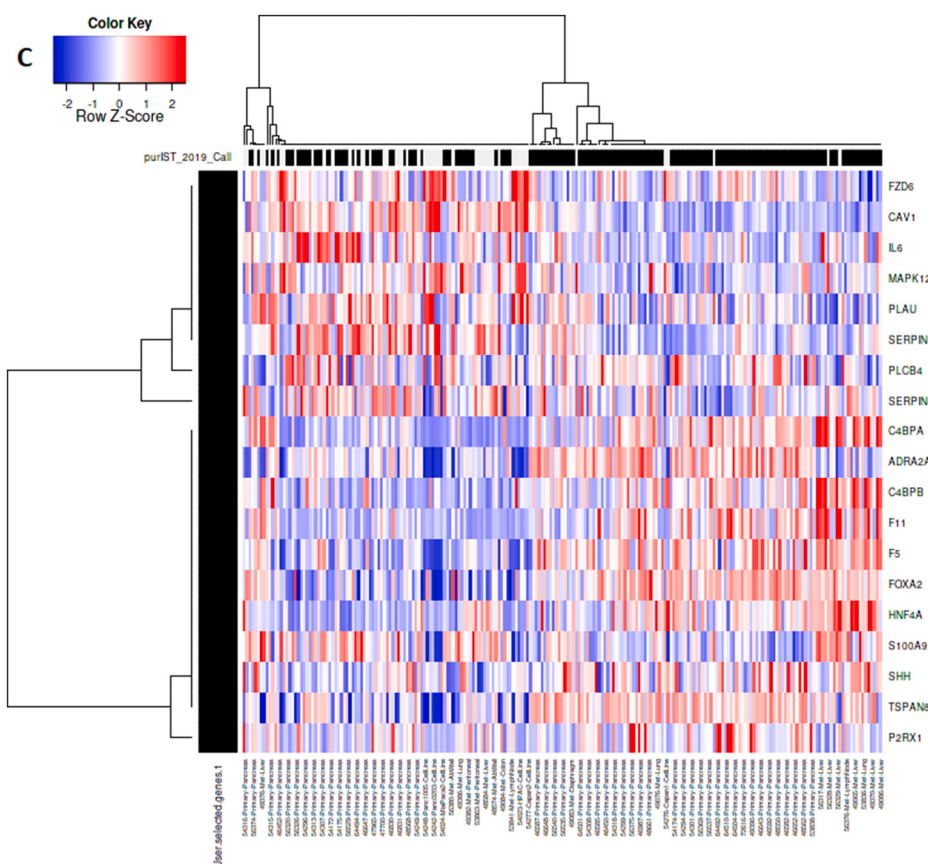


Fig. 4. (continued).

associated with VTE in patients with classical subtype. This gene is reported to be associated with pancreatic tumor growth and is associated with poor outcome or tumor progression in many cancer types including lung, gastric, bladder, breast, brain and colorectal cancer [42,43]. The differential expression of genes associated with poor outcome or tumor progression is in line with the higher recurrence rate in patients with VTE (70 %) compared to those without VTE (43 %). These early recurrences may have led to a hypercoagulable state resulting in a higher VTE incidence.

*KRT20* expression is also associated with poor overall survival, specifically in patients with pancreatic cancer [44]. It has been proposed to be used as biomarker for poor prognosis after R0 resection [45]. Interestingly, *KRT20* was significantly associated with no VTE with the highest log2FC in patients with classical molecular subtype only. Additionally, *MYO1A* and *CDH17*, were significantly downregulated in patients with VTE and classical type. The fact that expression of these three genes, all signature genes for classical subtype according to the original manuscript by Moffitt et al., was significantly lower in patients with classical subtype and VTE compared to those with classical subtype and no VTE, suggests a potentially important difference in genetic tumor properties in these patients. Validation in external PDAC datasets with VTE as outcome need to confirm a causal effect between differential expression of the genes and VTE risk.

Since VTE in cancer patients, especially in pancreatic cancer, is likely a multifactorial disease due to patient-related risk factors, tumor properties, and treatments, we hypothesize that molecular subtypes of specific tumors could be a better predictor of VTE risk than a small set of differentially expressed genes. While we could not confirm a significant risk difference between classical and basal-like subtypes, possibly due to limited power, we did find important differences in expression of coagulation-related genes. These findings were subsequently confirmed in three external PDAC datasets. Upregulation of several prothrombotic

genes was significantly associated with classical subtype, including *F5*, *F11*, and *C4BPB*, a protein involved in protein S inhibition [27], while other genes associated with fibrinolysis or inhibition of thrombin formation were significantly upregulated in basal-like subtype, including *PLAU*, the gene for urokinase-type plasminogen activator, an important protein for fibrinolysis [47], and *SERPINE2*, which functions as an inhibitor of thrombin. [34] While these findings suggest a more thrombogenic phenotype in patients with classical PDAC, the expression of other genes associated with potentially opposite effects were associated with basal-like subtype, such as plasminogen activator inhibitor-1 (*SERPINE1*), which is associated with VTE specifically in patients with pancreatic cancer [10]. Importantly, while several coagulation related genes are differentially expressed, for most genes it is not known in what way they contribute to thrombosis. For example, increased levels of *PAI-1* and activated *F11* are associated with an increased risk of VTE in patients with pancreatic cancer, but whether increased levels of *HNF4a* or *CAV1* contribute to the risk of thrombosis in these patients is unknown [10,49]. These data show that genes associated with blood coagulation are highly and differentially expressed across different PDAC subtypes. What the overall pro- or anticoagulant effect of all these differentially expressed genes is on the occurrence of VTE in these and other subtypes in clinical practice, remains uncertain.

Since enrolment in this study was restricted to patients undergoing surgery with curative intent, the results cannot readily be extrapolated to patients with advanced pancreatic cancer. Additionally, as clinical data were collected retrospectively, outcome events may have been missed resulting in an underestimation of the event rate and bias towards the null in the gene expression analyses. Therefore these findings need to be assessed in prospectively collected cohorts with PDAC patients, preferably in patients with advanced pancreatic cancer, in which the VTE incidence is higher [50].

Genetic heterogeneity within pancreatic ductal adenocarcinoma is

substantial and can affect the course of the disease and response to therapy. Although the association between this molecular landscape and cancer prognosis is increasingly being explored [51,52], data on tumor genes and tumor gene expression in relation to VTE is relatively scarce [11,52,55]. Additionally, as we assessed gene expression in bulk mRNA, studies assessing differences in PDAC tumor cells and stromal cells could provide more insights into the origins of increased VTE risk in these patients. Studies in this field may increase our understanding of the pathogenesis of cancer-associated VTE and potentially improve prediction of VTE in cancer patients, with the ultimate goal to select ambulatory high-risk patients for thromboprophylaxis. The present study suggests that such an approach may be possible. Additional studies are needed to assess VTE risk in patients with advanced pancreatic cancer with differences in tumor gene expression, including differential expression of *ATP6V0A4*, *ZNF114*, *SYT14*, *EREG*, *KRT20*, *MYO1A* and *CDH17*, to confirm or disprove a causal relation between single tumor gene expression levels and thrombosis risk. These studies can also be performed to confirm the hypothesis that VTE risk is different per molecular subtypes. Finally, identified genes need to be functionally validated in vitro and in vivo experiments to improve our understanding of the pathogenesis of VTE related to pancreatic cancer.

### CRedit authorship contribution statement

**Floris T.M. Bosch:** Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Frederike Dijk:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Saskia Briedé:** Writing – review & editing, Data curation. **Jesse V. Groen:** Writing – review & editing, Data curation. **Randa G. Hanna-Sawires:** Writing – review & editing. **Hans Halfwerk:** Writing – review & editing, Formal analysis, Data curation. **Frederikus A. Klok:** Writing – review & editing. **Karin A.H. Kaasjager:** Writing – review & editing. **Lodewijk A.A. Brosens:** Writing – review & editing. **Quintus Molenaar:** Writing – review & editing. **Bert A. Bonsing:** Writing – review & editing. **Sven Mieog:** Writing – review & editing. **Marc G. Besselink:** Writing – review & editing. **Olivier R. Busch:** Writing – review & editing. **Joanne Verheij:** Writing – review & editing. **Arantza Farina Sarasqueta:** Writing – review & editing. **Hanneke W. Wilmink:** Writing – review & editing. **Jan Koster:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Maarten F. Bijlsma:** Writing – original draft, Methodology, Data curation, Conceptualization. **Henri H. Versteeg:** Writing – review & editing. **Nick van Es:** Writing – original draft, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Jeroen T. Buijs:** Writing – original draft, Supervision, Project administration, Methodology, Conceptualization.

### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: FAK reports research support from Bayer, BMS, BSCI, AstraZeneca, MSD, Leo Pharma, Actelion, Farm-X, The Netherlands Organisation for Health Research and Development, The Dutch Thrombosis Foundation, The Dutch Heart Foundation and the Horizon Europe Program, all outside this work and paid to his institution. JWW reports grants made to the institution by MSD, Nordic, Servier and consultancy fees by Astra, Servier and MSD. MFB has received research funding from Celgene, Frame Therapeutics, and Lead Pharma, and has acted as a consultant to Servier, Olympus, and Wholomics. None of these parties were involved in the design of this study or drafting of the manuscript. NE reports receiving advisory board honoraria from Daiichi-Sankyo, LEO Pharma, and Bayer, which were transferred to his institution. All other authors report no conflicts of interest.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.thromres.2024.109240>.

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