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# RNA-sequencing to discover genes and signaling pathways associated with venous thromboembolism in glioblastoma patients: A case-control study

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ABSTRACT

*Background:* Glioblastoma patients are at high risk of developing venous thromboembolism (VTE). Tumor-intrinsic features are considered to play a role, but the underlying pathophysiological mechanisms remain incompletely understood.
*Objectives:* To identify tumor-expressed genes and signaling pathways that associate with glioblastoma-related VTE by using next generation RNA-sequencing (RNA-Seq).
*Methods:* The tumor gene expression profile of 23 glioblastoma patients with VTE and 23 glioblastoma patients without VTE was compared using an unpaired analysis. Ingenuity Pathway Analysis (IPA) core analysis was performed on the top 50 differentially expressed genes to explore associated functions and pathways. Based on full RNA-Seq data, molecular glioblastoma subtypes were determined by performing cluster analysis.
*Results:* Of the 19,327 genes, 1246 (6.4 %) were differentially expressed between glioblastoma patients with and without VTE (unadjusted $P < 0.05$). The most highly overexpressed gene was *GLI1*, a classical target gene in the Sonic Hedgehog (Shh) signaling pathway (log2 fold change: 3.7; unadjusted $P < 0.0001$, adjusted $P = 0.219$). In line, Shh signaling was among the top canonical pathways and processes associated with VTE. The proportion of patients with the proneural/neural glioblastoma subtype was higher among those with VTE than controls.
*Conclusion:* Shh signaling may be involved in the development of glioblastoma-related VTE.

## 1. Introduction

Cancer patients have a nine-fold increased risk of venous thromboembolism (VTE) in the first year after cancer diagnosis compared to individuals without cancer [1]. Moreover, thromboembolic events are an important cause of death in chemotherapy-treated cancer patients besides cancer progression itself [2]. Particularly, patients with glioblastoma suffer from a highly increased risk of VTE (10–30 %) [3–5].

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Tumor-intrinsic features in glioblastoma are thought to contribute to VTE, but the exact underlying pathophysiological mechanisms remain incompletely understood.

Prolonged thromboprophylaxis (beyond the perioperative period) is not generally prescribed in glioblastoma patients due to the increased risk of major bleeding, such as intracranial hemorrhage [6,7]. Risk stratification, to identify glioblastoma patients with the highest risk of VTE, could contribute to personalized thromboprophylaxis. However, the VTE risk assessment score that is currently recommended, the Khorana score, performs sub-optimal in patients with primary brain cancer [8,9]. The discovery of genes or pathways involved in VTE could potentially lead to novel candidate biomarkers to risk stratify high-risk glioblastoma patients for thromboprophylaxis.

Genomic and transcriptomic analyses of tumors are promising avenues for the identification of tumor-expressed genes and somatic mutations associated with VTE. At the genomic level, Ades et al. were the first to describe an association between *KRAS* mutational status and VTE in patients with metastatic colorectal cancer using PCR-based allele-specific amplification data [10]. More recently, Dunbar and colleagues used DNA next-generation sequencing (NGS) data derived from >14,000 solid tumors to identify somatic driver mutations that associate with VTE [11]. Tumor-specific mutations in a set of 6 genes were found to significantly increase the risk of cancer-associated thrombosis (CAT) independent of tumor type. In a comparable approach of targeted DNA-sequencing, we recently identified *CDKN2A* deletion as potential biomarker for glioblastoma-related VTE [12].

At the post-transcriptional level, case-control studies with lung cancer [13] and colorectal cancer [14] showed that next-generation RNA sequencing (RNA-seq) could identify tumor-expressed genes that associate with the development of VTE. Similarly, a selection of 19 tumor-expressed microRNAs was found to associate with VTE in colorectal cancer [15], thus proving the concept of using both genomics as well as transcriptomics in the search for novel, predictive biomarkers of CAT.

In this case-control study, by comparing the gene expression profiles of 23 tumors from glioblastoma patients with VTE to 23 tumors from glioblastoma patients without VTE, we aimed to identify genes or genetic mechanisms that are linked to VTE in patients with glioblastoma.

## 2. Methods

### 2.1. Patient cohort

A retrospective cohort study was performed including 324 consecutive patients with targeted DNA sequencing data available, who were diagnosed with glioblastoma according to the 2021 WHO criteria (IDH-wildtype) in two Dutch hospitals between February 2017 and August 2020 [12]. From all these patients, formalin-fixed paraffin-embedded (FFPE) tumor samples were obtained at the moment of glioblastoma diagnosis. mRNA-sequencing was conducted using tumor material of 25 glioblastoma patients with VTE and 25 glioblastoma patients without VTE (see below), who were individually matched on age, sex and minimal follow-up time. This study was approved by the Medical Ethics Research Committee (LUMC: #B19.039; HMC: #2019–089). The need for informed consent was waived by the institutional review board because of the retrospective study design and the fact that the majority of the patients had died at the start of data collection. Nonetheless, all patients that were alive at the start of this study were contacted and asked for informed consent. The STROBE reporting guidelines were used for reporting the data [16].

### 2.2. Chart review

Clinical and demographic information was retrospectively collected from three months before glioblastoma diagnosis until VTE, death, becoming lost to follow-up, or the end of the maximum observation period of 27 months (i.e., until two years after glioblastoma diagnosis). Patient VTE status, comprising symptomatic or incidental pulmonary embolism (PE; diagnosed by Computed Tomography Pulmonary Angiogram (CTPA) or VQ scan) and distal or proximal deep vein thrombosis (DVT; diagnosed by ultrasonography, conventional venography or CT-venography), as well as patient demographics were retrospectively assessed by chart review. All thromboembolic events were independently adjudicated by two experts who were blinded for any patient characteristics. Cerebral vein thrombosis was not included, due to the potential role of tumor resection and local compression as underlying cause. History of VTE between 12 months and 3 months before glioblastoma diagnosis was set as exclusion criterium, but no VTE events were reported in this time frame.

### 2.3. RNA sequencing

FFPE tumor samples from all patients were collected and prepared for fully automated total nucleic acid extraction as described previously (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) [17]. Subsequently, samples were treated with DNase and mRNA quality was analyzed with a fragment analyzer. rRNA depletion for RNA-Seq library preparation was performed using the QIAseq FastSelect – rRNA HRM kit, followed by mRNA sequencing with the Illumina NovaSeq 6000 system according to the manufacturer's instructions at GenomeScan B.V. (Leiden, The Netherlands). A sequencing depth of 60 million paired-end reads per sample was used.

### 2.4. Analysis of RNA sequencing data

RNA-Seq files were processed using the opensource BIOWDL RNA-seq pipeline v5.0.0 (https://zenodo.org/record/5109461#.Ya2yLFPMJhE). This pipeline performs FASTQ preprocessing (including quality control, quality trimming, and adapter clipping), RNA-Seq alignment, read quantification, and optionally transcript assembly. FastQC was used for raw FASTQ read quality control. Adapter clipping was performed using Cutadapt (v2.10) with default settings. RNA-Seq reads' alignment was performed using STAR (v2.7.5a) on GRCh38 human reference genome. UMI based deduplication was performed using UMI-tools (v1.1.1). The gene read quantification was performed using HTSeq-count (v0.12.4) with setting "–stranded = yes". The gene annotation used for quantification was Ensembl version 105. Using the gene read count matrix, counts per million (CPM) were calculated per sample on all annotated genes. 19,327 genes with a CPM > 1 in at least 25 % of all samples were kept for downstream analysis. Since the paired samples (matched on age, sex and minimal follow-up time as described before) did not show clustering when performing principal component analysis (PCA), subsequent analyses were performed unpaired to avoid bias resulting from the objective sample matching.

For differential gene expression analysis, dgeAnalysis R-shiny application (https://github.com/LUMC/dgeAnalysis/tree/v1.4.4) was used. EdgeR (v3.34.1) with TMM normalization was employed to perform differential gene expression analysis. Benjamini and Hochberg False Discovery Rate (FDR) was computed to adjust P-values, obtained with the quasi-likelihood (QL) F-test, for each differentially expressed gene.

Ingenuity Pathway Analysis (IPA) core analysis (Ingenuity Systems, Qiagen, Venlo, The Netherlands) was used on the top 50 differentially regulated genes to identify the main pathways involved [18]. Cluster analysis was performed using a 500 Gene Model which was previously shown to be sufficient for recapitulation of the three glioblastoma subtypes: classical, mesenchymal and proneural/neural [19]. The raw read counts of the 500 genes were normalized using TMM after which k-means clustering was performed with R v4.1.0. Clusters were assigned to the specific subtypes by examining the expression profile of subtype-specific genes that were previously identified by Teo et al [19]. After

defining the clusters, PCA and t-distributed stochastic neighbor embedding (t-SNE) have been performed, coloring the subtypes on the earlier created clusters.

### 2.5. Statistical analysis

The a priori sample size calculation was based on a standardized effect size of 0.617, and an 80 % power to detect 2.5 % of the total number of genes with a 2-fold regulation, resulting in 25 patients per group. FDR adjustment was performed with the Benjamini-Hochberg method [20]. Fisher's Exact Test was used for IPA core analysis and to evaluate the association between glioblastoma subtypes and development of VTE in our cohort. Univariable Cox regression models were used to calculate Hazard Ratios (HR) with corresponding 95 % confidence intervals (CI), describing associations between the risk of VTE and available clinical variables. Time-dependent Cox regression analysis was performed to determine the association between VTE and survival. Data were analyzed using GraphPad Prism version 9 and SPSS software version 25.0.0.2.

## 3. Results

### 3.1. Patient characteristics

Of the 324 patients with glioblastoma, 25 (7.7 %) developed VTE within three months before glioblastoma diagnosis until two years after [12]. mRNA sequencing was performed using FFPE tumor material obtained at the time of glioblastoma diagnosis. After strict RNA quality control, two VTE patients had to be excluded due to low complexity (Fig. 1). A nested case-control study was conducted with the remaining 23 patients with objectively confirmed VTE and 23 glioblastoma patients without VTE who were individually matched on age, sex and minimal follow-up time.

At baseline, the median age of the total study population was 68 years (IQR: 61–72) and the minority of patients was female (34.8 %, 16/46; Table 1; Table S1). The performance status of VTE patients (39.1 % with ECOG score 1 and 34.8 % with ECOG score 2) was reported to be worse in comparison to control patients (52.2 % with ECOG score 1 and 13.0 % with ECOG score 2). In both the control and the VTE group, most patients underwent tumor resection at the time of glioblastoma diagnosis (65.2 % and 56.5 %, respectively). Primary glioblastoma treatment consisted mostly of concomitant radio-chemotherapy (regular or short-course; 56.5 % in control patients and 60.8 % in VTE patients). Recurrence was observed in 69.6 % of the control group and 43.5 % of the VTE group. At the end of data collection, 73.9 % of control patients and 65.2 % of VTE patients had died. In both groups, one patient (4.3 %) was lost to follow-up.

Of the patients with VTE, fifteen had PE (65.2 %), seven DVT (30.4 %) and one both (4.3 %) in the period between three months before glioblastoma diagnosis until one year after. The median time between diagnosis and VTE was 2.7 months (IQR: 1.1–3.9). None of the patients

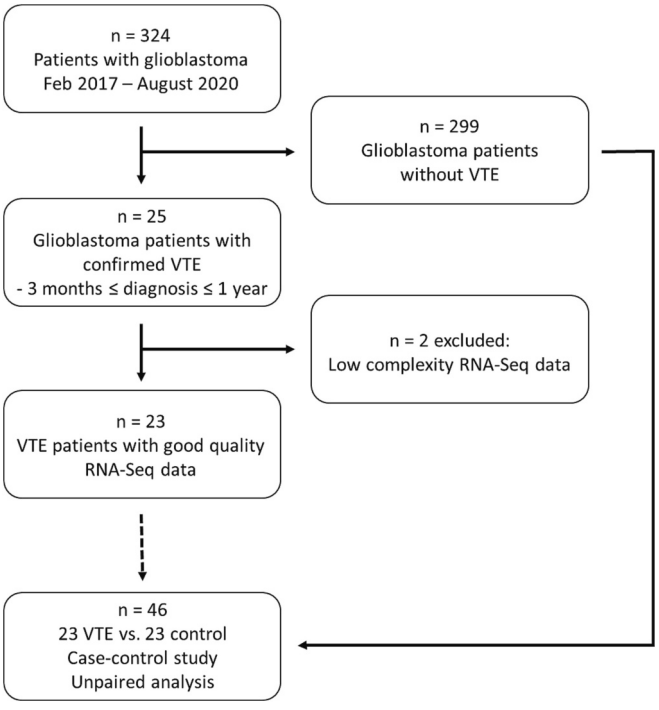


**Fig. 1.** Flowchart showing the design and inclusion of the study.
Abbreviations: Feb, February; VTE, venous thromboembolism; RNA-Seq, RNA sequencing.

**Table 1**
Patient characteristics.

| | | Control (n = 23) | VTE (n = 23) |
|---|---|---|---|
| Age in years (IQR) | | 68 (60–71) | 68 (61–72) |
| Sex | *Male* | 15 (65.2 %) | 15 (65.2 %) |
| | *Female* | 8 (34.8 %) | 8 (34.8 %) |
| *MGMT* promoter methylation | *Yes* | 4 (17.4 %) | 9 (39.1 %) |
| | *No* | 19 (82.6 %) | 13 (56.5 %) |
| | *Unknown* | – | 1 (4.3 %) |
| ECOG performance status | *0* | 6 (26.1 %) | 5 (21.7 %) |
| | *1* | 12 (52.2 %) | 9 (39.1 %) |
| | *2* | 3 (13.0 %) | 8 (34.8 %) |
| | *3* | 2 (8.7 %) | 1 (4.3 %) |
| | *4* | – | – |
| Type of surgery | *Biopsy* | 8 (34.8 %) | 10 (43.5 %) |
| | *Resection* | 15 (65.2 %) | 13 (56.5 %) |
| Therapy | *Concomitant TMZ/RT* | 12 (52.2 %) | 9 (39.1 %) |
| | *Concomitant TMZ/RT (short-course)* | 1 (4.3 %) | 5 (21.7 %) |
| | *RT only* | 6 (26.1 %) | 5 (21.7 %) |
| | *Chemotherapy only* | 1 (4.3 %) | 1 (4.3 %) |
| | *None* | 3 (13.0 %) | 3 (13.0 %) |
| Recurrence | | 16 (69.6 %) | 10 (43.5 %) |
| Status at end of data collection | *Follow-up period < 2 years* | 4 (17.4 %) | 6 (26.1 %) |
| | *Lost to follow-up* | 1 (4.3 %) | 1 (4.3 %) |
| | *Stable disease* | – | – |
| | *Progressive disease* | 1 (4.3 %) | 1 (4.3 %) |
| | *Died* | 17 (73.9 %) | 15 (65.2 %) |
| Type of VTE | *PE* | – | 15 (65.2 %) |
| | *DVT* | – | 7 (30.4 %) |
| | *Both* | – | 1 (4.3 %) |
| Moment of VTE | *Within 3 months before glioblastoma diagnosis* | – | 2 (8.7 %) |
| | *Within 6 weeks after glioblastoma diagnosis* | – | 6 (26.1 %) |
| | *During primary treatment of glioblastoma* | – | 8 (34.8 %) |
| | *After primary treatment of glioblastoma* | – | 6 (26.1 %) |
| | *During treatment of glioblastoma recurrence (n = 10)* | – | 1 (4.3 %) |

Baseline characteristics (age, sex, MGMT promoter methylation, ECOG performance status, type of surgery) were determined at the time of glioblastoma diagnosis.
Abbreviations: IQR, interquartile range; MGMT, O6-methylguanine-DNA methyltransferase; ECOG, eastern cooperative oncology group; TMZ, temozolomide; RT, radiotherapy; VTE, venous thromboembolism; PE, pulmonary embolism; DVT, deep vein thrombosis.

received anticoagulant treatment prior to a VTE event. The clinical variables *MGMT* promoter methylation, ECOG performance status and type of surgery did not significantly associate with VTE in our study population (Table S2). Also, no significant association was observed between VTE and poor survival (time-dependent Cox regression analysis: HR: 1.55; 95 % CI: 0.76–3.14; P = 0.225).

### 3.2. RNA-Seq analysis

Unpaired next generation RNA-Seq analysis revealed differential expression of 1246 out of 19,327 genes (6.4 %) in the tumor of glioblastoma patients with VTE as compared to control patients (unadjusted P < 0.05; Fig. 2). Of these differentially expressed genes, 707 (56.7 %) were overexpressed and 539 (43.3 %) were underexpressed. All genes in the top 10 showed a positive correlation with VTE, except for *FRZB* (log2 fold change (FC): −1.8, unadjusted P = 1.59E-04, adjusted P = 0.294; Table 2). The most highly upregulated genes were *GLI1*, the classic Sonic Hedgehog (Shh) target gene (log2 FC: 3.73, unadjusted P = 4,94E-05, adjusted P = 0.219), *IGF2*, which encodes the growth hormone Insulin-like Growth Factor 2 (log2 FC: 3.33, unadjusted P = 9.87E-05, adjusted P = 0.272) and *MYO15A* (log2 FC: 3.31, unadjusted P = 4.29E-05, adjusted P = 0.219), encoding an unconventional myosin protein involved in hair cell actin organization inside the cochlea. None of the genes were found to be statistically significant after adjustment for FDR. See Table S3 for the top 50 of differentially expressed genes.

### 3.3. IPA analysis

In addition to *GLI1*, four other genes in the top 10 of differentially expressed genes are engaged in axonal guidance signaling, e.g. through Shh (*NHLH1*) or Wnt signaling pathways (*DRAXIN*, *TMEM88B*, and *FRZB*). Indeed, when performing core analysis with IPA software based on the top 50 of differentially expressed genes, Shh signaling was widely represented in the top canonical pathways that were identified (*Sonic Hedgehog Signaling*: P = 1.19E-03; *Basal cell carcinoma signaling*: P = 7.15E-03; *Osteoarthritis pathway:* P = 8.26E-03). Furthermore, IPA analysis showed *SUFU*, a negative regulator of the Shh pathway, among the top upstream regulators, and Shh-related gene sets associated with cancer, organismal abnormalities/diseases, cellular development, cell growth, and cell-cell interactions. In addition to Shh signaling, the Wnt signaling pathway was also highlighted by IPA core analysis (*FRZB*, *Osteoarthritis pathway,* top upstream regulator *TCF7L2*). A summary of all IPA results can be found in Table 3.

### 3.4. Molecular subtype analysis

Molecular subtype analysis showed that most glioblastomas in our case-control study were classified as mesenchymal (18/46, 39.1 %), followed by classical (15/46, 32.6 %), and proneural/neural (13/46, 28.3 %) (Fig. 3A, Table 4). The proneural/neural glioblastoma subtype was a risk factor for VTE when compared to the other molecular subtypes (odds ratio: 3.05; 95 % CI: 0.81–10.17; P = 0.19; Fig. 3B, Table 4).

## 4. Discussion

The risk of VTE is highly increased in patients with glioblastoma compared to other cancer types, which suggests a role for tumor-intrinsic features such as the tumor gene expression profile. Next generation RNA-Seq has been shown a promising tool for biomarker prediction over the past decade. This is the first case-control study on glioblastoma-related VTE, in which the RNA-Seq based tumor expression profile of 23 glioblastoma patients with and 23 glioblastoma patients without VTE was compared. Due to the relatively rare cancer type in combination with the high number of VTE events included in the current study, the prognostic value of this exploratory study may be of significance once externally and functionally validated.

The top-regulated pathway in glioblastoma-related VTE - the Shh signaling pathway - was first discovered in *Drosophila melanogaster* to play a role in early development and segmentation [21]. In humans, Shh signaling is also essential in embryogenesis, including brain development, and adult tissue maintenance [22]. Aberrant Shh activation has been observed in many solid tumors, including brain cancer [23]. In fact, the Shh target gene *GLI1* has initially been discovered in human malignant glioma [24], hence the name *Glioma-associated oncogene homolog 1*. Although being identified as an amplified gene, the incidence of *GLI1* amplification in human glioma was originally reported to be

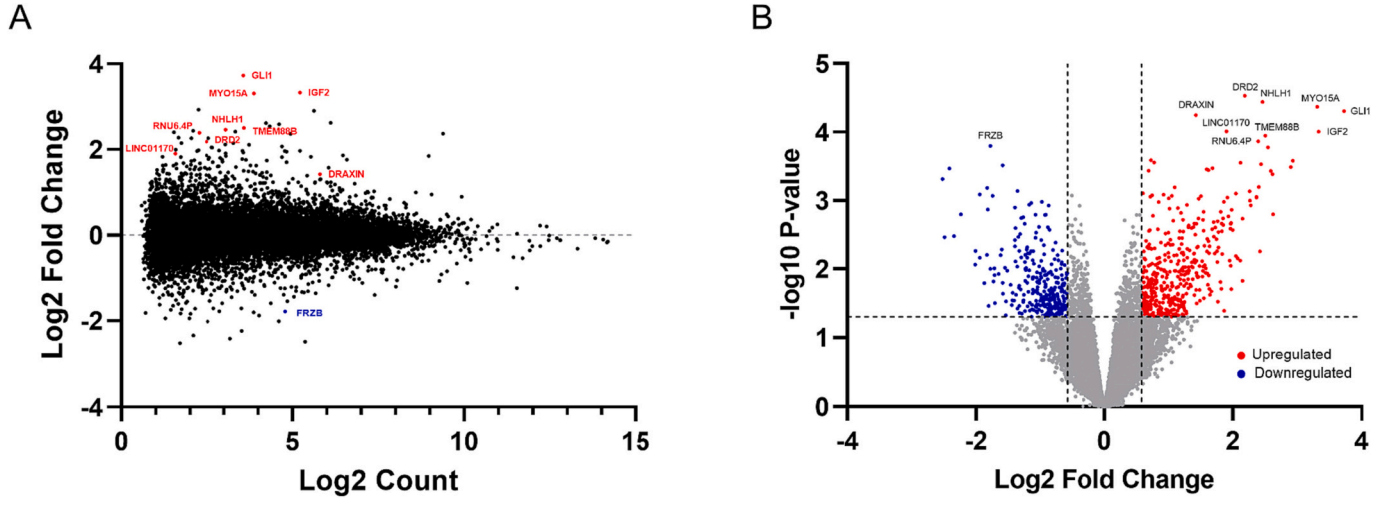


**Fig. 2.** Differentially expressed genes in tumor tissue from glioblastoma patients with and without VTE.
A. MA Bland-Altman plot showing the log2 fold change against the log2 average count for analyzed genes in this study (n = 19.327; CPM > 1 in at least 25 % of all samples). The names of the top 10 differentially expressed genes are shown. Red indicates upregulated genes and blue indicates downregulated genes (based on unadjusted P-values).
B. Volcano plot illustrating the distribution of individual log10 P-values and log2 fold changes of all analyzed genes. All samples above the horizontal dashed line are differentially expressed (unadjusted P < 0.05; n = 1246). The names of the top 10 differentially expressed genes are shown. Red indicates upregulated genes and blue indicates downregulated genes (based on unadjusted P-values). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Top 10 differentially tumor-expressed genes in glioblastoma patients with VTE compared with those without VTE.

| Gene | Full gene name | Average log2 fold change | P-value | Adjusted P-value | Function |
|---|---|---|---|---|---|
| *DRD2* | *Dopamine receptor D2* | 2.184 | 2.93E-05 | 0.219 | Dopamine receptor |
| *NHLH1* | *Nescient helix-loop-helix 1* | 2.460 | 3.65E-05 | 0.219 | Transcription factor growth/development, Hedgehog signaling |
| *MYO15A* | *Myosin XVA* | 3.310 | 4.29E-05 | 0.219 | Myosin: actin conformation ear |
| *GLI1* | *Glioma-associated oncogene homolog 1* | 3.728 | 4.94E-05 | 0.219 | Transcription factor development, Hedgehog signaling |
| *DRAXIN* | *Dorsal inhibitory axon guidance protein* | 1.423 | 5.67E-05 | 0.219 | Axon guidance protein, development CNS, antagonist Wnt signaling |
| *LINC01170* | *Long intergenic non-protein coding RNA 1170* | 1.901 | 9.79E-05 | 0.272 | lncRNA, cell-cycle regulation |
| *IGF2* | *Insulin-like growth factor 2* | 3.330 | 9.87E-05 | 0.272 | Protein hormone similar to insulin, fetal growth factor |
| *TMEM88B* | *Transmembrane protein 88B* | 2.503 | 1.13E-04 | 0.273 | Cardiomyocyte development, suppressor Wnt signaling |
| *RNU6.4P* | *RNA, U6 small nuclear 4, pseudogene* | 2.393 | 1.37E-04 | 0.294 | ncRNA, pseudogene |
| *FRZB* | *Frizzled-related protein (Wnt-binding protein)* | −1.782 | 1.59E-04 | 0.294 | Bone development, antagonist Wnt signaling |

**Table 3**
Summary of Ingenuity Pathway Analysis (IPA) core analysis based on the top 50 differentially expressed genes.

| Top canonical pathways | P-value |
|---|---|
| Sonic Hedgehog signaling | 1,19E-03 |
| Basal cell carcinoma signaling | 7,15E-03 |
| Osteoarthritis pathway | 8,26E-03 |
| Regulation of the epithelial mesenchymal transition in development pathway | 1,03E-02 |
| Zymosterol biosynthesis | 1,05E-02 |

| Diseases and disorders | P-value range |
|---|---|
| Cancer | 7.02E-03 to 5.02E-08 |
| Organismal injury and abnormalities | 7.02E-03 to 5.02E-08 |
| Neurological disease | 7.02E-03 to 9.82E-06 |
| Endocrine system disorders | 7.02E-03 to 3.02E-05 |
| Reproductive system disease | 7.02E-02 to 3.02E-05 |

| Molecular and cellular functions | P-value range |
|---|---|
| Cell morphology | 7.02E-03 to 3.02E-05 |
| Cellular response to therapeutics | 4.68E-03 to 6.32E-05 |
| Cellular development | 7.02E-03 to 1.88E-04 |
| Cellular growth and proliferation | 7.02E-03 to 1.88E-04 |
| Cell-to-cell signaling and interaction | 7.02E-03 to 4.08E-04 |

| Top upstream regulators | P-value overlap |
|---|---|
| TCF7L2 | 2.74E-09 |
| SH3TC2 | 2.33E-07 |
| EGR2 | 1.20E-05 |
| GJB6 | 2.00E-05 |
| SUFU | 2.00E-05 |

relatively low [25,26]. Nevertheless, a more recent study from 2010 identified *GLI1* amplifications in 22.6 % out of 31 glioblastoma samples based on genome-wide copy number analysis [27]. Shh/*GLI1* signaling has been associated with glioma stemness and survival [28]. In glioblastoma, GLI1 expression correlates with tumorigenicity and therapy resistance due to affecting stem cell activity [29].

Additionally, a recently discovered gain-of-function alternative splice variant of *GLI1*, truncated *GLI1* (*tGLI1*), was found in 67 % of glioblastoma cell lines, xenografts and primary specimens, while being undetectable in normal brain tissue [30]. This cancer-specific *GLI1* isoform induces glioblastoma migration, invasion and angiogenesis by also regulating non-GLI1 target genes, thus providing additional evidence for the importance of GLI1 in glioblastoma [31,32].

Shh signaling has been associated with decreased survival in several cancer types [33–35]. However, in glioblastoma, data are contradictory. Rossi et al. described that high GLI1 staining in glioblastoma tumor tissue correlated with poor survival [36]. A similar effect was seen in *PTEN*-expressing human glioblastoma tumors, in which hyperactive Shh/GLI1 signaling resulted in reduced survival time [37]. In contrast, Kim et al. demonstrated that low nuclear GLI1 expression was associated with worse progression-free survival time [38]. However, using the cBioPortal for Cancer Genomics, we found no association between GLI1 upregulation and decreased overall and progression-free survival time in the Glioblastoma Multiforme dataset of the PanCancer Atlas (TCGA, see Fig. S1) [39]. Moreover, VTE was not significantly associated with poor survival in our study population. Taken together, it is unclear at the moment whether the association between Shh signaling and VTE in glioblastoma could be partially explained by increased tumor aggressiveness.

Interestingly, non-canonical Shh signaling has been associated with thrombogenicity in human platelets in a non-cancer setting. That is, Tiwari and colleagues detected synthesis of the Shh ligand from pre-existing mRNAs in thrombin-stimulated platelets [40]. This resulted in Shh surface expression and secretion via extracellular vesicles, thereby establishing autocrine/paracrine feed-forward loops that stimulated platelet activation and thrombogenesis in vitro. Moreover, arterial thrombus formation in mice was impaired upon administration of Shh antagonists, thus providing evidence for a role of Shh signaling in arterial thrombosis in vivo [40]. Furthermore, GLI1 is known to mediate the epithelial-to-mesenchymal transition (EMT) in cancer cells [41], which contributes to tumor cell intravasation into the vasculature. Since EMT triggers a procoagulant state in tumor cells [42], GLI1 could contribute to the increased risk of glioblastoma-related VTE by inducing EMT-mediated procoagulant activity in glioblastoma. However, experimental validation is essential to explore a potential molecular relationship between *GLI1* expression and the procoagulant state of the glioblastoma tumor.

An association has also been described between GLI1 and growth hormone signaling in glioblastoma stem cells. Hsieh et al. reported a decrease in IGF1-dependent stem cell self-renewal, proliferation and

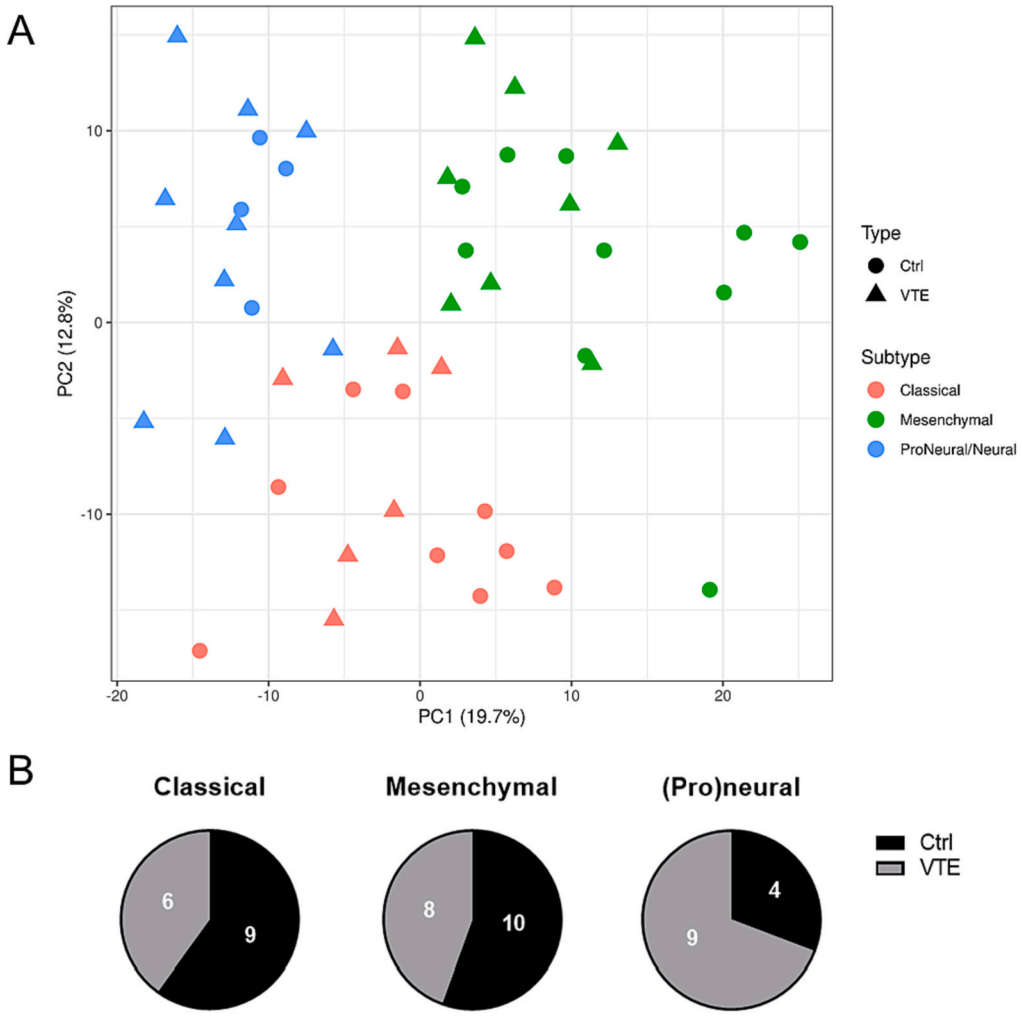


**Fig. 3.** The link between molecular glioblastoma subtype and development of VTE.
A. PCA plot showing the distribution of the glioblastoma subtypes classical (red), mesenchymal (green) and proneural/neural (blue) within the study population. Glioblastoma patients with VTE are indicated with a triangle, whereas control patients are indicated with a circle.
B. Pie charts showing the number of glioblastoma patients with VTE (gray) and glioblastoma patients without VTE (black) per subtype.
Abbreviations: VTE, venous thromboembolism. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 4**
The link between molecular glioblastoma subtype and development of VTE.

| | Total patients | VTE patients | P-value | OR (95 % CI) |
|---|---|---|---|---|
| Classical | 15/46 (32.6 %) | 6/15 (40.0 %) | 0.53 | 0.55 (0.17–1.80) |
| Mesenchymal | 18/46 (39.1 %) | 8/18 (44.4 %) | 0.76 | 0.69 (0.22–2.15) |
| (Pro)neural | 13/46 (28.3 %) | 9/13 (69.2 %) | 0.19 | 3.05 (0.81–10.17) |

Abbreviations: VTE, venous thromboembolism; OR, odds ratio; CI, confidence interval.

angiogenesis following GLI1 suppression, suggesting a role for combined Shh/GLI1 and IGF1 signaling in glioblastoma stem cell migration and invasion [43]. Indeed, stem cell chemoresistance could be terminated by blockade of the GLI1 and IGF1 pathways. Moreover, *IGF1* has been associated with platelet aggregation [44,45], indicating a potential role for growth hormone signaling in thrombogenesis. Since *IGF2* was identified as the second highest upregulated gene in our dataset after *GLI1*, our data underline the relation between Shh/GLI1 and IGF signaling in glioblastoma. However, more research is warranted to determine the potential combined involvement of GLI1 and IGF2 in glioblastoma-related VTE.

*GLI1* and *IGF2* are the two genes with the highest fold change in the top 10 of differentially expressed genes in our study. The top 10 also includes the transcription factor *NHLH1*, another component of the Shh signaling pathway, thus underlining the role of Shh signaling in glioblastoma-related VTE. Furthermore, in agreement with the IPA core analysis, several members of the Wnt signaling pathway are found in the top 10, including *TMEM88B*, *FRZB* and *DRAXIN*. Interestingly, Shh and Wnt signaling are both essential for cell proliferation and cancer stemness, resulting in a complex interaction between both pathways regulating tumorigenicity [46]. However, no link has been described between the Wnt signaling pathway and CAT so far.

Additionally, the top 10 gene *MYO15A*, which is often mutated in patients with hearing loss, was suggested to independently play a role in glioblastoma [47] as well as non-cancer-associated VTE [48], but more research is warranted to explore its role in glioblastoma-related VTE. Another gene in our top 10, the dopamine receptor *DRD2,* also associates with several cancer types including glioblastoma, being involved in tumor growth and stemness [49]. No direct link has been described with (cancer-associated) VTE.

Finally, the top 10 consists of the pseudogene *RNU6.4P* and the long non-coding RNA *LINC01170*, which both do not encode a functional protein. Their exact function in glioblastoma is unknown.

In this study, we performed cluster analysis to explore a potential link between the different glioblastoma subtypes and the risk of VTE. Interestingly, the proportion of patients with proneural/neural glioblastoma was higher among those with VTE than controls, which was not observed for the other molecular subtypes (Fig. 3). This suggests that proneural/neural glioblastoma is a risk factor for VTE, although our findings were not statistically significant. Combined expression of a set of genes, captured in a molecular subtype, may be a greater determinant for a particular outcome (VTE) than the expression of single genes. However, the relative small sample size for this subgroup analysis may have hampered a statistically significant output.

Previously, Magnus et al. determined the so-called coagulome of the different human glioblastoma subtypes by studying the expression of several coagulation-related genes based on 202 patient samples [50]. The mesenchymal subtype, characterized by angiogenesis and EMT, showed the highest upregulation in comparison to the other subtypes. On the other hand, the proneural subtype, which is characterized by stem cell markers, demonstrated a less procoagulant coagulome, as opposed to our results. However, the study by Magnus et al. only examined gene expression levels and not actual VTE events. Considering the association between Shh signaling and stemness in glioblastoma, and the observed link between the Shh pathway and glioblastoma-related VTE in our cohort, an increased risk of VTE in the proneural/neural subtype, in which stem cell markers are upregulated, could be plausible. However, the VTE risk of the different glioblastoma subtypes should be determined at a larger scale in order to be beneficial for prophylactic decision making in the future.

We are the first to describe a case-control study in which the tumor gene expression profile of a relatively large group of glioblastoma patients with VTE was compared to the gene expression profile of glioblastoma patients without VTE. However, our study also has several limitations. First of all, none of the differentially expressed genes in our dataset remained significant after FDR correction. Therefore, the conclusions and implications stated here need to be carefully considered and externally validated first. Nevertheless, as stated by Goeman & Solari, the exploratory nature of our study may allow for a less strict interpretation of the FDR results [51]. The lack of significance in our cohort could be explained by the high degree of inter- and intratumor molecular heterogeneity, which is a well-established hallmark of glioblastoma [52]. Consequently, the presented data derived from our 23 × 23 case-control study may provide less statistical power than a smaller case-control study with a more homogeneous cancer type. Single-cell RNA sequencing would be a good alternative to dissect cellular heterogeneity in glioblastoma and the potential consequences for glioblastoma-related VTE in the future.

Furthermore, it cannot be ruled out that the potential link between Shh signaling and glioblastoma-related VTE as described here is a consequence of Shh signaling being upregulated in glioblastoma per se, and that the association found is merely an epiphenomenon rather than a causal one. Functional studies are essential to investigate a potential mechanistical relation between the Shh pathway and VTE in glioblastoma. Nevertheless, since we specifically compared the tumor expression profile of glioblastoma patients with and without VTE, identification of the Shh target gene *GLI1* among the most highly upregulated genes does suggest involvement of Shh signaling in glioblastoma-related VTE, either by inducing a more aggressive phenotype or by triggering a procoagulant state.

Additionally, not all possible confounders may have been identified due to the retrospective study design. Also, immortal time bias was introduced due to the inclusion of three months before glioblastoma diagnosis. However, since the observed case fatality rate was low, we expect this to be of little effect on the data presented in this study.

Finally, in order to perform glioblastoma subtype analysis, a previously identified set of subtype-specific genes was used [19]. However, since this set only consisted of five genes per subtype and because of the small sample size of this study, careful interpretation is warranted concerning these results.

In this study, we aimed to identify genes or genetic mechanisms involved in glioblastoma-related VTE. Comparison of the RNA-Seq based tumor expression profile of glioblastoma patients with and without VTE resulted in the identification of Shh signaling as one of the potential underlying pathways. Future investigation should involve external validation in another glioblastoma cohort and functional validation to investigate a molecular link between *GLI1* overexpression and increased procoagulant activity in glioblastoma. Novel studies regarding a potential role for *GLI1* as biomarker for glioblastoma-related VTE are of interest to explore the possibilities of personalized thromboprophylaxis for glioblastoma patients with the highest risk of thrombosis.

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## CRediT authorship contribution statement

M.Y. Kapteijn: Study design, data collection, data analysis and statistical evaluation, data interpretation, manuscript writing first draft, manuscript revision and approval.

V.R. Lanting: Data collection, data analysis and statistical evaluation, data interpretation, manuscript revision and approval.

F.H.J. Kaptein: Data collection, manuscript revision and approval.

N.A.M. Guman: Data collection, data interpretation, manuscript revision and approval.

E.H. Laghmani: Data collection, manuscript revision and approval.

T.B. Kuipers: Data analysis and statistical evaluation, manuscript revision and approval.

H. Mei: Data analysis and statistical evaluation, manuscript revision and approval.

J.J. Goeman: Data analysis and statistical evaluation, manuscript revision and approval.

F.I. Mulder: Data collection, manuscript revision and approval.

S.G. van Duinen: Study design, manuscript revision and approval.

M.J.B. Taphoorn: Study design, manuscript revision and approval.

L. Dirven: Study design, manuscript revision and approval.

M.L.D. Broekman: Study design, manuscript revision and approval.

N. van Es: Data interpretation, manuscript revision and approval.

F.A. Klok: Study design, data interpretation, manuscript revision and approval.

J.A.F. Koekkoek: Study design, data collection, data interpretation, manuscript revision and approval.

H.H. Versteeg: Study design, data interpretation, manuscript revision and approval.

J.T. Buijs: Study design, data analysis and statistical evaluation, data interpretation, manuscript writing first draft, manuscript revision and approval.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: F. A.K. has received research support from Bayer, BMS, BSCI, MSD, Leo Pharma, Actelion, Farm-X, The Netherlands Organization for Health Research and Development, The Dutch Thrombosis Foundation, The Dutch Heart Foundation and the Horizon Europe Program, all paid to his institution and outside the submitted work. The other authors have no conflicts of interest to declare.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2023.10.018.

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