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Full Length Article

Targeted DNA sequencing to identify genetic aberrations in glioblastoma that underlie venous thromboembolism; a cohort study☆

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ABSTRACT

Background and objectives: Patients with glioblastoma have a high risk of developing venous thromboembolism (VTE). However, the role of underlying genetic risk factors remains largely unknown. Therefore, the aim of this study was to discover whether genetic aberrations in glioblastoma associate with VTE risk.
Methods: In this cohort study, all consecutive patients diagnosed with glioblastoma in two Dutch hospitals between February 2017 and August 2020 were included. Targeted DNA next-generation sequencing of all glioblastomas was performed for diagnostic purposes and included mutational status of the genes *ATRX*, *BRAF*, *CIC*, *FUBP1*, *H3F3A*, *IDH1*, *IDH2*, *PIK3CA*, *PTEN* and *TP53* and amplification/gain or deletion of *BRAF*, *CDKN2A*, *EGFR*, *NOTCH1* and *PTEN*. The primary outcome was VTE within three months before glioblastoma diagnosis until two years after. Cumulative incidences were determined using competing risk analysis adjusting for mortality. Univariable Cox regression analysis was performed to determine hazard ratios.
Results: From 324 patients with glioblastoma, 25 were diagnosed with VTE. Patients with a *CDKN2A* deletion had a 12-month adjusted cumulative incidence of VTE of 12.5 % (95%CI: 7.3–19.3) compared with 5.4 % (95%CI: 2.6–9.6) in patients with *CDKN2A* wildtype ($p = 0.020$), corresponding to a HR of 2.53 (95%CI: 1.12–5.73, $p = 0.026$). No significant associations were found between any of the other investigated genes and VTE.
Conclusion: This study suggests a potential role for *CDKN2A* deletion in glioblastoma-related VTE. Therefore, once independently validated, *CDKN2A* mutational status may be a promising predictor to identify glioblastoma patients at high risk for VTE, who may benefit from thromboprophylaxis.

1. Introduction

Cancer patients have a nine-fold increased risk of developing venous thromboembolism (VTE) compared to the general population, greatly affecting disease course and outcome [1]. The exact risk is dependent on patient-related (e.g. age, loss of motility) and tumor-associated risk factors (e.g. tumor type and tumor stage) [2,3]. Patients with a primary brain tumor are among the cancer patients with the highest incidence of VTE [4], implying that intrinsic, tumor-related features drive hypercoagulability in primary brain cancer.

Although it is of great importance to prevent VTE, thromboprophylaxis is not generally prescribed to cancer patients as it increases the risk

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of complications such as major bleeding (MB). Therefore, risk models are developed to identify cancer patients at high risk of VTE in order to provide personalized prophylactic anticoagulation. The currently recommended VTE risk assessment model for patients with solid tumors (Khorana score) is based on tumor type and laboratory variables, i.e. low hemoglobine, high platelet and high leukocyte levels [5], but did not include sufficient numbers of patients with primary brain tumors during its development to be reliable for this population. Therefore, this risk stratification model performs sub-optimal and is poorly validated in patients with brain cancer [6].

Glioblastoma is the most frequent and the most aggressive type of primary brain malignancy [7]. Histopathologically, glioblastomas are characterized by microvascular proliferation, necrosis and intratumoral heterogeneity, demonstrating rapid de novo development without clinical or histological evidence of a lower grade precursor lesion [8]. However, following combined histological and molecular grading of the 2021 WHO classification of tumors of the central nervous system [9], the presence of wildtype *IDH* genes is now included in classification of glioblastoma - a WHO grade 4 tumor. Importantly, glioblastoma is accompanied by a hypercoagulable state, resulting in a high incidence of both local (micro)thrombi within the tumor [10] as well as systemic VTE in 10–30 % of the patients [2,11,12]. The risk of VTE is particularly high in the postoperative period, but remains increased throughout the full disease trajectory, thereby worsening the clinical course of glioblastoma patients. Interestingly, in contrast to the Khorana score, low platelet counts were found to associate with VTE in glioblastoma, suggesting a different risk profile for glioblastoma-related VTE as compared with other cancer types [13].

Two thrombogenic proteins have been suggested to contribute to glioblastoma-related VTE. Upregulation of tissue factor (TF), the initiator of the extrinsic coagulation pathway, and increased levels of TF-bearing extracellular vesicles (EVs) have been observed in glioblastoma patients [14]. In addition, overexpression of podoplanin, a potent inducer of platelet activation via platelet receptor CLEC-2 [15], is often seen in glioblastoma, also being accompanied by an increase in podoplanin-expressing EVs [13,16].

Interestingly, common tumor driver genes and mutations in glioblastoma have been shown to induce expression of TF. While high levels of the gene *EGFR* correlate with TF upregulation [17], the oncogenic *EGFRvIII* mutation leads to increased TF expression and activity in glioma cell lines, influencing glioblastoma tumor progression [18,19]. Other tumor driver genes commonly found in glioblastoma, such as *PTEN* and *TP53*, were also found to induce TF expression in vitro, in glioma and colorectal cancer cells, respectively [20–22]. However, it has not yet been addressed whether these driver mutations that are commonly found in glioblastoma clinically associate with glioblastoma-related VTE.

In the last decade, next generation sequencing (NGS) has been a useful tool to find novel genetic associations between the patient- or tumor-derived genome and cardiovascular diseases, such as coronary heart disease and (cancer-associated) VTE [23–25]. Both germline as well as tissue-specific somatic mutations have been identified as genetic predictors of VTE in cancer [25,26]. Recently, we have performed a large cohort study consisting of 967 glioblastoma patients diagnosed between 2004 and 2020 in two Dutch hospitals, to determine the incidence and prognostic impact of glioblastoma-related VTE and MB [12]. Currently, we present a genetic follow-up study in which we have included all consecutive patients with targeted DNA NGS data available from clinical practice, to identify whether genetic aberrations in glioblastoma associate with VTE as primary outcome and MB as secondary outcome.

2. Materials and methods

2.1. Study population

In this cohort study, we included all adult patients (≥18 years) that were histopathologically diagnosed with glioblastoma in two hospitals in the Netherlands, the Leiden University Medical Center (LUMC) and Haaglanden Medical Center (HMC), between February 2017 and August 2020. The standard of care for glioblastoma patients consisted of pursuing maximum 'safe' resection in all patients, followed by concomitant radio-chemotherapy and adjuvant chemotherapy with temozolomide. If tumor location or patient condition did not allow for surgical resection, a biopsy was performed for histological confirmation. Postoperatively, the final diagnosis and treatment plan of all patients were discussed and documented at the multidisciplinary tumor board meeting of both hospitals. All patients with glioblastoma diagnosis were manually selected for the study. According to the 2021 WHO criteria, IDH-mutant grade 4 gliomas were classified as 'Astrocytoma, IDH-mutant', and not considered glioblastoma [9], and were therefore not included in this study.

For diagnostic purposes, targeted DNA NGS using a neuro-oncology gene panel was routinely performed on all glioblastoma tumors (n = 328) derived from tumor resection or biopsy at glioblastoma diagnosis.

This study was approved by an accredited Medical Ethics Research Committee (LUMC: #B19.039; HMC: #2019-089) and performed under guidelines of Good Clinical Practice (GCP). The need for informed consent was waived by the institutional review board due to the retrospective study design and the fact that the majority of the study population was already deceased at the start of the study. The STROBE reporting guidelines were used for reporting the data [27].

2.2. Chart review

The primary outcome of this study was development of objectively confirmed VTE, which was the composite of symptomatic or incidental pulmonary embolism (PE), diagnosed by Computed Tomography Pulmonary Angiogram (CTPA) or VQ scan, and distal or proximal deep vein thrombosis (DVT), including catheter-associated thrombosis, diagnosed by ultrasonography, conventional venography or CT-venography, within three months before glioblastoma diagnosis until two years after [28–30]. Patients with cerebral vein thrombosis (CVT, $n = 3$) were excluded, as tumor-related non-genetic risk factors such as resection could likely be an underlying cause.

The secondary outcome was major bleeding (MB), also assessed in the period between three months before glioblastoma diagnosis until two years after, defined as 1) fatal bleeding, and/or 2) symptomatic bleeding in a critical site, such as intracranial, intraspinal, intraocular, retroperitoneal, pericardial, intra-articular or intramuscular with compartment syndrome, and/or 3) bleeding associated with a decrease in hemoglobin of 20 g/L or more, or leading to transfusion of two or more units of blood or red blood cell concentrates, based on the criteria of the International Society on Thrombosis and Haemostasis (ISTH) [31]. All thromboembolic and bleeding events were adjudicated by two independent experts who were blinded for the NGS outcomes, without any discrepancies.

All patients were followed from three months before the date of glioblastoma diagnosis until primary/secondary outcome, death or throughout the maximum observation period of 27 months (i.e. until two years after the date of glioblastoma diagnosis). Data regarding patient demographics, tumor and VTE/MB characteristics, Eastern Cooperative Oncology Group (ECOG) performance status, treatment details and death (if applicable) were manually collected by in-depth chart review by three medically trained data collectors and pseudonymized. At the end of data collection, 36 patients were lost to follow-up and 63 patients had not yet reached the end of the indicated follow-up period. All patient records were updated at January 1, 2021, to allow for a minimum follow-up of four months. An a priori exclusion criterium was

history of VTE between 12 months and 3 months before glioblastoma diagnosis, but no VTE events were reported in this time frame.

2.3. Tissue collection and isolation

Based on H&E staining, regions with the highest percentage of neoplastic cells were defined for tumor cell estimation by a neuropathologist and tissue cores, whole sections or micro-dissected formalin-fixed paraffin-embedded (FFPE) tumor tissue were prepared for fully automated total nucleic acid extraction as described previously (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) [32].

2.4. Targeted DNA NGS data

All tumor tissue derived from diagnostic surgery was subjected to targeted neuro-oncology gene panel sequencing. Over time, three consecutive versions of the panel (custom ampliseq NEUR, NEURv1 and NEURv2) were designed, of which NEUR was previously described [33]. Detailed panel description tables with genomic information are prepared in Javascript scripts which are made publicly available (see Table S1 for NGS panel details) [34]. To maintain consistency, only genes that were included in all three panel versions were selected in the current study. Mutational analysis of all exons was performed on *ATRX*, *CIC*, *FUBP1*, *PTEN* and *TP53*. Specific mutation hotspots of *BRAF*, *H3F3A*, *IDH1*, *IDH2* and *PIK3CA* were investigated. Copy Number Variant (CNV) analysis, to assess gene amplification/gain or deletion/loss, was conducted for *BRAF*, *CDKN2A*, *EGFR*, *NOTCH1* and *PTEN* [35].

Sequencing libraries were prepared according to the manufacturer's recommendations and sequenced on the Ion Torrent Platform (ThermoFisher Scientific). Variant and copy number analysis was performed as described in [36].

The defined NGS quality criteria were a minimum of 1.5 million reads per sample, a minimum depth of 100 independent reads per locus of interest and a minimum variant allele frequency (VAF) of 0.1. A binary mutation model was used for all genes (mutated yes/no). One patient was excluded due to poor overall NGS quality. Mutational analysis was found to be unreliable in two patients, resulting in a total of 322/324 (99.4 %) patients for the analysis of mutational data, whereas nine patients had to be excluded because of low quality CNV data, leading to 315/324 (97.2 %) patients in total for CNV analysis. Since *PTEN* and *BRAF* were included for both mutational as well as CNV analysis, overall mutational status of these genes could be analyzed in 313/324 (96.6 %) patients.

2.5. Statistical analysis

Gene variants with a frequency of >2.5 % in the total cohort were included for further analyses. Univariable Cox regression models were used to calculate Hazard Ratios (HR) with corresponding 95 % confidence intervals (CI), describing associations between all individual genes or available clinical variables and the risk of VTE/MB. Time-dependent Cox regression analysis was performed to distinguish between VTE/anticoagulant-dependent and -independent effects on development of MB. Follow-up time was calculated from three months before the date of glioblastoma diagnosis until development of VTE/MB, death, becoming lost to follow-up, January 1st, 2021 in case of follow-up <2 years, or completing 27 months of follow-up, whichever came first. Because of the poor prognosis of glioblastoma patients, adjustment for competing risk of death was conducted by performing a Cumulative Incidence Competing Risk (CICR) analysis in RStudio [37], resulting in adjusted *p*-values and cumulative incidence curves of VTE/MB to compare glioblastoma patients with and without specific mutations. Since all VTE events and most MB events (33/36, 91.7 %) occurred in the period between three months before glioblastoma diagnosis until one year after, cumulative incidences for both events were determined at one year after diagnosis. Adjustment for multiple testing was not performed because of the exploratory nature of the study. SPSS software version 25.0 (SPSS Inc., Chicago, IL, United States) and RStudio software version 4.0.2 (PBC, Boston, MA, United States) were used for statistical analyses.

3. Results

3.1. Study population

In two Dutch hospitals (LUMC and HMC), 328 consecutive patients were diagnosed with glioblastoma, IDH-wildtype between February 2017 and August 2020 (Fig. 1). All glioblastoma tumor samples were subjected to targeted DNA NGS for diagnostic purposes. Of the 324 patients eligible for analysis, 25 VTE events (7.7 %) and 36 MB events (11.1 %) were reported within the indicated follow-up period of three months before glioblastoma diagnosis until two years after.

Baseline characteristics and disease outcome at the end of follow-up are reported in Table 1. The median age of the study population was 68 years old (IQR: 58–74) and slightly more men (195/324, 60.2 %) were included. Diagnosis of glioblastoma was based on tumor tissue obtained from resection (66.7 %, 216/324) or biopsy (33.3 %, 108/324). The majority of patients (249/324, 76.9 %) reported good (ECOG score = 1) to moderate (ECOG score = 2) performance status at time of diagnosis. The first therapy after resection/biopsy consisted either of regular (117/324, 36.1 %) or short-course (30/324, 9.3 %) concomitant radiochemotherapy, or radiotherapy (66/324, 20.4 %) or chemotherapy alone (9/324, 2.8 %). Recurrence was observed in 133 patients (41.0 %), with a median time to recurrence of 6.8 months (IQR: 4.7–11.2) after glioblastoma diagnosis. At the end of data collection, 18 patients (5.6 %) were still alive, 207 (63.9 %) had died, 36 (11.1 %) were lost to follow-up and 63 (19.4 %) reached the end date of the study before having completed two years of follow-up. The median observation time was 9.2 months (IQR: 5.5–14.6). The majority of deaths was reported to be cancer-associated (187/207, 90.3 %; Table 1). Autopsies had not been performed.

3.2. VTE events

Of the 25 patients with VTE, 17 suffered from PE (68.0 %), 7 from DVT (28.0 %) and 1 from both (4.0 %; Table 2). All events were reported in the period between three months before glioblastoma diagnosis until one year after, with a median time to VTE of 3.0 months (IQR: 1.2–4.1) from the moment of diagnosis. Two patients (8.0 %) developed VTE within one month before glioblastoma diagnosis and six (24.0 %) within the first six weeks afterwards (i.e. after resection/biopsy, but prior to further glioblastoma treatment). Most patients (16/25, 64.0 %) were diagnosed with VTE during or after the first round of glioblastoma treatment as mentioned in Table 1. Of all patients with glioblastoma recurrence ($n = 133$), only 1 was diagnosed with VTE after the reported recurrence date, whereas the other 11 patients developed VTE prior to recurrence. None of the patients received therapeutic anticoagulation prior to a VTE event. In all cases, anticoagulant therapy was initiated from the moment of VTE diagnosis, with either low molecular weight heparin (LMWH) (16/25, 64.0 %) or a direct oral anticoagulant (DOAC) (9/25, 36.0 %). VTE recurrence was seen in one patient despite anticoagulant therapy. None of the events was fatal.

The clinical variables Age, Sex, *MGMT* promoter methylation and ECOG performance status did not significantly associate with VTE in our cohort (Table S2). Tumor resection was found to be significantly associated with a decreased risk of VTE compared with tumor biopsy (HR: 0.41, 95%CI: 0.18–0.94, $p = 0.035$).

3.3. Major bleeding events

The overall majority of the 36 MB events in our cohort was

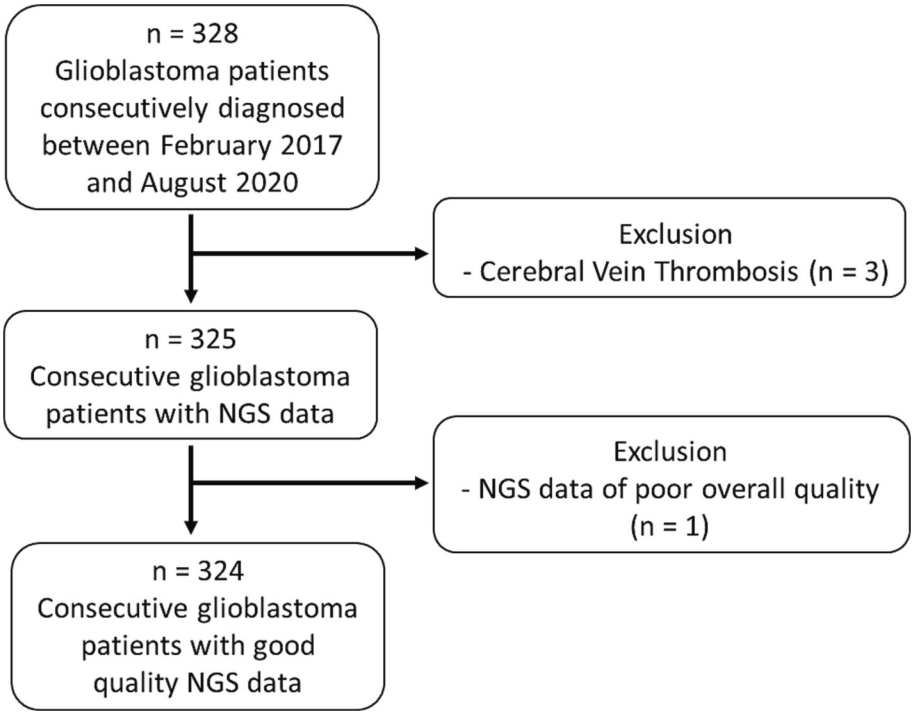

Fig. 1. Flowchart showing the design and inclusion of the study.

intracranial (34/36, 94.4 %; Table S3). Most events were reported within one year after glioblastoma diagnosis (33/36, 91.7 %). In 17 patients (47.2 %), MB occurred within six weeks after glioblastoma diagnosis, of which 2 events (11.8 %) at the moment of surgery and 10 events (58.8 %) within one week after (11 patients with tumor resection, 1 patient with a biopsy). Eight patients (22.2 %) were already using anticoagulants at the time of MB, of which seven (87.5 %) receiving anticoagulant treatment because of a prior VTE event during the indicated follow-up period (Fig. S1). The patient with non-VTE related thromboprophylaxis was receiving long-term anticoagulant treatment because of a mechanical heart valve. Bleeding recurrence was reported four times (11.1 %) and four MB events were found to be fatal (11.1 %).

3.4. Genes associated with VTE in glioblastoma

Of the 13 genes included in all gene panels, six were found to have a mutation frequency above the predefined threshold of 2.5 %, varying between 3.4 % (*ATRX*, 11/322) and 44.7 % (*PTEN*, 140/313) (Table 3, Table S4). Of these six genes, *CDKN2A* most strongly associated with VTE. Patients with a *CDKN2A* deletion were found to have a 12-month adjusted cumulative incidence of VTE of 12.5 % (95%CI: 7.3–19.3), compared with 5.4 % (95%CI: 2.6–9.6) in patients with *CDKN2A* wild-type ($p = 0.020$, Fig. 2). This corresponded to a HR of 2.53 (95%CI: 1.12–5.73, $p = 0.026$). Mutational status of *CDKN2A* was not significantly associated with poor survival (Kaplan-Meier log rank test, $p = 0.461$).

3.5. Genes associated with MB in glioblastoma

Of all 13 genes tested, *CDKN2A* deletion was also found to most strongly associate with MB (Table S5A), with a 12-month adjusted cumulative incidence of 15.9 % (95%CI: 10.0–22.9) compared with 6.5 % (95%CI: 3.4–10.8) in case of *CDKN2A* wildtype ($p = 0.017$). This correlated with a HR of 2.36 (95%CI: 1.18–4.72, $p = 0.015$). However, development of VTE and the consequent use of anticoagulant treatment is known to increase the risk of MB. In fact, seven patients with MB were receiving secondary thromboprophylaxis because of a prior VTE event (Fig. S1). Indeed, a significant association was found between VTE within the indicated follow-up period and development of MB in our cohort (HR: 2.61, 95%CI: 1.14–5.97, $p = 0.023$). This could drive the relation between *CDKN2A* deletion and MB, because of the described association between *CDKN2A* deletion and VTE. To correct for this, we performed time-dependent Cox regression analysis with VTE as time-dependent covariate. In this additional analysis, the significant relation between *CDKN2A* deletion and MB was lost (HR: 1.96, 95%CI: 0.96–3.97, $p = 0.063$; Table S5B).

3.6. Linking CDKN2A deletion to glioblastoma-associated VTE

Finally, we aimed to investigate how the presence of a *CDKN2A* deletion might contribute to the development of VTE, by studying its association with two procoagulant proteins in glioblastoma: TF and podoplanin. Using the Glioblastoma Multiforme dataset of the Pan-Cancer Atlas (TCGA, $n = 592$), retrieved from the cBioPortal for Cancer Genomics [38,39], we determined the association of *CDKN2A* deletion with podoplanin (*PDPN*) and tissue factor (*F3*) mRNA expression.

From 592 patients in the dataset, 145 samples were available with data on both mRNA expression scores of *PDPN* or *F3* as well as *CDKN2A* CNV status. Deletion of *CDKN2A* was found to significantly associate with high *PDPN* mRNA expression z-score compared with diploid *CDKN2A* status ($p = 0.009$, Fig. 3A), using batch normalization relative to all samples (log RNA Seq V2 RSEM) and a z-score threshold of ± 2.0. A similar effect, with a p-value of 0.058, was observed for *F3* expression (Fig. 3B).

4. Discussion

Glioblastoma patients suffer from a highly increased risk of developing VTE, which is associated with increased morbidity and mortality. Therefore, improved risk prediction of patients that would benefit most from anticoagulant prophylaxis is urgently needed. In this study, we examined whether mutational status of specific genes in glioblastoma associate with VTE or MB, and could be candidate biomarkers for risk stratification of individual glioblastoma patients.

Table 1
Cohort characteristics and patient status at the end of follow-up, including cause of death.

Cohort characteristics		All patients (%)	VTE patients (%)	MB patients (%)
N		324 (100 %)	25 (100 %)	36 (100 %)
Age (median, IQR)		68 (58–74)	68 (60–72)	64 (57–73)
Sex	Male	195 (60.2 %)	17 (68.0 %)	26 (72.2 %)
	Female	129 (39.8 %)	8 (32.0 %)	10 (27.8 %)
MGMT promoter methylation	Yes	97 (29.9 %)	9 (36.0 %)	13 (36.1 %)
	No	222 (68.5 %)	15 (60.0 %)	22 (61.1 %)
	Unknown	5 (1.5 %)	1 (4.0 %)	1 (2.8 %)
ECOG performance status[a]	0	56 (17.3 %)	5 (20.0 %)	6 (16.7 %)
	1	150 (46.3 %)	10 (40.0 %)	19 (52.8 %)
	2	99 (30.6 %)	9 (36.0 %)	7 (19.4 %)
	3	19 (5.9 %)	1 (4.0 %)	4 (11.1 %)
	4	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
Type of surgery	Biopsy	108 (33.3 %)	10 (40.0 %)	8 (22.2 %)
	Resection	216 (66.7 %)	15 (60.0 %)	28 (77.8 %)
Therapy	Concomitant TMZ/RT	117 (36.1 %)	10 (40.0 %)	12 (33.3 %)
	Concomitant TMZ/RT (short-course)	30 (9.3 %)	5 (20.0 %)	4 (11.1 %)
	RT only	66 (20.4 %)	5 (20.0 %)	6 (16.7 %)
	Chemotherapy only	9 (2.8 %)	1 (4.0 %)	1 (2.8 %)
	Other	16 (4.9 %)	1 (4.0 %)	1 (2.8 %)
Recurrence		133 (41.0 %)	12 (48.0 %)	16 (44.4 %)
Status at end of follow-up	Follow-up period < 2 years	63 (19.4 %)	7 (28.0 %)	7 (19.4 %)
	Lost to follow-up	36 (11.1 %)	1 (4.0 %)	4 (11.1 %)
	Stable disease	14 (4.3 %)	0 (0.0 %)	2 (5.6 %)
	Progressive disease	4 (1.2 %)	1 (4.0 %)	1 (2.8 %)
	Died	207 (63.9 %)	16 (64.0 %)	22 (61.1 %)
Cause of death	Cancer associated (expected)	181 (87.4 %)	12 (75.0 %)	16 (72.7 %)
	Cancer associated (unexpected)	6 (2.9 %)	1 (6.3 %)	1 (4.5 %)
	Pulmonary embolism (PE)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
	Arterial cardiovascular event	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)
	Bleeding	4 (1.9 %)	1 (6.3 %)	4 (11.1 %)
	Other	6 (2.9 %)	1 (6.3 %)	0 (0.0 %)
	Unknown	10 (4.8 %)	1 (6.3 %)	1 (4.5 %)

Abbreviations: IQR, interquartile range; MGMT, O^6-methylguanine-DNA methyltransferase; ECOG, eastern cooperative oncology group; TMZ, temozolomide; RT, radiotherapy.

[a] ECOG performance status: 0) Fully active; 1) restricted in physically strenuous activity, but able to carry out work of a light nature; 2) capable of all selfcare, but unable to carry out any work activities; 3) capable of only limited selfcare (confined to bed or chair for >50 % of waking hours); 4) completely disabled.

Table 2
Characteristics of VTE events.

VTE events		N (%)
N		25 (100 %)
Type of VTE	Pulmonary embolism (PE)	17 (68.0 %)
	Deep vein thrombosis (DVT) lower extremity	7 (28.0 %)
	PE & DVT	1 (4.0 %)
Moment of VTE	Before glioblastoma diagnosis	2 (8.0 %)
	Within 6 weeks after glioblastoma diagnosis	6 (24.0 %)
	During treatment of glioblastoma	9 (36.0 %)
	After treatment of glioblastoma	7 (28.0 %)
	During treatment of glioblastoma recurrence (n = 12)	1 (4.0 %)
ECOG performance status at time of VTE[a]	0	5 (20.0 %)
	1	10 (40.0 %)
	2	9 (36.0 %)
	3	1 (4.0 %)
	4	0 (0.0 %)
Anticoagulant treatment started after VTE	LMWH	16 (64.0 %)
	DOAC	9 (36.0 %)
VTE recurrence		1 (4.0 %)
VTE fatal		0 (0.0 %)

Abbreviations: VTE, venous thromboembolism; ECOG, eastern cooperative oncology group; LMWH, low molecular weight heparin; DOAC, direct oral anticoagulant.

[a] ECOG performance status: 0) Fully active; 1) restricted in physically strenuous activity, but able to carry out work of a light nature; 2) capable of all selfcare, but unable to carry out any work activities; 3) capable of only limited selfcare (confined to bed or chair for >50 % of waking hours); 4) completely disabled.

By using targeted DNA NGS data derived from tumor tissue of glioblastoma patients, we have identified *CDKN2A* deletion as a possible novel prognostic factor of glioblastoma-related VTE. Glioblastoma patients with a deletion in the *CDKN2A* gene were found to have a 2.53-fold increased risk of developing VTE, which resulted in a cumulative incidence of VTE of 12.5 % at one year after glioblastoma diagnosis (Fig. 2). Furthermore, time-dependent Cox regression analysis with VTE as time-dependent covariate showed that *CDKN2A* deletion is not significantly associated with MB in our cohort ($p = 0.063$; Table S5B), suggesting that patients with a *CDKN2A* deletion may benefit from thromboprophylaxis. However, despite correcting for VTE-related anticoagulant therapy, we still found an elevated HR of 1.96 (95%CI: 0.96–3.97), which asks for caution when considering anticoagulant prophylaxis for glioblastoma patients based on *CDKN2A* mutational status. Furthermore, external validation is required because of the small sample size and low number of events in our study.

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) is located at the second most frequently inactivated locus in cancer after *TP53*: the *INK4a/ARF/INK4b* locus [40]. Homozygous deletion of *CDKN2A* has been associated with increased carcinogenesis and poor survival in several cancer types, such as pancreatic adenocarcinoma, melanoma, colorectal cancer, non-small cell lung cancer and, interestingly, IDH-mutant glioma [41,42]. In glioblastoma, homozygous deletion of *CDKN2A* is found in ~50 % of the patients [43,44], slightly higher than observed in the current cohort (43.3 %). It represents one of the three main genetic alterations, together with amplification or activating mutations of *EGFR* and loss of *PTEN* [43].

CDKN2A encodes two proteins, $p16^{INK4a}$ and $p14^{ARF}$, which are the

Table 3
Associations between glioblastoma-related VTE and all genes with a mutation frequency of >2.5 %.

Gene	Type of variant	Total cohort	Cumulative incidence competing risk analysis			Univariate Cox regression analysis	
			Cum. incidence VTE (1y after diagnosis)		*p*-Value	HR (95%CI)	*p*-Value
			Wildtype	Mutant			
CDKN2A	D	43.2 % (136/315)	5.4 %	12.5 %	0.020	2.53 (1.12–5.73)	0.026
TP53	M	25.5 % (82/322)	9.3 %	5.0 %	0.282	0.58 (0.20–1.70)	0.321
PIK3CA	M	3.7 % (12/322)	8.5 %	0.0 %	0.328	0.05 (0.00–705.12)	0.534
EGFR	A	31.7 % (100/315)	7.5 %	10.6 %	0.527	1.27 (0.56–2.87)	0.569
ATRX	M	3.4 % (11/322)	8.2 %	9.1 %	0.889	1.74 (0.23–12.91)	0.588
PTEN	M, D	44.7 % (140/313)	8.7 %	8.2 %	0.894	1.10 (0.50–2.41)	0.816

Abbreviations: D, deletion; M, mutation; A, amplification; VTE, venous thromboembolism; HR, hazard ratio; CI, confidence interval.

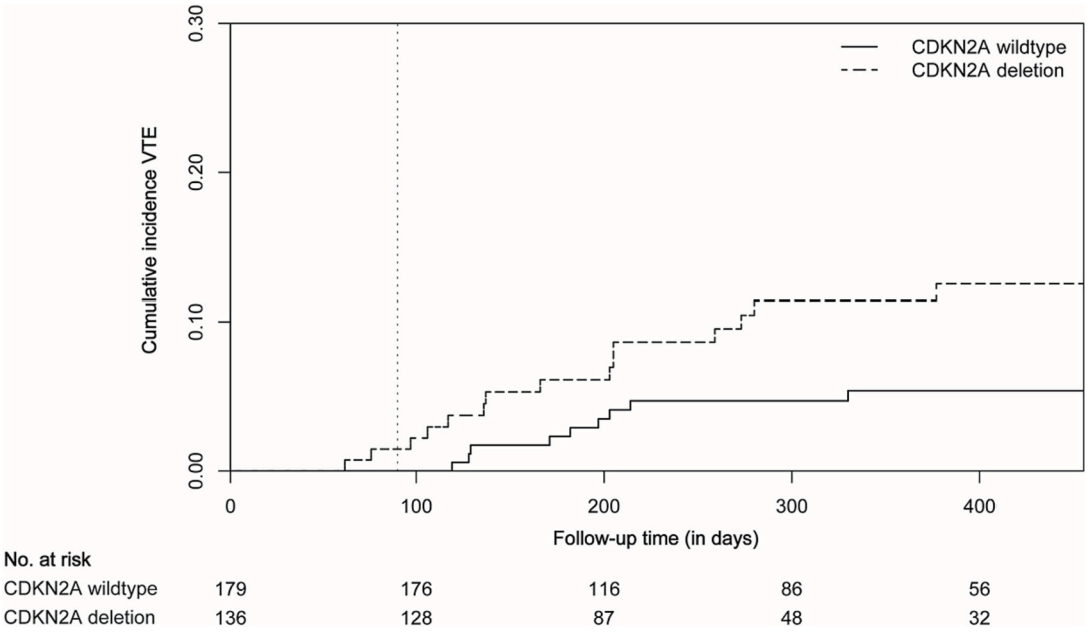

Fig. 2. *CDK2NA* mutational status associates with VTE in glioblastoma.
Adjusted cumulative incidence of VTE of patients with a wildtype *CDKN2A* gene (solid line) or a *CDKN2A* deletion (dashed line) from three months before glioblastoma diagnosis until one year after. The date of glioblastoma diagnosis is indicated with the dotted line.
Abbreviations: VTE, venous thromboembolism.

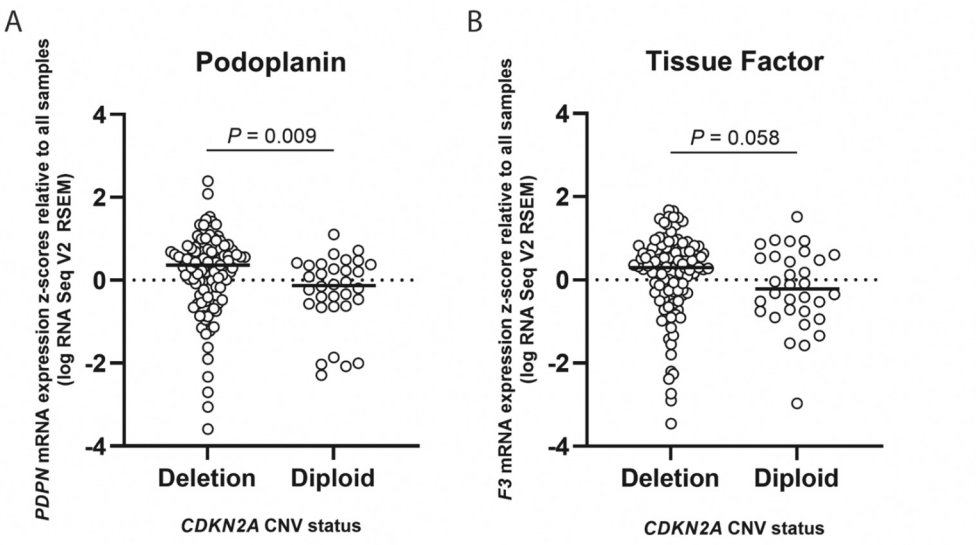

Fig. 3. Association of *CDKN2A* deletion with Podoplanin or Tissue Factor mRNA expression in a glioblastoma dataset from the cBioportal of Cancer Genomics.
mRNA expression z-scores relative to all samples (log RNA Seq V2 RSEM) of *PDPN* (A) and *F3* (B) in relation to *CDKN2A* copy number variant (CNV) status in a Glioblastoma Multiforme dataset from the cBioPortal of Cancer Genomics (TCGA Pan-Cancer Atlas). Data are shown as individual patient samples (n = 145) with the corresponding median (black line). Mann-Whitney *U* test was used for statistical evaluation.
Abbreviations: CNV, copy number variant

products of an alternative reading frame [45]. $p16^{INK4a}$ inhibits cell cycle progression by binding to the cyclin-dependent kinases CDK4/6, eventually resulting in G1-phase arrest of the cell cycle by retention of the transcription factor E2F [46]. $p14^{ARF}$, on the other hand, is functionally linked to p53 by inhibiting its negative regulator E3 ubiquitin-protease ligase Mdm2, thus allowing p53-mediated cell cycle control and preventing tumorigenesis. Dysregulation of the *TP53* signaling pathway, i.e. through disruption of $p14^{ARF}$ activity, is often seen in glioblastoma [43].

The *CDKN2A* gene was shown to be associated with coronary heart disease in a non-cancer setting [23,24], although its functional role herein remained elusive. We are the first to describe a potential association of somatic *CDKN2A* deletion and VTE in glioblastoma. Interestingly, by screening 341 genes in a large PanCancer cohort using a targeted DNA sequencing approach, *CDKN2A* was recently found to be in the top 10 of somatic mutations that associate with cancer-associated thrombosis in patients with solid tumors [25]. These data support the findings described in the current study, and suggest that genetic mechanisms in glioblastoma also relate to other tumor types.

To study the functional effects of *CDKN2A* deletion and to evaluate the implications of the association described here, we used the Pan-Cancer Atlas Glioblastoma Multiforme dataset derived from the cBioPortal for Cancer Genomics [38,39] to show that *CDKN2A* deletion has a biologically small, but statistically highly significant effect on genetic expression of the procoagulant proteins podoplanin ($p = 0.009$) and, to a lesser extent, TF ($p = 0.053$; Fig. 3). As podoplanin-positive EVs contribute to VTE in glioblastoma [3,16], we speculate that deletion of *CDKN2A* is an underlying genetic cause that leads to development of systemic VTE via increased levels of podoplanin-positive EVs. However, a causal role for *CDKN2A* deletion in upregulation of podoplanin and an associated hypercoagulable state awaits exploration in more fundamental studies.

Patients with podoplanin-positive primary brain tumors have been shown to have lower peripheral blood platelet counts [13]. Because of the link between podoplanin expression and *CDKN2A* deletion in the current study, we also explored the association between *CDKN2A* deletion and blood platelet count prior to glioblastoma surgery in our cohort. Interestingly, we observed a trend towards a lower platelet count in the presence of a *CDKN2A* deletion ($273*10^9$/L; 95%CI: 226–$323*10^9$/L) compared with *CDKN2A* wildtype ($288*10^9$/L; 95%CI: 239–$327*10^9$/L), although not statistically significant and clinically irrelevant ($p = 0.118$, data not shown). More research is required to investigate the link between *CDKN2A* deletion and podoplanin, and the subsequent effect on blood platelet counts in glioblastoma patients.

There may also be a connection between *CDKN2A* deletion and coagulation through *CDKN2A*-encoding proteins $p16^{INK4a}$ and $p14^{ARF}$. Indeed, *CDKN2A-p14*^{ARF} was shown to suppress TF-mediated coagulation in glioblastoma cells in vitro by inducing transcription of tissue factor pathway inhibitor-2 (TFPI2) independently of p53 [47]. These results are consistent with our findings, showing that deletion of *CDKN2A*, which will lead to loss of *CDKN2A-p14*^{ARF}, increases the risk of glioblastoma-related VTE, possibly by preventing TFPI2 activity within the tumor.

Furthermore, deletion of *CDKN2A-p14*^{ARF} results in loss of p53 activity, which together with mutations in K-Ras is known to induce TF upregulation in colorectal cancer cells and tumors [22,48]. A similar trend was seen in biopsies from non-small cell lung cancer patients with mutational expression of p53 together with PTEN [49]. Although mutations in *TP53* were not significantly associated with VTE in our dataset, p53 inactivation via deletion of *CDKN2A-p14*^{ARF} may have contributed to increased risk of VTE through upregulation of TF.

Additionally, $p16^{INK4a}$ is involved in regulation of the cell cycle complex by binding to CDK4/6. A similar function was described for $p15^{INK4b}$, a protein encoded by the *CDKN2A*-related gene *CDKN2B* that is also present at the *INK4a/ARF/INK4b* locus [40]. Interestingly, somatic mutations in *CDKN2B* were recently found to highly associate with an increased risk of VTE both independent of tumor type as well as in a high-grade glioma sub-cohort [25]. Since $p16^{INK4a}$ and $p15^{INK4b}$ have overlapping functions, it is tempting to speculate about a similar role for *CDKN2A-p16*^{INK4A} in glioblastoma-related VTE, which would be in agreement with data from our study. Moreover, redundancy of $p16^{INK4a}$ and $p15^{INK4b}$ may very well explain oncogenic co-deletion rather than specific inactivation of either one [40]. Unfortunately, *CDKN2B* was not included in the neuro-oncology gene panel used in the current cohort, hence the exact interrelation between *CDKN2A* and *CDKN2B* in glioblastoma-related VTE remains to be elucidated.

In addition to VTE, we used our targeted DNA NGS data to look into genetic aberrations that associate with MB in our study population. When correcting for preceding VTE events and subsequent anticoagulant treatment, we found no significant association between *CDKN2A* deletion and MB in our cohort ($p = 0.063$), although we still found an elevated HR of 1.96 (95%CI: 0.96–3.97). This may open the door towards personalized thromboprophylaxis for glioblastoma patients at the highest risk of VTE. Moreover, the observation that most VTE events occurred within 6 months after glioblastoma diagnosis (Fig. 2) could be used as an argument for limited duration of therapeutic anticoagulation in glioblastoma patients with *CDKN2A* deletion. However, the elevated HR of 1.96 and low power of our study still require precaution, calling for additional research to explore the effect of prophylactic anticoagulation in glioblastoma patients with a *CDKN2A* deletion.

A limitation of our study is the retrospective approach, in which data collection is dependent on in-depth chart review. Another potential restriction is the three month immortal timeline that was established by including the period of three months before glioblastoma diagnosis. Consequently, there would be a potential bias of missing an early event, which would have led to an underestimation of the observed HR. However, this is not likely as the case fatality rate is low, with no fatal events within the observed 23 VTE events in the period until two years after glioblastoma diagnosis.

Furthermore, the custom-made targeted DNA neuro-oncological gene panels used contain some of the most commonly mutated genes (*CDKN2A*, *EGFR*, *PTEN*) in glioblastoma, but otherwise consist of a very limited number of genes. Exact copy-numbers (gains, amplifications) and zygosity status (e.g. to distinguish between homozygous or heterozygous *CDKN2A* deletions) were not available, nor did we have information on the occurrence of *EGFRvIII* mutations in our cohort. We used a binary model (unmutated vs. mutated) to get a clear overview of genes involved in glioblastoma-related VTE and MB. A more detailed method, by sub-classifying between different mutations within one gene, could have led to a specific association, but probably would have fragmented the results. Nevertheless, the employed gene panels are routinely used in clinical practice, which will contribute to and enhance the implementation of *CDKN2A* deletion as potential biomarker for glioblastoma-related VTE.

Finally, we did not correct for multiple comparisons, as this was an exploratory study in which we wanted to determine the effect of the genetic aberrations included in the neuro-oncological gene panels available. However, with a cohort of only 324 patients of which 25 with VTE, the probability of making a type I error (incorrectly rejecting a true null hypothesis) is high. Therefore, external validation is necessary to thoroughly test the true association between glioblastoma-related VTE and *CDKN2A* deletion in an independent cohort.

With this study, we report a potential link between *CDKN2A* deletion and VTE in glioblastoma, associated with an increased cumulative incidence of VTE of 12.5 % at one year after glioblastoma diagnosis in our data. If externally validated, this may allow for personalized risk stratification, particularly for management decision on thromboprophylactic measures. Furthermore, we have described a potential molecular link between the presence of a *CDKN2A* deletion and increased expression of the procoagulant protein podoplanin, which may directly increase the risk of both local as well as systemic VTE in glioblastoma. Altogether, our results may lead to a better understanding of

glioblastoma pathophysiology in relation to VTE and may establish more accurate treatment strategies with regard to glioblastoma-related VTE in the future.

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.

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

M.A.M. Stals: Data collection, manuscript revision and approval.

E.E. Klaase: Data collection, manuscript revision and approval.

I. García-Ortiz: Data collection, manuscript revision and approval.

R. van Eijk: Data collection, data interpretation, manuscript revision and approval.

D. Ruano: Data collection, data interpretation, manuscript revision and approval.

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

S.C. Cannegieter: Study design, data analysis and statistical evaluation, manuscript revision and approval.

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

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

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

F.A. Klok: 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.

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Declaration of competing interest

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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.2022.11.013.

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