Mechanistic insights into cancer-associated thrombosis in brain cancer

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ABSTRACT

Cancer-associated thrombosis (CAT) is an umbrella term describing multiple forms of deregulated hemostasis and clotting related to cancer progression or therapy. Venous thrombo-embolism (VTE) is the most prevalent and widely studied systemic manifestation of CAT, associated with considerable morbidity and risk of life-threatening pulmonary embolism (PE). While some aspects of CAT may be unspecific or iatrogenic, the biology of cancer cells contributes to this condition through expression of the procoagulant phenotype often attributable to upstream oncogenic mutations, state of cellular epigenome and influences of the tumor microenvironment (TME). High grade astrocytic brain tumors (HGGs) are among the most procoagulant neoplastic disease states. Podoplanin (PDPN) and tissue factor (TF) have been implicated in HGG-associated VTE risk, while Isocitrate Dehydrogenase (IDH) mutations protect from thrombosis in HGG. Highly procoagulant, IDH-wild type HGGs, such as glioblastoma (GBM) represent a cluster of disease states each comprising cellular populations endowed with different repertoires of TF and PDPN expression. GBM cells project their influence systemically, at least in part, through release of extracellular vesicles (EVs) carrying TF, PDPN and other factors. The specific roles of these mechanisms in GBM-associated VTE are under investigation. Given the intense interest in developing new treatment strategies in GBM the interplay between these interventions and the activity of the hemostatic system is of paramount importance. Indeed, a better understanding of mechanisms driving CAT in distinct molecular and therapeutic contexts of specific brain tumor subtypes could inform more individualized approaches to management of thrombosis in patients.

Key words: cancer; glioblastoma; thrombosis; oncogenes; coagulome; extracellular vesicles.

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Contributions: JR conceived the study and wrote the manuscript.

Conflict of interest: the author declares no competing interests.

Funding: this work was supported by grants to JR from Canadian Institutes for Health Research (CIHR PJT 183971), Fondation Charles Bruneau (FCB) and Fondation CIBC, Cancer Research Society and New Frontiers for Research Fund (NFRF) – Exploration, as well as Canadian Foundation for Innovation (CFI10). JR is the recipient of the Jack Cole Chair in Pediatric Hematology/Oncology.

Received: 26 December 2025.
Accepted: 13 February 2026.

Bleeding, Thrombosis and Vascular Biology 2026; 5(s1):430
doi:10.4081/btvb.2026.430

Highlights

- Clinical and biological aspects on cancer associated thrombosis (CAT) are linked.
- The emerging pathways of CAT are targets of oncogenic transformation.
- CAT in high grade astrocytic brain tumours epitomizes systemic nature of cancer.
- Biological complexity of high-grade brain tumors impacts CAT-driving mechanisms – contextualizing the role of podoplanin and IDH mutations.
- Coagulant phenotypes of individual brain cancer cells are diverse and may act in concert.

Cancer-associated thrombosis as a clinical and biological problem

There is a tension between the search for more effective medical measures in the management of cancer associated thrombosis (CAT) and the quest to understand the biological causes, mechanisms and pathways through which cancers can deregulate the hemostatic system. While the former is often based on increasingly powerful and consequential, but inherently correlative, clinical science, the latter seeks strict causality and mechanistic understanding, albeit within reductionist confines of experimental concepts, assays and model systems. Such models may offer conceptual rigor and clarity, but often reflect but a fragment of a larger, more complex and variable clinical reality, thereby posing a risk of mismatch between mechanistic predictions and the realities of life.[1,2] This dichotomy, while often debated from both perspectives, can also be seen as an es-

sential force of progress, on the one hand stimulating biologically-based approaches to CAT prediction, prevention, treatment and post-event care, and on the other clinical verification of these premises to advance the clinical practice.

CAT is generally understood as a cumulative term comprising a multitude of hemostatic system disorders occurring in patients in conjunction with cancer and/or cancer treatment. Cancer-induced pro-coagulant conditions often occur within the venous macrocirculation, such as in cases of venous thromboembolism (VTE), including peripheral deep vein thrombosis (DVT), superficial vein thrombosis (SVT), or pulmonary embolism (PE). Somewhat less frequently, thrombosis may affect the arterial vasculature leading to arterial thromboembolism (ATE). Cancer related coagulopathies may also manifest themselves as complex systemic conditions, such as disseminated intravascular coagulation (DIC), or may preferentially affect the microcirculation, such as in thrombotic microangiopathy (TMA) in cases of anti-VEGF therapy,[3] or tumor microvascular thrombosis.[4-7] The diversity of these conditions reflects, at least to some extent, the spectrum of underlying biological mechanisms and that of their triggering disease states. These considerations led to the concept of cancer-associated and often highly context-specific 'coagulome', understood as a distinct set of molecular (and cellular) conditions and effectors that converge upon functions of the hemostatic system through a range of mechanistic links.[8,9]

VTE is perhaps the most prevalent and most studied form of CAT. The focus on VTE in cancer patients stems from the burden of associated morbidities and risks, and also from the fact that this condition occurs with a much higher frequency in cancer patients than in the general population.[4] While the numbers may differ between specific studies, recent reports estimate that approximately 4.6% of cancer patients develop VTE, which only affects 0.4% of cancer-free subjects.[4] According to other studies the estimated risk of cancer-related thrombosis could be as high as 15%,[10] and even higher in specific types of cancer.[7,11] As mentioned, such a high prevalence of CAT poses considerable clinical challenges due to significant morbidity, mounting medical needs, and demand for resources.[4] Importantly, CAT is associated with poorer overall outcomes and survival in cancer patients,[12] including responses to modern anticancer therapies, such as immunotherapy.[4] In subsets of cancer patients, thrombosis may become a frequent, even the second leading, cause of cancer-associated mortality.[13]

These are formidable challenges which, quite appropriately, are confronted with increasingly refined, evidence-based practice guidelines,[14] risk assessment tools[15] and relatively effective pharmacological countermeasures, such as protocols of low molecular heparin (LMWH), direct oral anticoagulants (DOACs), or antiplatelet agents, each associated with side effects including the risk of bleeding.[4] Moreover, these measures are based largely on hematological considerations even though there is a growing body of knowledge pertaining to CAT-associated processes that may act upstream of the hemostatic system, and are yet to be incorporated into cancer care paradigms.

Understanding the biology of CAT

It could be argued that understanding CAT at a deeper biological level could lead to expanded repertoire of biomarkers and interventions, potentially tailored to specific patients and not restricted to conventional anticoagulants acting at the very end of the chain of pathogenetic causation. Perhaps, acting on upstream events that trigger common coagulation pathway, or at the level of cancer and stromal cell populations that ultimately elicit thrombosis (if known) could lead to improved and more personalized, or tumor-specific, approaches to CAT management.[16]

As it stands, though, the risk of cancer-associated VTE is typically assessed according to several clinical circumstances with implied degree of causality in precipitating thrombosis, including patient-related characteristics (e.g. age, sex, history of VTE, genetic predisposition) and treatment-related factors (bed rest, stasis, catheterization, surgery, hormone therapy, chemotherapy and some targeted agents).[4] Importantly, VTE risk is also inferred from the hematological state of susceptibility to thrombosis, as revealed by certain biomarkers (leukocytosis, platelet counts) and, to a degree, from the specifics related to the underlying cancer (stage, type and molecular hallmarks of the disease).[4,17,18] Some of these factors are often grouped as elements of the Virchow's triad to signify their frequent co-existence and possible interdependence. While these considerations are of great importance in specific clinical circumstances, the ultimate causative trigger of CAT is, implicitly, the underlying malignancy and its biological nature.

Cancer is associated with several processes that may influence CAT in seemingly unspecific ways, including tissue damage, vascular invasion, angiogenesis, vascular permeability, impact of anticancer therapies, or other aspects of care, such as surgery or prolonged bed rest. It is also important to keep in mind that the threshold for cancer-associated VTE may change with physiological factors and host/genetic predispositions, such as factor V Leiden or prothrombin G20210A mutations.[19] However, these determinants offer only a partial explanation of the causality in CAT.

CAT does not appear to be a simple, unspecific, or random aftermath of external circumstances. For example, distribution of the VTE risks between different tumor sites is clearly non-random, with pancreatic, brain, ovarian or stomach cancers being associated with high risk of thrombosis, in contrast to breast, prostate and skin cancer, which are less prothrombotic, in spite of profound vascular perturbations they may elicit.[11,20] Disease specific anticancer treatments may also change the coagulome of cancer and stromal cell populations and alter their ability to trigger CAT.[21]

Even within the same tissue of origin the procoagulant potential of the neoplastic disease may differ depending on the cancer subtype,[8] or evolve over time.[22] Indeed, while it is generally accepted that more advanced cancers, especially at the metastatic stage, are more likely to trigger CAT, it is less understood whether and how the evolution of the disease may be paralleled by the evolution of mechanisms through which cancer interacts with the hemostatic system.[23] Within a given cancer site, different pathological and molecular subtypes of the disease may present with different levels of VTE risk.[7] Not only risks, but also CAT outcomes are influenced by the nature of the underlying malignancy.[24] Of course, these examples do not explain why some cancer patients with seemingly the same diagnosis experience thrombotic events, while others do not, but they point to a mechanistic link between cancer biology and the occurrence, as well as biological drivers, of CAT in different contexts.[25]

The foundational tenet of cancer biology is that malignancies originate as a result of cellular transformation events driven by

inheritable somatic genetic (mutational) and epigenetic alterations in pathways responsible for crucial cellular functions.[26] Consequently oncogenes and tumor suppressors[26] trigger cascades of biological responses leading to changes in cellular phenotypes, fate, function, and interactive apparatus (e.g. secretome) impacting cellular 'communities' and the tumor microenvironment (TME). The latter aspect includes inflammatory processes, immune responses, complement and the vasculature.[1,27-29] While oncogenic mutations may be primary causes of CAT their existence is not necessarily tantamount to the specific risk of thrombosis regardless of other biological circumstances. It should be noted, for example, that sustained oncogenic signaling may sometimes be required for 'tumor maintenance',[30] but some consequences of these events may persist even when oncogenic circuitry is genetically or pharmacologically disrupted. This may occur, as a consequence of irreversible changes in tissue homeostasis, intracellular molecular redundancies and other factors.

It could be argued that, as with the formation of the TME as a whole, the upstream causation of CAT lies with oncogenic mutations,[31] a notion for which there is currently a sizeable body of experimental data[8,16,32-35] and mounting clinical evidence.[7,36,37] For example, in experimental systems modulation of the status of mutant KRAS oncogene was shown to alter the coagulant phenotype of colorectal cancer (CRC) cells,[32] while KRAS mutation was also associated with elevated incidence of VTE in CRC patients.[37] In large patient cohorts, mutations of STK11, KRAS, CTNNB1, KEAP1, CDKN2B, and MET genes predicted increased VTE risk across multiple cancer types whereas mutations of IDH1/2 genes impacted thrombosis in a cancer-specific manner.[7,36]

Although the emerging link between oncogenic mutations and CAT is of considerable biological interest, it does not automatically imply that one can make direct deterministic clinical predictions.[17] Not all patients with cancer harboring a specific mutation develop thrombosis. This is understandable given the microenvironmental modifiers, as well as redundant, multistep and dynamic nature of effector mechanisms through which oncogenic mutations may exert their cellular, multicellular and systemic (body-wide) effects,[25] including on CAT. The case in point is the seemingly paradoxical notion that targeted agents developed to disable cancer 'driver' genes are not necessarily able to prevent thrombosis, and in some cases may even exacerbate the risk.[38]

Several complexities may explain this observation. For example , the effects of targeted agents may include prolongation of patients survival and competing mortality risks,[38] or the non-curative outcomes due to acquisition of drug resistance.[39] Other confounders may encompass changing cellular dependencies on specific oncogenic signaling pathways, influences of additional mutations, or compensatory signaling, loss of extrachromosomal DNA carrying oncogenic sequences under therapeutic pressure, epigenetic reprogramming of cancer cells, cell survival modifying effects of the TME, activation of pathways dependent on cell-cell communication, or inflammation, among others.[40] These processes may contribute to the dynamic nature of CAT-inducing tumor micro- and macro-environments.[20]

Thus, while oncogenic mutations and epigenetic alterations may contribute to the upstream causation of CAT, their downstream effector mechanisms triggering thrombosis are often modulated by several additional cues and a more predictable chain of events is often difficult to establish.

Examples of effector pathways operative in CAT

CAT is among the most startling manifestations of the systemic impact of cancer, even if it presents as a localized disease.[1] Among the most striking examples of this notion are high-grade astrocytic brain tumors, such as glioblastoma (GBM; as discussed below), which occur almost exclusively as intracranial lesions, while driving a high risk of peripheral VTE.[7,41,42]

Several cellular and molecular mechanisms have been implicated, as mediators of thrombosis in various cancer settings,[43] but the proposed common denominators are controversial, and the existence of several different pathways of CAT (effectively different CATs) remains likely (Figure 1). Therefore, identification of mechanisms operative in specific clinical contexts is important, especially if CAT diagnostics was to move beyond common hematological manifestations. A better understanding of such upstream events could lead to design and deployment of more targeted, causality-driven, biologically-based and potentially more personalized biomarkers and therapeutic countermeasures.[44]

While cellular and molecular mechanisms contributing to CAT have been extensively reviewed in the recent literature[4,18,20] it may be worth revisiting briefly some of the main tenets (Figure 1). Thus, apart from unspecific or iatrogenic factors, the biology of CAT often revolves around the concept of hypercoagulability (prothrombotic state) of the circulating blood, which in cancer patients is believed to create a heightened probability of an overt thrombotic event. It is unclear whether the systemic nature of hypercoagulability can be attributed to the local generation of high levels of procoagulant activity within the tumor mass and the 'spill over' effect into the circulation, or it arises systemically, as a result of shedding tumor-derived procoagulant material (e.g. circulating cancer cells or their products).

Several studies point to the change in phenotype of cancer cells and their pro-inflammatory surroundings as major triggers of local coagulant activity, which may then spread systemically.[20] This includes the expression by cancer cells of molecules with prothrombotic activity, such as tissue factor (TF), podoplanin (PDPN), or plasminogen activator inhibitor 1 (PAI-1),[34,45,46] superimposed with other hemostatic perturbations at the tumor site, including hemorrhage, microvascular thrombosis and intravascular aggregates of platelets.[7,46-48] In one study experimental tumors in mice generated extremely high levels of FXa locally, while only a fraction of this activity could then be detected in the peripheral vasculature.[16]

How procoagulant activity arises within the tumor mass is a subject of some debate. As mentioned, tumor cells may acquire high intrinsic expression of TF,[49] PDPN,[46] PAI-1,[50] factor VII[51] or thrombin[52] and may also expose phosphatidylserine (PS) on their surfaces.[53] This phenotype may locally contribute to triggering the coagulation cascade, platelet activation, reduced fibrinolytic activity, often acting downstream of mutant oncogenic drivers.[16,32-34] However, single cell sequencing studies suggest that the coagulant phenotype is not uniformly distributed among cancer cells, but instead the expression of different coagulation effector genes (e.g. TF or PDPN) may be associated with distinct cancer cell subpopulations, within the same tumor, with some cells co-expressing multiple factors while others being negative

for such expression.[16] Tumor-associated inflammatory and immune cells may, similarly, adopt ostensibly procoagulant phenotypes marked by the expression of TF, PAI-1, formation of neutrophil extracellular traps (NETs), secretion of cytokines able to activate endothelial cells, and other features that may contribute to cancer associated immune-thrombosis.[29,54,55] PDPN, for instance, is also often expressed by cancer associated fibroblasts[56] and pro-coagulant phenotype including TF could be triggered in activated, or injured endothelial cells.[57,58]

While the ability of cancer cells and TME constituents to generate procoagulant milieu is highly suggestive, as to their causative role in CAT, the expression levels of TF and other effectors within the tumor mass are often not predictive of the systemic VTE risk.[59] Moreover, it still remains unclear how could tumor-associated and localized procoagulant activity trigger a systemic hypercoagulable state, or VTE in remote vascular beds. The frequently invoked explanation in this regard is the existence of tumor-derived 'carriers' of the procoagulant activity, either in a form of soluble molecules (e.g. cancer procoagulant[60]) or multimolecular cellular fragments known as extracellular vesicles (EVs), previously often referred as microparticles (MPs).[61] Indeed, EVs released by cancer cells[32,45,62-64] or their inflammatory counterparts[65-67] have been implicated as carriers of TF, PDPN, PS, mucins and other mediators.[16,61] Upon release into the circulation, such procoagulant EVs could potentially engage circulating components of the coagulation cascade directly.[68] Since, EVs can transfer TF between cells[65] it is also possible that such TF-EVs could be taken up by other cells in the circulation, or the vessel wall, resulting in formation of procoagulant complexes on the surfaces of EV recipient cells.

While these are plausible scenarios supported by some experimental data, the presence of circulating TF-EVs not always accompanies, or predicts, the increased VTE risk in the clinic. One factor that may confound EV-related studies is the frequent lack of uniformity between protocols used for isolation and analysis of EVs, including those carrying coagulant cargo. Differences in collection of plasma samples, EV isolation protocols and cargo detection may result in disparities between studies.[69-71] Interna-

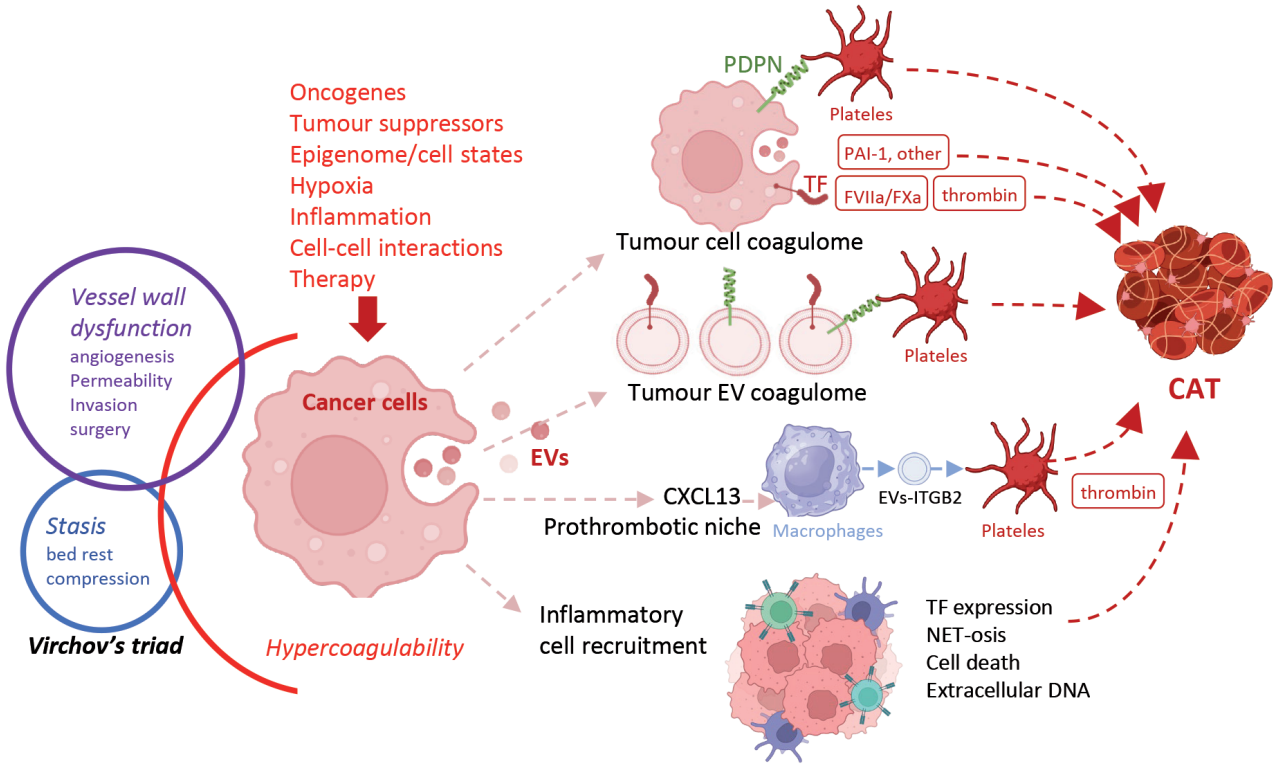

Figure 1. Constituents of cancer-associated thrombosis. Examples of processes implicated in cancer-associated thrombosis (CAT) including the expression procoagulant phenotype (coagulome) by cancer cells, stroma and their derived extracellular vesicles (EVs). Some of the related effector molecules include podoplanin (PDPN), tissue factor (TF), coagulation factors (e.g. FVII) or inhibitors of fibrinolysis (e.g. PAI-1). These factors activate platelets directly (PDPN) or indirectly (TF pathway), which may contribute to local and systemic thrombosis. It has been proposed that cancer cells may also induce formation of prothrombotic niche (PTN) by releasing CXCL13, which activates lung macrophages, which in turn release ITGB2 positive EVs able to activate platelets thereby leading to triggering coagulation cascade. Recruitment of inflammatory cells may bring additional mediators into the tumor site, leading to formation of neutrophil extracellular traps (NETs), cell death and release of extracellular DNA and other processes that contribute to CAT. These different responses may impact preferentially the intratumoral activation of the coagulation pathway or lead to systemic hypercoagulability, which combined with other elements of the Virchow triad could precipitate VTE. The upstream regulators of these events include genetic drivers of cancer progression (oncogenes and tumor suppressors), epigenetic factors, elements of the tumor microenvironment (TME), therapy effects and other biological variables. Images were prepared using BioRender.

tional collaboration and consensus development along with new technologies may render this effort more conclusive.[72]

However, these inconsistencies may also suggest the existence of additional, or alternative mechanisms driving systemic hypercoagulability. In this regard, a recent experimental study suggested that tumor derived EVs do not need to possess a direct prothrombotic activity to activate coagulant responses. Instead, tumor-derived CXCL13 may be released into the circulation and stimulate lung macrophages to produce ITGB2-positive EVs. These EVs, in turn, bind to, and activate, platelets and trigger formation of platelet-rich microthrombi, in what is referred to as prothrombotic niche (PTN) in the lung.[73] This process also activates TF pathway and contributes to metastatic disease,[73] as suggested also by earlier studies.[74,75] While there is no evidence that PTN contributes to large vessel VTE in cancer patients, its role in experimental DIC-like lung microthrombosis highlights one possible pathway of hemostatic perturbations that may lead to formation of CAT-like states irrespectively of the coagulant phenotype of cancer cells themselves.[73] It is of interest that activation of platelets, coagulation cascade or formation of microthrombi are closely linked to metastasis, which may be both the cause and effect of exacerbated CAT in advanced cancers.[73-75]

It should be noted that VTE risk may also be correlated with the profile of gene expression and proteome of patient plasma outside of the canonical hemostatic system.[76,77] While ultimately clot formation would entail coagulation and platelet activating mechanisms, non-canonical factors and genes correlating with VTE in cancer patients may represent wider systemic responses to the disease.

CAT in brain cancer

Brain microcirculation accounts for disproportionally high fraction (20%) of the cardiac output,[78] a property that is paralleled by robust hemostatic defenses, including high expression of TF in astrocytes surrounding the cerebral capillaries.[79] While this might suggest that tumours arising from the astrocytic lineage (gliomas) would be automatically associated with high propensity to trigger CAT, both the extent of microvascular thrombosis[7,80] and VTE risk are not uniformly distributed among gliomas.[81] Instead, it is mainly a subset of high grade (grade IV) gliomas, especially GBM, that is strongly linked with elevated VTE risk in a range of between 20% to upward of 25%, especially within the first six months post diagnosis.[41,82-84] While GBM patients are routinely offered surgery as a part of standard treatment, the elevated VTE risk continues beyond the perioperative period at a rate or 1.5-2.0% per month of survival.[85]

In this context the judicious use of thromboprophylaxis and anticoagulant treatment with LMWH, or DOACs are under investigation with recommendations conditional upon the risk of intracranial hemorrhage (ICH).[41] In addition, safer profiles of inhibitors acting upon factor XI are in the research pipeline.[41]

In keeping with these findings, GBM patients often receive high VTE risk scores according to several tools developed in the field thus far, including the Khorana score,[86] harboring non-0 blood group, status of circulating markers, clinico-pathological status of the disease and other criteria.[41] A recent prospective study proposed a ten-point scale to assess the VTE risk in diffuse glioma patients.[42] According to this model positive predictors of thrombosis in these patients include: i) prior history of VTE, ii) hypertension, iii) asthma, iv) white blood cell count, v) WHO tumor grade, vi) patient age, and vii) body mass index.[42] Notably, three factors were found to negatively correlate with VTE in this cohort, including: hypothyroidism, promoter methylation of O6-methylguanine-DNA methyltransferase (MGMT) and mutations in the coding sequence of Isocitrate Dehydrogenase 1/2 (IDH1/2) genes.[42] The latter two features signify the link between molecular biology of high-grade brain tumors and the associated risk and possibly the nature of the underlying thrombosis.

It is of note that while CAT represents a significant co-morbidity associated with subsets of high grade brain tumors it also plays an important role in shaping the biology of the disease.[87] For example, intra-tumoral, vaso-occlusive microvascular thrombosis was suggested to be functionally linked with patterns of perivascular necrosis and enhanced invasive features in GBM.[88] Non canonical features of CAT effector molecules, such as TF and PDPN, were implicated as regulators of vascular and cancer cell signaling along with biological and therapeutic responses,[89-91] including exit of cancer cells from the state of dormancy.[92] To better position these mechanisms in the context of the disease it is useful to discuss the increasingly complex biological landscape of high-grade gliomas.

The evolving understanding of high-grade brain tumor complexity

High grade gliomas (HGG), including GBM, pose enormous clinical challenges due to largely cryptic clinical course prior to diagnosis,[93] infiltrative and aggressive growth pattern at presentation, and dismal prognosis marked by approximately fifteen months of median overall survival.[94] While relatively rare (3.0-3.2 cases per 10^5 persons, annually), GBM is therapeutically intractable in spite of aggressive standards of care involving surgery followed by an intense chemo-radiation protocol with consolidating follow up courses of temozolomide. Unfortunately, this regimen (Stupp protocol) offers only a transient respite followed by inevitable recurrence of therapy resistant, molecularly altered[95] and highly aggressive disease.[94] While there is no established second line treatment for recurrent GBM, and multiple experimental protocols involving targeted agents, antiangiogenics and immunotherapeutics are still to deliver better outcomes, transient benefits were associated with targeting the tumor vasculature with bevacizumab.[94] GBM patients may also receive tumor treating fields[96] and supportive care including glucocorticoids.[94] These interventions carry certain levels of prothrombotic risks, which are superimposed on the impact of cellular and molecular complexity of the disease itself, all contributing to the cumulatively high VTE risk.[41]

The molecular understanding of HGG, including GBM, has undergone a profound revision over the past two decades. What was once regarded as a single disease entity marked by histological hallmarks, such as pseudopalisading necrosis, microvascular proliferation and high incidence of microvascular thrombosis, has progressively undergone nosological fragmentation, as a result of a rapid advent of precision molecular diagnostics powered by emerging tools of next generation sequencing (NGS), multiomics and, more recently, single cell transcriptomics.[97,98] These approaches revealed that rather than being a uniform disease, HGG

consists of several transcriptionally and biologically distinct molecular subtypes (Proneural, Classical and Mesenchymal), which formed the basis of the Cancer Genome Atlas (TCGA)-based GBM classification system.[94,99,100]

It soon became clear that pediatric high grade gliomas (pHGGs), in spite of some histological resemblance of GBM, possess a unique repertoire of molecular characteristics, such as oncohistone mutations (H3K27M, H3G34V), chromatin remodeler mutations (ATRX), deregulation of growth factor receptor genes (PDGFRa) and changes in tumor suppressors (TP53).[97] These changes coincide with profoundly altered epigenetic landscapes, stalled cell differentiation processes and topographic features that separate pHGG from their adult HGG/GBM counterparts.[101,102] This notion is further underscored by the unique methylation profiles of disease subgroups across the brain tumor landscape.[103] Pediatric HGG patients typically experience a relatively low incidence of VTE (up to 2.8%), but a recent retrospective study suggests that this complication could be exacerbated by such tumor features as Grade 4, epidermal growth factor receptor (EGFR) positivity, p53 mutations, glucocorticoid therapy and central venous catheter placement (CVCP). On the other hand, anticoagulation therapy, IDH1 mutations, MGMT methylation, radiotherapy, chemotherapy, and prolonged bed rest are reported to lower the VTE risk in this group of patients.[104] This subject requires further study given the role of oncohistones that drive a large proportion of pHGG in regulating vascular features[105] and EV landscapes[106] of these tumors, ostensibly without a major rise in the VTE risk.

In contrast to their pediatric counterparts adult HGGs were found to be associated with distinct repertoires of genetic events, such as chromosomal aberrations (e.g., amplification of chromosome 7 in GBM, 1q19p codeletion in oligodendroglioma and others) along with mutations affecting telomerase promoter, EGFR gene and its highly oncogenic forms (EGFRvIII), as well as changes in genes encoding PDGFRa, neurofibromin 1 (NF1), cell cycle regulators (CDK4), PTEN and other traits.[97] Interestingly, the levels of intra-tumoral microvascular thrombosis in GBM patients have been linked to elevated EGFR expression by cancer cells,[47] which, however, does not seem to affect the overall risk for systemic VTE.[107] The suggested link between EGFR expression and microvascular thrombosis is notable as EGFR/EGFRvIII carrying EVs have been reported to trigger a non-angiogenic vascular growth process (vasectasia) in models of mesenchymal GBM in mice[28] with yet unknown consequences for thrombosis. A subset of HGGs also carry methylation in the promoter of the MGMT gene, which blocks its expression in cancer cells rendering them susceptible to chemotherapy with temozolomide[94] along with alteration of other biological properties, such as invasiveness.[108] Some of these features could be captured by patient derived xenograft models of GBM.[16,109,110]

Important recent studies led to a better understanding of the defining role of IDH1/2 gene mutations in the biology and presentation of HGGs.[94] These advances ultimately resulted in the separation of mutant IDH bearing HGG tumors from the GBM diagnosis under recent WHO 2021 classification.[94] This classification now confines GBMs to grade IV gliomas with IDH1/2 wild type genotype.[111] This separation also altered the composition of the aforementioned proneural (PN) subtype of HGG (as per TCGA classification), which initially included the majority of IDH mutant tumors.[42] The composition of classical and mesenchymal GBM subtypes within the TCGA classification of GBM remained largely unaffected by the exclusion of IDH mutant lesions.[94] It should also be noted that these descriptions are mainly related to newly diagnosed GBMs, while the molecular characteristics of recurrent lesions undergo progressive changes, a drift that includes more pronounced mesenchymal-like features[99] and multiple molecular alterations.[95]

The advent of single cell RNA sequencing technologies during the past decade led to another major change in perspective as to the GBM complexity.[98] Thus, single cell studies revealed that the transcriptomic profiles of the aforementioned GBM subtypes reflect merely global phenotypic equilibria of constituent cancer cells, which are otherwise highly heterogeneous. Indeed, GBM masses were found to contain subsets of cells molecularly reminiscent of proneural, classical or mesenchymal tumors, amidst those that exhibited mixed gene expression profiles.[98] This complexity is typically superimposed with rich microglial and astrocytic TEM of GBMs, often accounting for 30-40% of the tumor mass.[112] At the same time TEM of these gliomas is depleted for immune effector cells (below 1% cells),[113] which could potentially be one of the reasons for lack of responsiveness of GBMs to immune checkpoint inhibitors (ICIs).[114]

Moreover, at the single cell level the GBM cell phenotypes were shown to exhibit a degree of plasticity forming transitory states with biases toward either astrocyte (AC)-like, oligodendrocyte progenitor cell (OPC)-like, neural progenitor cell (NPC)-like or mesenchymal-like tumors, emanating from increasingly well-defined stem-like/progenitor cell populations.[115] The extension of this analysis led to the identification of alternative pathway-based cellular phenotypes including glycolytic/plurimetabolic (GPM), mitochondrial (MTC), neuronal (NEU) and proliferative/progenitor (PPR) states.[116] The impact of this heterogeneity on clinical manifestation of CAT has not been rigorously studied and is of great interest.[41] While single cell phenotyping and reassembly of complex cellular landscapes has been illuminating it should be kept in mind that the samples subjected to such analysis typically comprise less than 10-30 thousands of cells[28] and therefore may not be fully representative of the totality of the tumor mass, or its dissemination throughout the brain.

Functionally, GBM growth and relapse is believed to be driven by tumor initiating glioma stem cells (GSCs). The diversity of such cells physically isolated from surgical specimens of GBM further supports tumor complexity in that subsets of these cells may transcriptionally resemble either proneural-like or mesenchymal-like tumors.[117] These GSC subtypes exhibit distinct biological,[118] vascular[28,119] and coagulant propertie[16] but their role in CAT remains poorly defined.

Impact of biological complexity of high-grade gliomas on CAT

The cellular and molecular complexity underlying the GBM/HGG biology and its progression is yet to be fully projected upon the corresponding nexus of coagulant effectors, processes and VTE risks.[41] Notable milestones in this regard, as mentioned, include the discovery of the protective role of IDH mutations in brain tumor-associated thrombosis[7] and the emerging role of some of the effector mechanisms implicated in VTE, especially PDPN,[46] along with possible and more complex contributions of the TF pathway (Table 1).[42]

As alluded to earlier, GBM is the case in point for a malignant disease confined to a single organ site (brain), while having multifaceted systemic impact, including the high risk of peripheral thrombosis.[120] The mechanistic interrelation between events occurring locally within the tumor mass and systemically in the circulation still remains to be fully elucidated. It is also of note that while GBM lesions often contain high degree of microvascular thrombosis, which tends to correlate with more aggressive course of the disease,[47] it is not formally demonstrated that these local vascular features are driven by the same mechanisms as the systemic VTE risk[16,107] even though recent studies reported some convergence.

In this regard an important study by Riedl et al examined the expression of PDPN in high grade astrocytic brain tumors, as a correlate and possible trigger of VTE.[46] These investigators described a significant association between high levels of PDPN expressed by cancer cells and the rising VTE risk in HGG patients.[46] PDPN is a cell surface-associated sialomucin that binds to the CLEC2 receptor on the surface of platelets and directly triggers their activation. It is suggested that this interaction could represent a primary event resulting in platelet driven microvascular and peripheral thrombosis in GBM patients, who also frequently experience paradoxically lowered platelet counts, possibly due to consumption.[41,46]

It is presently unclear how could GBM cell-associated PDPN activate platelets (intravascularly) leading to thrombosis and whether this would occur mainly within the tumor microcirculation and/or systemically. In experimental systems involving engineered human GBM cells expressing PDPN, these cells were able to release PDPN-carrying EVs with platelet activating potential, both in vitro and in mouse xenograft models.[16] While PDPN was also detected in blood of a small sample of GBM cases,[16] a subsequent larger study found no correlation between VTE and circulating PDPN in a cohort of HGG patients.[42] Thus, the mechanistic link between intratumoral PDPN expression and peripheral activation of platelets leading to thrombosis remains to be investigated further.

Another molecular candidate effector of GBM-associated thrombosis is TF.[33,92,121] This is for several reasons. TF is expressed at high levels in the brain microenvironment[79] and in GBM cells, notably with preponderance of cytoplasmic staining over that of the plasma membrane.[122] In experimental settings TF along with other elements of the coagulation signaling pathway (factor VII, thrombin receptor) are regulatory targets of GBM associated common oncogenic alterations, such as EGFR/EGFRvIII,[35,121] or PTEN.[33] In keeping with these findings TF/F3 mRNA expression is elevated in GBM subtypes enriched for EGFR (classical and mesenchymal) relative to their EGFR-negative (proneural) counterparts.[8] These effects are further amplified by hypoxia.[33] In GBM models in mice the lysates

Table 1. Reported determinants of thrombosis in high grade brain tumors.

Factor	Effect	Observations	References
IDH mutation	IDH mutation in is associated protection from thrombosis in brain tumors	IDH mutant HGG exhibit reduced VTE risk and lower level of microvascular thrombosis	7,127
Podoplanin (PDPN)	PDPN expression is elevated in GBM cells increases VTE risk	PDPN upregulation correlates with elevated VTE risk in GBM patients; GBM cell-associated PDPN activates platelets and correlates with thrombocytopenia; PDPN is detectable in plasma of GBM patients; PDPN gene is unmethylated in IDH wild type brain tumors PDPN is released as cargo of EVs in experimental GBM models	16,46
Tissue factor (TF)	TF expression in GBM cells	TF is upregulated in cultured GBM cells expressing mutant oncogenes (e.g. EGFR) and detected in GBM samples; elevated F3 mRNA is detected in classical and mesenchymal GBM; TF is downregulated and F3 is methylated in IDH mutant tumors; TF is detectable in plasma of GBM patients; TF levels in GBM or blood do not correlate with VTE risk in GBM	7,8,42,59,121, 124,127
Risk factors for VTE in HGG patients	Ten factor VTE risk assessment tool in diffuse glioma patients	i) Pro-thrombotic factors: prior history VTE, hypertension, asthma, white blood cell count, WHO tumor grade, patient age, and body mass index ii) Anti-thrombotic factors: IDH mutation, hypothyroidism, MGMT promoter methylation	42
Extracellular vesicles (EVs)	EVs carry coagulant activities in GBM	EVs carry PDPN and TF in GBM models; EVs carrying TF are detectable in plasma of GBM patients; EVs from platelets and endothelial cells are elevated in blood of GBM patients; EV-associated TF activity does not correlate with VTE risk in some but not all studies	16,124,148-150
Cell-free mitochondria	Mitochondria induce neutrophil-rich thrombi	Circulating cell-free mitochondria are associated with thrombosis in GBM patients, they carry cardiolipin and induce neutrophil-rich thrombi in mice	151

of the tumor mass were found to be highly positive for FXa, which was in line with the correspondingly high expression of TF by cancer cells.[16] TF is also released by cancer cells,[32,64] including experimental GBM cells, as cargo of EVs,[16,123] which were readily detected in the circulating blood of GBM patients.[7,42] Collectively, these circumstances, along with the ability of decrypted TF and TF-EVs to activate the coagulation cascade and drive thrombosis in cancer models in mice,[45] suggest that TF could be a part of the CAT-driving circuitry in GBM, acting both locally and systemically. However, this notion is inconsistent with other studies reporting no correlation between TF expression and levels of circulating TF in blood and the VTE risk in GBM patients.[59,124] This is at variance with more recent studies suggesting an association between circulating TF activity (as cargo of EVs) and VTE risk in GBM patients[42] and it is clear that this question requires further analysis, potentially with patient stratification into GBM subtypes.[8]

Since GBM cells reportedly express TF mainly in the cytoplasm and not on the cell surface (unlike in epithelial cancers)[122] it is possible that EVs released from these cells harbor TF, but in a form that is not fully exposed on the cell surface. It is possible that such luminal (concealed) TF could be recycled to the cell surface in the TF-EV recipient cells. It is also possible that EVs carrying TF in this case are generated in the endosomal compartment (exosomes) and not on the cell surface (ectosomes/microparticles).[125] Such small EVs may require an adapted isolation technique and may be poorly recovered from plasma under common centrifugation schemes. Whether such small EVs could still transfer TF to other cells in the peripheral circulation and render them procoagulant[63] remains to be studied.

One important consideration stemming from recent studies involving instruments operating at a single vesicle resolution (Nano-flow cytometry, ExoView, ONI) revealed that subsets of individual EVs may carry both PDPN and TF, possibly along with other effectors.[1,16] This finding may suggest that correlating individual factors associated with the EV compartment (e.g., either PDPN or TF) and the VTE risk may be insufficient, and the analysis of multifunctional procoagulant EVs may be worth exploring going forward.[1] Also, standard coagulation assays which rely on TF, PS and other effectors may not capture platelet activating potential of EVs carrying both TF and PDPN.[16]

It should be noted that both TF and PDPN are targets of methylation-dependent epigenetic down-regulation in IDH mutant HGGs, due to generation by cancer cells of the oncometabolite, D-2-hydroxyglutarate (D2HG). D2HG inhibits dioxygenases that normally demethylate DNA and histones[126] resulting in wide spread changes in methylation landscapes of cancer cells, including downregulation of TF and PDPN expression.[16,127,128] In contrast, in IDH-wild type grade IV HGGs (GBMs) *TF/3* and *PDPN* genes are unmethylated and, in addition, some of these factors could be deregulated by oncogenic pathways including RAS, EGFR, PI3K, PTEN loss and hypoxia.[33,121,129] Furthermore, EV biogenesis, cargo loading, ability to mediate intercellular communication and other EV properties are also regulatory targets of oncogenic mutations including EGFRvIII,[130] PTEN and others.[131,132] It follows that these mechanisms may converge upon the release of coagulant EVs from cancer cells and thereby represent a part of the molecular landscape underlying CAT in GBM and other cancers.

Cellular, spatial and temporal determinants of glioma coagulome

While the aforementioned studies dissect the prevailing CAT mechanisms across different HGG subtypes, analyses of coagulant processes at the single cell level are only beginning to emerge.[1,16,133] Thus, as mentioned, a survey of previously published single cell transcriptomes obtained from surgical specimens of GBM with different subtype characteristics illustrated a considerable heterogeneity of individual cancer cells in terms of their expression profiles of coagulome-related genes, including F3/TF, PDPN, F2R, PROS, PLAT and others.[134] In this setting the expression of oncogenic EGFR was found in both TF/F3 high and low cellular populations, suggesting that this oncogenic pathway is not a sole determinant of cancer cell coagulome *in vivo*. In another series of single cell analyses which involved clustering of GBM cells around neurodevelopmental differentiation pathways,[135] F3/TF expression was enriched in cells with astrocytic signatures, while PDPN mRNA was enriched in the mesenchymal cell cluster. In this dataset progenitor cells expressed low level of either one of these prothrombotic transcripts.[16] The latter observation was borne out in the phenotype of human GSCs isolated from GBM patients, in that both proneural and mesenchymal stem cells expressed low levels of TF and PDPN unless placed under induced differentiation conditions.[16] The levels of corresponding proteins found in GSC isolates were consistent with these findings[16] and may suggest that in different cancers tumor initiating cells may differ with respect to their coagulant profiles and interactions with the hemostatic system.[136-138]

While TF and PDPN are mainly expressed by GBM cells, the emerging evidence suggests that reactive astrocytes or microglia may also contribute to procoagulant milieu of brain tumor tissues, at least to some extent.[139] Nonetheless, the global profiles of putative effectors of VTE, as determined by bulk transcriptomic or proteomic studies, appear to represent a cumulative summation of more intricate and diverse landscapes of single cell coagulomes. These early studies point to the notion that coagulant effects of individual cancer cells is unequal, and only some, but not all of them may express functional pro-thrombotic phenotypes.[1] These cells could be seen as 'superdrivers' of GBM-associated CAT and their content in surgical specimens could, conceivably, be a part of the anticipated VTE risk.[16]

The cellular complexity of GBM also has important structural and temporal dimensions. For example, spatial analysis of single GBM cell transcriptomes revealed that cells in specific phenotypic states are organized as distinctive, interactive microdomains.[140] It is very likely that these multicellular and interactive features impact cellular phenotypes and undergo major changes in the course of disease progression, therapy and relapse. Whether and how such cellular microdomains in GBM come into contact with the hemostatic system, or whether they regulate the coagulant phenotype of cancer and stromal cells, impact microvascular thrombosis or VTE risk remains to be determined. Similarly, there is a strong likelihood of longitudinal changes in coagulant processes that accompany the natural history of GBM, and these factors may alter not only the risk, but the very mechanism of CAT over time. Again, little is known about these aspects of the disease at the present time. While the grim course of GBM places therapeutic emphasis on the control of the main disease, these interventions

affect coagulant mechanisms[141] and the vascular system,[94] and consequently biologically-driven adjustments of VTE prevention and treatment may also be worth considering.

Closing remarks

Recent progress in studies on the biology of GBM at the molecular and cellular level have unmasked an astounding diversity of disease processes and multiplicity of pathogenetic facets. Whether and how the expression of coagulant phenotypes by cancer cell populations are linked to VTE risk is beginning to be explored in the earnest.[16] The reciprocal interrelationships between hemostatic system effectors (coagulation factors, fibrinolytic machinery, platelets) and mechanisms driving GBM progression has also attracted recent attention.[91,92,142]

The emerging data coalesce around a model (Figure 1) in which oncogenic transformation acts in a tumor specific manner to activate downstream mediators of CAT either emanating directly from cancer cells, from activated elements of the TME, or related to systemic hemostasis. These factors could potentially act in concert to overcome the threshold of thrombosis in specific disease contexts. In other words, predicting VTE may require development of a multimolecular 'CAT signature' instead of a search for single 'markers', even as compelling as TF or PDPN. In this setting tracing 'dominant' procoagulant mediators (or their combinations) associated with defined molecular subtypes of cancer, including GBM could lead to establishing more tailored intervention strategies with potentially improved efficacy. For example, targeting PDPN/platelet pathway might be expected to lessen the burden of thrombosis in IDH-wild type HGGs, where the role of PDPN is strongly implicated.[46] At the same time, it should be evaluated whether introduction into HGG subsets driven by mutant IDH1/2 anticancer agents targeting this oncogene (vorasidenib)[143] may reverse the thrombo-protective effects of IDH mutation and increase the risk of VTE. It is noteworthy that in contrast to brain cancer, IDH mutations do not seem to have thrombo-protective effects in hepatobiliary tumors.[36] These examples illustrate the potential appeal of taking a tumor-specific approach in studies on CAT.

In this rapidly evolving landscape, many questions still remain unanswered. For example, it is still unclear whether mechanisms of thrombosis are affected by the formation of complex multicellular structures where cancer cells are connected by a web of tumor microtubes discovered in experimental HGG models.[144] Similarly, function changing interactions between GBM cells and neurons,[145] or endothelial cells[63,119] has not been fully investigated as potential factors influencing the interface between cancer and the hemostatic system. How various pathways of cell cooperation, communication and molecular exchange, including through EVs,[146] intersect with oncogenic regulation of the cellular coagulome, and how they influence the cross-talk with the hemostatic system represents another area of great interest, especially in light of recent reports suggesting that FVII signaling may impact cellular vesiculation processes.[147]

How should expression of putative pro-thrombotic factors by cancer cells (e.g. TF, PDPN and others) be interpreted? Is CAT driven by coagulant phenotypes of subsets of tumor cells expressing highest levels and widest repertoires of pro-thrombotic factors, or is this a collective feature of GBM cell populations? How do these cells project their effects systemically? What is the role of EVs and tumor interactions with the coagulation system, platelets and the peripheral vasculature? What are the key pathways of CAT (or different CATs?) operative in different subtypes of GBM and across the disease trajectory? Addressing some of these biological questions may add to the present mostly hematological perspective on CAT in brain tumors.[41]

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