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PhD Thesis

Development and validation of target therapies for patients with brain cancer, through the modulation of angiogenesis, invasiveness, and pharmacological sensitivity/resistance, in the era of precision medicine

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"Chi dice che impossibile
non dovrebbe disturbare
chi ce la sta facendo"
Albert Einstein

A mio marito,
Alla mia famiglia,
Ai miei colleghi

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SUMMARY

Brain tumors represent a group of heterogeneous neoplasms which, despite originating in the same anatomical region, differ in morphology, etiology, molecular biology and especially in clinical behavior. It has been estimated that brain tumors affect about 200,000 people worldwide every year, representing approximately 2% of cancer deaths. Among all primary human brain tumors, glioblastoma (GBM) is the most malignant and frequent (~70%), with a median survival of about 14 months and a 5-year survival rate at 5%, thus representing an extreme therapeutic challenge. GBM is characterized by sustained proliferation and survival, immune system escape, intense angiogenesis, invasion, cell infiltration, rapid progression, resistance to radio- and chemotherapies, with high frequency of relapse. In cancer pathogenesis, particularly in high grade tumors as GBM, aberrant neo-angiogenesis is a vital process for the mass growth: it is driven by neoplastic cells in order to respond to the tumoral hypoxic environment in the necrotic core, which increases the demand for oxygen and nutrients by neoplastic cells, and is, therefore, essential to carry out the metabolic functions on which their survival is based. Several observations led to the knowledge that tumoral neo-angiogenesis gives rise to ultra-structurally abnormal vessels, larger than their normal counterparts, dilated, convoluted, irregularly branched and exceptionally permeable due to the presence of fenestrations and the lack of a complete basal membrane. Due to these destructive features, despite aggressive therapeutic treatment, consisting in surgical resection followed by chemotherapy with temozolomide (TMZ), most patients experience tumor recurrence in less than one year, suggesting the urgent need to implement clinical practice with novel prognostic and therapeutic strategies. To this aim, this research project has been focused on: i) aberrant angiogenesis mediated by endothelial cells (ECs), which promote tumor infiltration into surrounding tissues, with consequent compromission of cognitive skills, and ii) genetic instability, which characterize GBM with a high intra- and intertumoral heterogeneity. In particular, the underlying hypothesis is that a specific molecular signature characterizes “resistant” and “sensitive” GBM, and it may be responsible for patient overall survival. Indeed, although GBMs from short- and long-term survivors (STS and LTS) are histologically the same, their biological and molecular characteristics are remarkably different, suggesting that factors that contribute to patients’ longevity are important for precise diagnosis and correct clinical management of the disease. The genetic profiling obtained by array CGH on STS and LTS revealed a high number of copy number variation (CNVs) across chromosomes 1 to 22, among which several novel potential prognostic and predictive biomarkers have been described and discussed. In particular, aCGH highlighted the presence of an altered chromosomic pattern relative to calpain family genes. Calpains are a conserved family of cysteine proteinases that catalyze the controlled proteolysis of many specific substrates. Calpain activity is implicated in several

fundamental physiological processes, including cytoskeletal remodeling, cellular signalling, apoptosis and cell survival. Alterations of the calpain activity balance has been observed in numerous cancer types, as they can reduce apoptosis, increase cell proliferation and stimulate cell migration and invasiveness. The characterization of calpain expression in primary GBM endothelial cells (GECs) showed an upregulation of calpains and a positive correlation between expression level and patient survival. The blockade of calpain activation with natural and synthetic inhibitors resulted in the inhibition of GEC viability and proliferation, as well as in the promotion of caspase-induced cell apoptosis. Further, calpain inhibition led to the arrest of tumor angiogenesis and migration *in vitro.*
Finally, the molecular investigation revealed a downregulation of proliferative signalling as MAPK and anti-apoptotic regulators as Bcl-2, as well as an upregulation of proapoptotic mediators, as Caspases and Bax-family. From this evidence, calpains may be considered as novel potential therapeutic targets to treat cancer and to limit its progression.
Overall, the discovery of novel potential prognostic and predictive biomarkers, by the "omics" approach, will allow to optimize patient's management, as early prediction of those patients who are likely to be STS or LTS, and a full understanding of their clinical course can thus assist clinicians in providing tailored treatments and support patients and their families. However, beyond prognosis, what will really impact clinical management of GBM is if genomic analyses can lead to customized treatment and ultimately improved survival, following the concept of precision medicine.

AIM OF THE STUDY

The main purpose of the present study concerns the identification of a specific genetic signature characterizing "sensitive" and "resistant" glioblastomas, which may be responsible for patient's response to therapies and therefore progression free survival and overall survival. The exploration of genetic alterations impacting tumor aggressiveness means the potential identification of novel prognostic, predictive and targetable biomarkers, which may really impact clinical practice in terms of patient management and therapy optimization. Further, the discovery of novel cancer pathomechanisms may lead to the identification of unestablished molecular targets, whose investigation and screening may really improve the application of personalized treatments, in the era of precision medicine.

List of abbreviation

ANG angiopoietin

BBB blood brain barrier

CAPN1 calpain 1

CAPNS1 calpain small subunit 1

CNS central nervous system

CNV copy number variation

EC endothelial cells

ECM extracellular matrix

EGFR epidermal growth factor receptor

GBM glioblastoma

GEC glioblastoma endothelial cells

GO gene ontology

GSC glioblastoma stem cells

HIF1a hypoxia-inducible factor

IDH isocitrate dehydrogenase

KPS Karnofsky performance status

LTS long-term survivors

MGMT Isocitrate dehydrogenase

MMPs matrix metalloproteinases

OS overall survival

PPI protein-protein interaction

RTK receptor tyrosine kinase

STS short term survivors

TERT telomerase reverse transcriptase gene

TMZ temozolomide

VEGF vascular endothelial growth factor

VWF von Willebrand factor

WHO world health organization

1. INTRODUCTION

1.1 Central Nervous System Tumor

Central nervous system (CNS) tumors are a cluster of heterogeneous neoplastic entities that arise in a common anatomical region but differ from each other in morphology, etiology, site of onset, molecular biology and clinical behavior[1]. CNS tumors affect about 200,000 people worldwide every year, representing approximately 2% of cancer deaths[2,3]. The mortality and morbidity of CNS tumors is strictly related to their localization and their invasive growth rate[4]. The highest percentage of brain tumors that originate in the CNS are benign and occurs in the meninges, a structure consisting of three layers of protective tissue surrounding the cerebral cortex[5]. However, most malignant primary brain tumors occur in the cerebral cortex, with the highest percentage developing in the frontal lobe[5] (Figure 1). The frontal lobe constitutes almost two-fifths of the human brain and it provide the modulation of voluntary movement, fluency of speech and expression of emotion. Patients with a tumor in this area (26%) often experience seizures as an initial symptom. Due to its location near the ear, most of temporal lobe function are related to auditory processing. This means that surgical excision of tumors located in the temporal lobe (19%) is challenging for the region complexity. The left and the right hemisphere of the parietal lobe differs for role, thus patient with tumor in the left lobe often suffer for speech alteration, whereas patients with tumor in the right lobe often experience an altered perception of physical location of body parts and understanding of geographical location.

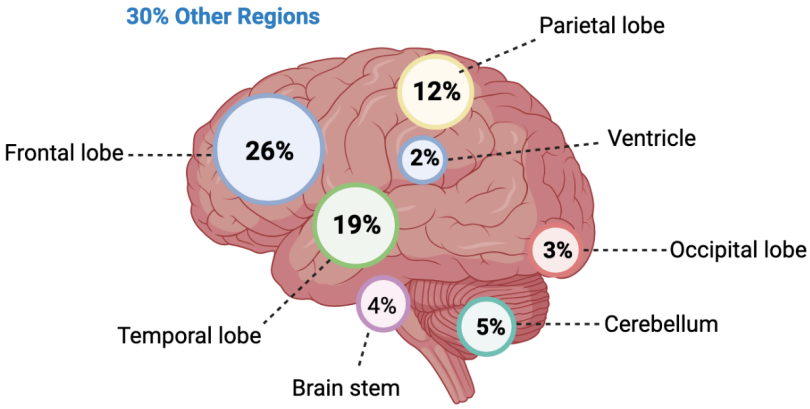

Figure 1. Frequency of tumor onset in different brain localizations.

Malignant primary brain tumors remain among the most difficult cancers to treat, with a 5-year overall survival (OS) no greater than 35%[6]. By the definition, primary tumors are those that start from cell within the brain, spreading to the other brain localization but rarely to other organs. Secondary

or metastatic brain tumors begin in another part of the body and then spread to the brain. These tumors are 5-10 times more common than primary brain tumors and are named by the location in which they begin[7]. The pie chart in Figure 2 represents the yearly incidence of brain tumors in adults.

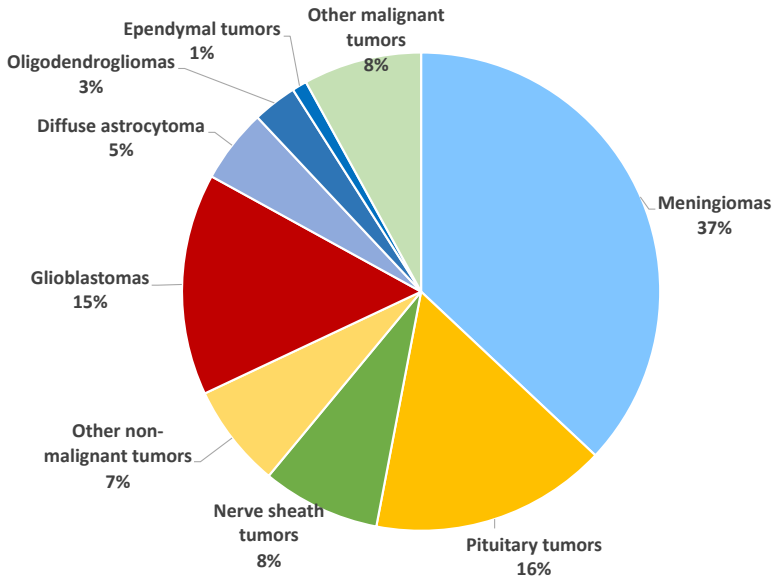

Figure 2. Graphic representation of yearly incidence of primary brain tumors in adults from CBTRUS 2010–2014

Most malignant primary tumors (about 75%) are classified as gliomas, tumors of neuroectodermal origin arising from glial or precursor cells, which include astrocytoma, oligodendrogliomas, and ependymomas. The World Health Organization (WHO) specifies a grading system for brain tumors ranging from grade I, the least aggressive with best prognosis, to grade IV, the most malignant with the worst prognosis. The WHO classification has undergone major restructuring in the 2016 with subsequent effects on diagnostic criteria, diagnostic testing approaches, grading, prognosis, and treatment planning. The major update of guidelines released in 2016 consists in the introduction and integration of molecular parameters into diagnostic procedure previously based only on histopathological features.

1.1.1 Clinical presentation of brain tumors

Patients with primary brain tumors can present with focal (i.e., headaches related to the specific location in the brain) or generalized symptoms (i.e., fatigue, behavioral alterations as hallucinations, depression, anxiety, decreased attention and concentration, memory problems)[8]. These symptoms

may last over days to weeks, or months to years, depending on the speed of growth and location of the tumour. Precise information about the wide range of experienced symptoms is almost lacking and when available, it is also quite contradictory. Commonly, six symptoms as fatigue, communication difficulties and headaches, motor difficulties, drowsiness and uncertainty about the future were reported in >50% in a sample of patients with glioma, with a considerably negative impact on patient's quality of life[9]. Generally, the clinical presentation of brain tumors may be related to the precise localization. Tumors in some functional areas of the brain will cause more obvious focal neurological deficits than in other areas and tend to be discovered sooner by imaging. Frontal lobe tumors might cause weakness or dysphasia; parietal lobe tumors might cause numbness, hemineglect, or spatial disorientation; and tumors involving the optic radiations anywhere in the temporal, parietal, or occipital lobe might cause visual field defects. Conversely, tumors located in the prefrontal lobe, temporal lobe, or corpus callosum often result in cognitive dysfunctions such as personality changes, mood disorders, and short-term memory deficits[6]. Patients affected by brain tumors can also present with generalized symptoms, not associated with the anatomic location. For example, 50–80% of patients might present with seizures[10], about 30% with headaches[11] and 15% with symptoms of increased intracranial pressure, such as progressive headaches worse at night, morning nausea and vomiting, drowsiness, blurred vision from papilledema, and horizontal diplopia from cranial nerve VI palsy[11].

1.1.2 CNS tumor classification

Historically, the classification of CNS tumors has relied exclusively on findings from microscopy and immunohistochemical (IHC) analysis. Since the recognition that various and specific "molecular signatures" may provide diagnostic utility[12], a major revision to the prior 2007 WHO CNS tumor classification was undertaken[13]. Therefore, the resulting WHO 2016 includes both microscopic and molecular parameters into CNS tumor classification, providing a major restructuring to several brain tumor groups, particularly diffuse gliomas. The combined phenotypic and genotypic classification generated new integrated diagnoses, where the histopathological name is followed by the genetic features (e.g., glioblastoma, *IDH*-wildtype; Table 1).

Table 1. Simplified WHO 2016 classification of selected neuroepithelial tissue tumors

Diffuse gliomas

WHO II

- Diffuse astrocytoma, *IDH*-mutant
- Diffuse astrocytoma, *IDH*-wildtype
- Diffuse astrocytoma, NOS
- Oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted
- Oligodendroglioma, NOS

WHO III

- Anaplastic astrocytoma, *IDH*-mutant
- Anaplastic astrocytoma, *IDH*-wildtype
- Anaplastic astrocytoma, NOS
- Anaplastic oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted
- Anaplastic oligodendroglioma, NOS

WHO IV

- Glioblastoma, *IDH*-wildtype
- Glioblastoma, *IDH*-mutant
- Glioblastoma, NOS
- Diffuse midline glioma, *H3 K27M*-mutant

Other astrocytic tumors

WHO I

- Pylocytic astrocytoma

WHO II

- Pleomorphic xanthoastrocytoma

WHO III

- Anaplastic pleomorphic xanthoastrocytoma

Ependymal tumors

WHO I

- Subependymoma
- Myxopapillary ependymoma

WHO II

- Ependymoma

WHO III

- Anaplastic ependymoma

Neuronal and mixed neuronal-glial tumors

- Diffuse leptomeningeal glioneural tumor

WHO I

- Dysembryoplastic neuroepithelial tumor
- Ganglioglioma
- Gangliocytoma
- Dysplastic gangliocytoma of cerebellum

WHO III

- Anaplastic ganglioglioma

IDH: Isocitrate Dehydrogenase; **NOS:** not otherwise specified.

Grade I gliomas represent tumors mostly regarded as benign and curable by complete resection. They often present *BRAF* mutation but not *IDH* mutation. Contrariwise, diffuse gliomas are almost never cured by resection alone and are graded using histopathological and molecular features. Histologically, grade II, known also as low-grade gliomas (LGG), comprises diffuse oligodendrogliomas and astrocytomas, present nuclear atypia, slow growing rate, and they may follow long clinical courses. Grade III, anaplastic oligodendrogliomas and astrocytomas, display increased mitotic activity, thus grow faster and more aggressively than grade II, exhibiting increased anaplasia and infiltration in neighboring tissues. Grade IV (glioblastomas, GBM) show additional microvascular proliferation, necrosis, or both. GBM is the most common and aggressive and can develop directly, as primary tumor, or evolve from lower grade tumors, as secondary GBM. The integration of molecular parameters in the traditional classification determined the further stratification of grades II–III gliomas into three diagnostic and prognostic subgroups based on their *IDH*, *ATRX*, and 1p/19q status[14,15] (Figure 3). Most grade II–III gliomas harbor a key driver mutation in the *IDH* gene and have typical *TP53* and *ATRX* mutations[13] and are called diffuse astrocytoma,

IDH-mutant. Grades II–III gliomas harboring *IDH1* mutation but no *ATRX* mutation, 1p/19q codeletion status assessment is required to distinguish astrocytomas from oligodendrogliomas[13,16].

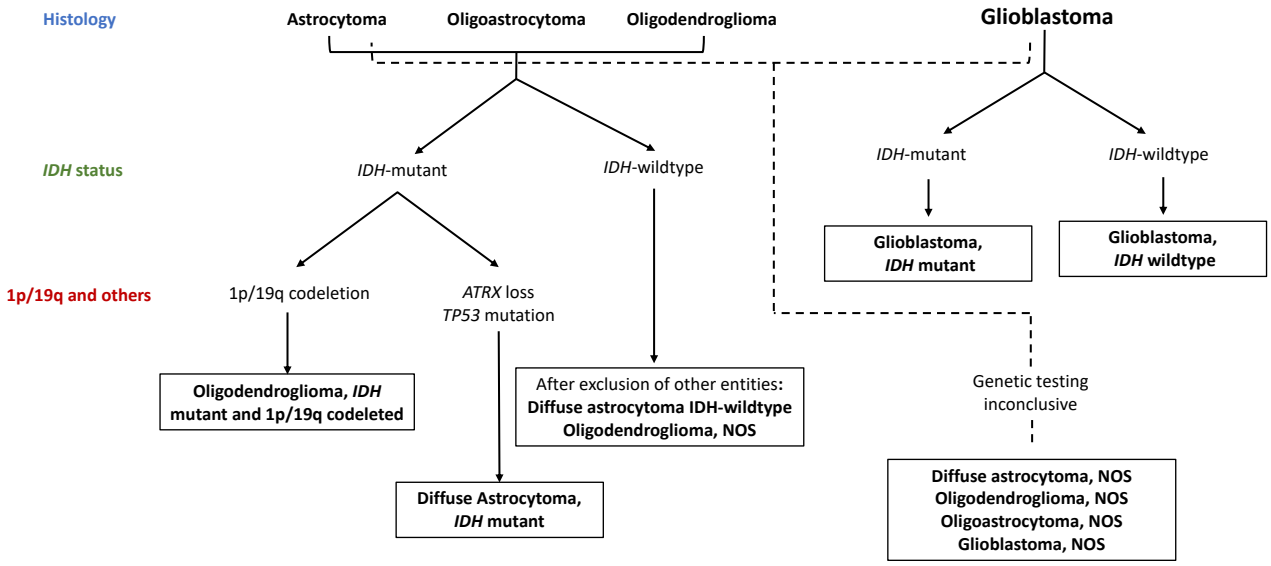

Figure 3. A simplified algorithm for classification of the diffuse gliomas based on histological and genetic features. **IDH:** Isocitrate Dehydrogenase; **NOS:** not otherwise specified.

Despite several studies[17,18] have shown a promising role for *TERT* promoter mutation in predicting clinical outcome among grade II–III gliomas with the same *IDH* status and among GBMs, independently of their *IDH* status, its prognostic impact remains controversial. The most commonly used molecular analyses for gliomas are described below are the most commonly used molecular analyses for gliomas.

1.1.3. Molecular markers

MGMT

DNA methylation is an epigenetic mechanism involving the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs)[19]. Abnormal methylation plays an important role in the tumors' development. Locus-specific hypermethylation usually occurs at promoter of CpG islands of tumor suppressor genes, resulting in transcriptional silencing. This is the case of O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair

protein involved in repairing damage induced by alkylating agents such as TMZ[20]. Methylation of the *MGMT* promoter (MGMTp) silences the *MGMT* gene and reduces the ability of tumour cells to repair such damage, increasing the mortality of glioma cells. MGMT can prevent the formation of DNA cross-linking, then reducing the cytotoxicity of alkylating agents and optimizing chemotherapy effects. MGMTp methylation predicts benefit from alkylating chemotherapies as TMZ (Temodal®, Temodar®, Merck & Co, White House Station, NJ, USA) in GBM patients[13,21,22], including elderly patients. However, the consideration by Mur et al. that MGMTp methylation is extensively heterogeneous in HGG patients, raised several doubts about the worth of *MGMT* for the management of patient's therapeutic regime, advancing the need for other biomarkers in the prognosis of glioma [23]. Therefore, its testing generally does not impact treatment as alkylating chemotherapy is the standard of care for all GBMs. MGMTp methylation is common in glioblastomas (30–50% of primary, *IDH*-wildtype, glioblastoma) and in oligodendrogliomas (>90%), but less common in lower grade astrocytomas[13,24].

IDH

Isocitrate dehydrogenase (IDH) is a cytosolic enzyme that catalyze the oxidative decarboxylation of isocitrate to alpha-ketoglutarate (α-KG) and plays an important role in the reduction of $NADP^+$ to reduced nicotinamide adenine dinucleotide phosphate NADPH. IDH has two forms of existence *in vivo*: NADP-dependent IDH (IDH1 and IDH2) and NAD-dependent IDH (IDH3)[25].

IDH is involved in a number of cellular processes, including regulation of cellular redox status, glutamine metabolism, glucose sensing, lipogenesis and mitochondrial oxidative phosphorylation[26,27]. In 2008, Parsons firstly sequenced 20661 protein-coding genes of 22 glioma samples, founding that 12% of GBM patients had repeated mutation in the *IDH1* active site, with *IDH1* mutation occurred mostly in young and secondary GBM[28]. A year later, De Carli et al. demonstrated that more than 70% of grade II and III astrocytomas and oligodendrogliomas were *IDH1*-mutant and that these patients experienced a better outcome than *IDH* wild-type ones[29]. Further, it has been observed that *IDH1* mutation occurs frequently in anaplastic oligodendroglioma (75%), secondary GBM (67%), oligodendroglioma (67%), anaplastic astrocytoma (62%), anaplastic ganglion tumor (60%) and ganglion glioma (38%), but in primary GBM the mutation rate is significantly lower, only 5% [30]. Functionally, the mutation of *IDH1* disrupts the stability of its active center, due to the changes in the structure of the enzyme from an open to a closed conformation[31]. Indeed, substrate recognition depends on the amino acid residues in the active site, whereas the frequent mutated active site residue in cancer is arginine 132 (R132)[32]. This mechanism leads to the reduction of IDH binding ability and the production high levels of 2-hydroxyglutaric acid (2-HG),

which promote glioma development and progression, by the upregulation of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α)[33].

Nowadays, *IDH1* and *IDH2* mutations are thought to be an early event of gliomagenesis and diffuse gliomas harboring *IDH1/2* mutations are associated with a better prognosis than diffuse gliomas, *IDH*-wildtype[34].

Chromosomes 1p/19q Deletion

The codeletion of chromosomes 1p and 19q results from a non-balanced centromeric translocation t(1:19) (q10:p10)[35]. Several studies have shown that 1p/19q deletion occurs mainly in low-grade oligodendrogliomas[36], with a deletion rate of about 70% in WHO II grade oligodendroglioma patients is about 70%, and 15% in WHO grade III oligodendroglioma patients is about 15%[37]. 1p/19q deletion's incidence rate is lower in astrocytoma and GBM, approximately about 12% and 3%[38]. Co-deletion of chromosome 1p/19q increased survival in patients with low-grade gliomas and oligodendrogliomas, with a median survival time twice more than the 1p/19q non-deletion patients[39], by promoting the sensitivity to chemotherapy[40]. Therefore, 1p/19q codeletion is now considered a predictive factor of an increased response to alkylating agents, conferring a favorable prognosis among diffuse gliomas[41,42]. Recently, Odia et al. found that 1p/19q deletion was secondary to the *IDH1* mutation[43], thus this codeletion, combined with IDH mutation, is now required for the diagnosis of oligodendroglioma *IDH*-mutant and 1p/19q codeleted[13].

TERT

The transcriptional activation of the telomerase reverse transcriptase (*TERT*) gene, which is normally silenced in somatic cells, is a key step in tumorigenesis[44], including GBM[45]. Mechanistically, *TERT* gene, located on chromosome 5p15.33, encodes the catalytic subunit of telomerase, which counteracts the shortening of telomeres by adding small, repeated segments of DNA to the ends of chromosomes each time the cell divides. *TERT* reactivation in cancer is thought to contribute to cell survival and immortalization, as it confers unlimited proliferation potential to cancer cells by stabilizing their telomere length[46]. Several studies demonstrated that inhibition of *TERT* expression enhances sensitivity of cells to DNA damage by radiation and chemotherapy, suggestive of a possible combination therapy for cancer treatment[47,48]. More recent, Zhou et al. observed that *TERT* promoter mutation is significantly associated with the risk of glioma, with the highest mutation rate in GBM, astrocytoma, and oligodendroglioma[18].

1.2 Glioblastoma

Glioblastoma (GBM, WHO grade IV) is the most frequent and malignant glioma in adults, and the prognosis for patients is often bleak. The median survival rate of patients diagnosed with GBM is 15–23 months and the 5-year survival rate is less than 6%, which is the lowest long-term survival rate of malignant brain tumors[49]. GBM is characterized by rapid progression, survival, invasiveness, angiogenesis, immune system escape, genetic instability, high frequency of relapse, and resistance to radio and chemotherapies (Figure 4). For these reasons, despite the current standard therapy, including maximal safe resection followed by radiotherapy in combination with temozolomide[21] and various salvage therapies once tumour progression occurs, the majority of patients succumb to the disease within 2 years of diagnosis[50]. Furthermore, the associated morbidity with progressive decline in neurologic function and quality of life can have a devastating impact on patients and caregivers[51].

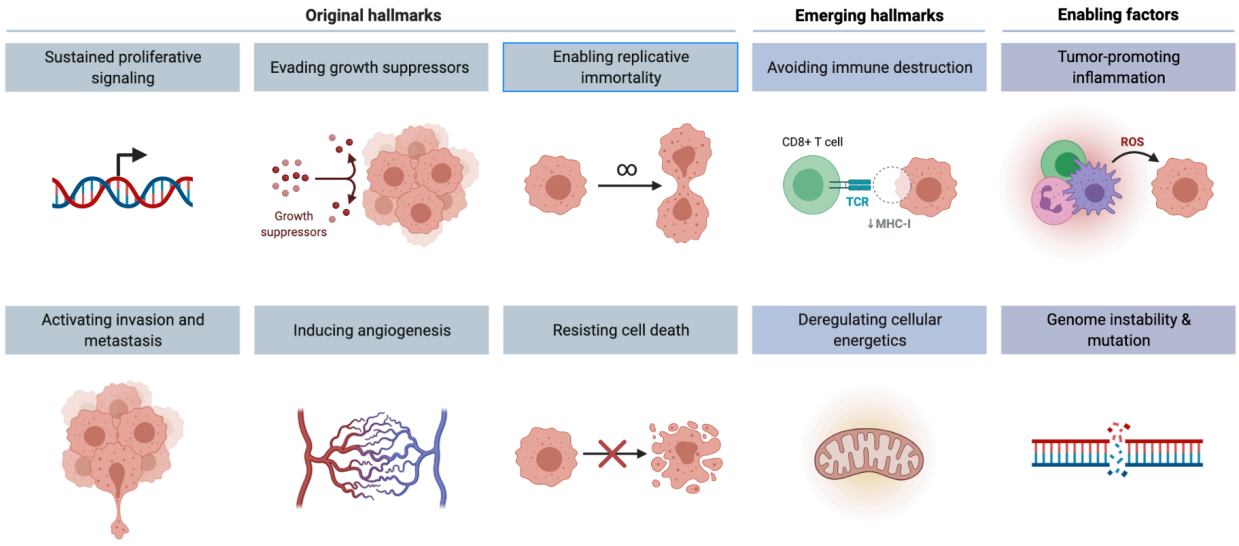

Figure 4. GBM cellular and molecular hallmarks, underlying its malignancy and rapid progression

1.2.1 Epidemiology of GBM

Glioblastoma accounts for 57% of all gliomas and 48% of all primary malignant CNS tumors[52]. Its annual incidence is about 3/100,000 people, very low when compared to that of several other cancers of the human body. The median age at diagnosis is 65 years, with rates highest in the group aged 75

to 84 years, for which annual incidence reach 15/100,000 individuals. GBM is 1.58 times more common in males than in females, with an annual age-adjusted incidence of 4.00 compared with 2.53 per 100,000 population, respectively, white Caucasians more than Blacks and Asians. Older age and lower performance status are associated with less aggressive care and shorter survival. Survival is inversely correlated with age: 5% of all patients who receive a diagnosis of GBM are alive after 5 years and this value decreases to 2% if we consider the population over 65 years[53]. There are few validated risk factors for GBM. Exposure to ionizing radiation is the strongest risk factor associated and it is also the only known potentially modifiable[54]. An inverse association between GBM and a history of allergies and other immune-related conditions has also been identified, but the exact underlying biological reasons have not been elucidated[55]. Genetic syndromes with autosomal dominant inheritance with incomplete penetrance such as neurofibromatosis types 1 and 2, Li Fraumeni and Turcot seem to lead to the development of GBM usually secondary to a previous lower grade tumor. In any case, this is a much lower fraction (around 5%) than the total[56].

1.2.2 Classification and Histopathogenesis

GBM, along with other gliomas, are thought to arise from neuroglial progenitor cells[57]. Histopathologically, GBM is characterized by vascular proliferation and necrosis. Other signs of malignancy, already present in anaplastic astrocytomas, regard anaplasia, the high mitotic index and invasiveness[41]. As for all diffuse gliomas, a defining biological feature of GBM is tumor cell infiltration. Neoplastic cells show the ability to move long distances from the place of origin by tracking neuropil structures. They may travel in the opposite hemisphere and may give rise to additional tumor foci, giving rise to multifocal GBM. Due to this biological behavior, a complete microscopic resection can never be achieved, and residual neoplastic cells are frequently the source for disease recurrence. Furthermore, GBM is characterized by pleomorphic cells, mitotic activity, intravascular microthrombi, necrosis with or without cellular pseudopalisading, and/or microvascular proliferation[58]. The 2016 revision of the WHO classification of CNS tumors restructured the classification of gliomas, predominantly with the integration of molecular features in addition to histopathologic appearance[59]. Tumor heterogeneities pose challenges to GBM diagnosis, prognosis and treatment, as histologic diagnosis often varies among clinicians and limits diagnostic reproducibility. Both histologically and genetically, GBM show significant inter-tumoral and intra-tumoral heterogeneity, differing mutations, and indistinct phenotypic and epigenetic states. This genomic instability leads to different promising target therapies, thus varying therapy choices and clinical outcomes[60,61]. Based on transcriptional profile, in 2010 Verhaak et al. offered more in-depth research and treatment possibilities for GBM, based on the four subtypes: Proneural, Neural, Classical

and Mesenchymal[62]. The proneural subtype is characterized by high PDGFRA gene expression and frequent IDH1 mutation and is found primarily in younger patients. Despite it shows no significant difference from other subtypes in response to chemotherapy and radiotherapy, the proneural subtype has the better survival rates[63]. The neural subtype has similar gene expression patterns compared with normal brain tissue and shows neural markers as *NEFL* (Neurofilament light polypeptide), *SLC12A5* (Solute carrier family 12 members 5), *SYT1* (Synaptotagmin 1) and *GABRA1* (Gamma-aminobutyric acid type A receptor alpha1). Notably, it often tends to be more responsive to radiation and chemotherapy. The classical subtype shows aberrant alterations, including amplification of Chr7, loss of Chr10, inactivation of the RB (Retinoblastoma-associated protein) pathway, and focal 9p21.3 homozygous deletion. In addition, Sonic hedgehog pathways, Notch signaling pathways and the neural precursor and stem cell marker NES are highly expressed in the Classical subtype. Importantly, patients with Classical subtype show a significant reduction in mortality with aggressive radiotherapy and chemotherapy. The Mesenchymal subtype is characterized by extensive necrosis and inflammation, upregulation of interstitial and angiogenesis genes, deletion of tumor suppressor genes *P53*, *PTEN*, and *NF1*, and high expression of genes in the tumor necrosis factor superfamily and the NF-κB pathway[64]. Although responsive to aggressive radiotherapy and chemotherapy, the prognosis of Mesenchymal subtypes is the worst among all subtypes[63]. Recently, Sharma et al. found that Vascular Endothelial Growth Factors (*VEGF-A*, *VEGF-B*), as well as Angiopoietin-1 and Angiopoietin-2 genes are highly expressed in the Mesenchymal subtype[65].

As described above, the mutational status of *IDH* is routinely used to classify GBMs, resulting in *IDH*-wild type GBM and *IDH*-mutated GBM. *IDH*–wild-type GBM correspond to the clinically defined primary GBM characterized by *de novo* development, with no identifiable lower grade lesion. This group represent the majority of GBM patients (90%) and is often found in older patients, with a more aggressive clinical course[66]. Conversely, *IDH*-mutant GBM are typically secondary GBM arising from a precursor anaplastic or diffuse astrocytoma. This group comprises about 10% of patients and is more frequent in younger patients with a median age of 44 years. *IDH* mutation generally determines a better prognosis than *IDH*-wild type[67].

1.2.3 Additional molecular markers of GBM

The clinical outcome of patients with GBM may be influenced by a variety of tumor molecular biomarkers, whose characterization provides the scientific basis for the development of novel targeted, antiangiogenic therapies and immunotherapies. Multiple chromosomic unbalances, as well as alteration in the expression levels of genes and/or proteins have been identified in GBM, including activation of oncogenes and/or silencing of tumour-suppressor genes. The Cancer Genome Atlas

(TCGA) research network confirmed three signalling pathways commonly disrupted in GBM, kinase (RTK)/Ras/PI3K, p53 and Rb signalling.

EGFR

Among the alterations involving RKTs, the amplification of the Epidermal Growth Factor Receptor (*EGFR*) gene is the most common oncogenic one and can be identified in approximately 40% of patients with GBM[68], most of which are classical GBM. In approximately 50% of tumors with amplified *EGFR*, a unique *EGFR* variant lacking exons 2-7 is present (*EGFRvIII*), which results in a constitutive activation of downstream signalling pathways[69].

EGFR is a major activator of physiological responses including proliferation, survival, migration, and tumorigenesis. In GBM, there can be tens additional copies of *EGFR*[59]. *EGFRvIII* is expressed by small extrachromosomal DNA fragments, known as "double minutes", dynamically regulated by unidentified mechanisms[70]. The value of *EGFR* amplification as indicator of poor survival is still controversial[71], but it is widely recognized that it may serve as predictive biomarker for response to RTK inhibitors. However, despite *EGFR*-amplified tumors initially respond to RTK inhibition, data suggests that they often become resistant to this form of treatment[72].

RTK/RAS/PI3K signalling

The co-activation of multiple RTKs, as the above-mentioned *EGFR*, the platelet-derived growth factor receptor (*PDGFR*) or *MET* has been reported as the cause of reduced tumor responsiveness to target therapy[73]. Interestingly, their co-amplification may reflect the presence of genetically distinct tumor populations within a neoplasm[74].

The activity of *PDGFRα*, whose amplification is a major event occurring largely in proneural GBM[68], is responsible for the activation of the Ras/Raf/MAPK pathway, ultimately regulating the activity of transcription factors operating in proliferation, survival, differentiation, and apoptosisal[75]. This pathway can also be directly or indirectly activated through mutations of downstream components. Of note, the upstream Ras antagonist, Neurofibromin 1 (*NF1*) is either deleted or mutated in about 20% of primary GBMs and this profile is typically associated with the mesenchymal GBM subtype[75].

The PI3K family initiates the activation mammalian target of rapamycin (mTOR), which affects tumour cell growth, proliferation, and survival[76]. PI3K activity is mainly counteracted by the phosphatase and tensin homolog (*PTEN*) gene, located on chromosome 10q, encoding a protein that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate[76]. *PTEN* mutations and homozygous deletions are found in 36% of GBMs, and often result in a truncated protein[62].

p53 signalling

To date, p53 in one of the most studied tumor suppressor protein, widely characterized in many cancer subtypes, including gliomas. The *TP53* tumour-suppressor gene, located on the short arm of chromosome 17, encodes a protein that causes cell-cycle arrest in the G1 and/or G2 phase of the cell cycle, and also promotes apoptosis upon DNA damage[77]. *TP53* mutation or deletion, as well as loss of 17p, induces genetic instability and confers a growth advantage, resulting in clonal expansion of glioma cells[78]. Aside the mutational status of *TP53*, its pathway is often tightly modulated by upstream mediators as MDM2, MDM4, and p14ARF as well as downstream effectors such as ATM and ATR[79]. TCGA data reveals that 78% of GBM present mutations in the p53 signaling pathway. In particular, inactivation of the p53 protein is also caused by *MDM2* amplification, which is found in high-grade gliomas in the absence *TP53* mutation[80]. Other frequent genetic alterations include loss of *p14ARF* through homozygous deletion of the cyclin-dependent kinase (CDK) inhibitor 2A (*CDKN2A*) gene, resulting in MDM2 overexpression and functional p53 loss[24]. However, because the p53 pathway functions in so many different cellular responses such as cell cycle regulation, apoptosis, differentiation, and DNA damage response, the prognostic and predictive values of p53 is still largely undetermined[71].

Rb signalling

The Retinoblastoma (Rb) pathway is commonly de-regulated in brain tumors. Rb is a negative regulator of the cell cycle, discovered because of its loss in retinoblastoma[81]. Its activity is mainly regulated though phosphorylation by cyclin D, CDK4 and CDK6. Despite only 20% of GBMs are Rb-mutated, inactivating mutations of the upstream regulator p16INK4a, a suppressor of CDK4, or activating mutations in the downstream factors CDK4 or cyclin D result in dysregulated control of the E2F1 transcription factor are very common[82]. Furthermore, promoter methylation of the Rb gene is 43% more prevalent in secondary GBM as compared to primaries. Rb alteration have not been described in low grade or anaplastic astrocytomas, suggesting that it may be a late event in astrocytoma progression[83].

1.2.4 Treatment Management

The standard initial approach for most primary CNS tumors is maximal safe surgical resection, which allows for accurate histological diagnosis, tumor genotyping, and a reduction in tumor volume. For GBM, the Stupp protocol published in 2005, consist in maximal resection followed by radiotherapy (60 Gray [Gy] over 6 weeks) with concomitant daily TMZ and a further 6 cycles of maintenance

TMZ. Compared with radiotherapy alone, in patients with good performance status (Karnofsky performance status ≥60), the median OS was 14.6 months for radiotherapy plus temozolomide versus 12.1 months for radiotherapy alone[21].

Molecular Target Therapy

According to cIMPACT (the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy), peculiar genetic alterations as *EGFR* amplification, *TERT* promoter mutation, together with whole chromosome 10 loss and whole chromosome 7 gain (-10/+7) can be considered as molecular markers for the diagnosis of *IDH*-wildtype GBM, also in absence of histological features. Many high throughput genomic, transcriptomic and epigenomic studies reported the existence of up to seven GBM molecular subtypes characterized by mutational and expression profiles, as well as different DNA methylation patterns[61,84,85]. The distinction of molecular subtypes may help to evaluate the specific therapeutic vulnerabilities, being relevant in terms of treatment strategy. Actually, the current standard of care consisting in concomitant and adjuvant TMZ seems to benefit mostly patients presenting aberrant CpG methylation of the *MGMT* gene promoter, considered a positive prognostic biomarker for newly diagnosed GBM[86]. In recent years, transcriptomic profiling, using Nanostring technologies, succeeded in performing targeted gene expression to characterize several GBM phenotypes and to correlate molecular signature with therapeutic response of various molecules[87]. The results of this investigation reported genes differentially expressed between drug responders and non-responders advancing the enormous potential benefit of personalized medicine. Nowadays, most of clinical trials targeting GBM focus on oncopromoter signaling, susceptibility to apoptosis and cell cycle control (Figure 5). Below, an overview of the most promising examples of molecular target therapy. Unfortunately, the poor effect of the multitargeted therapies described in increasing patient OS, validates the urgent need to discover novel paradigms, optimizing patient clustering and implementing precision medicine.

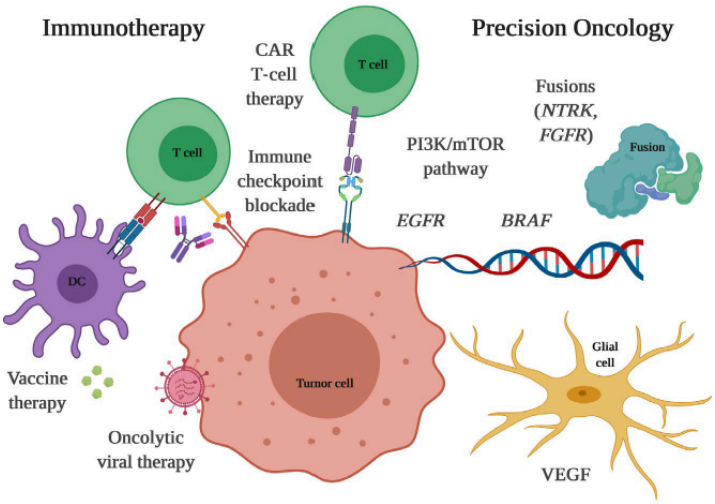

Figure 5. Novel Therapeutic Targets for GBM. CAR: chimeric antigen receptor; DC: dendritic cell; EGFR: epidermal growth factor receptor; FGFR: fibroblast growth factor receptor; mTOR: mammalian target of rapamycin; VEGF: vascular endothelial growth factor[67].

VEGF-target therapy

VEGF is the best characterized pro-angiogenic factor in cancer, of which it promotes angiogenesis, endothelial cell proliferation and survival. Bevacizumab (Avastin®, Genentech/Roche) is a monoclonal antibody against human VEGF, which was quickly approved by the US Food and Drug Administration (FDA) in 2009, as a single agent for patients with recurrent GBM. Despite the high initial radiographic response and the promising results reporting increased response rates and 6-month more progression-free survival (PFS)[88], bevacizumab effects proved to be only transitory and most GBM patients recurred after a median of 3–5 months[89].

More recently, the co-administration of bevacizumab with Lomustine (CCNU), an alkylating agent of the nitrosourea family in a phase 2 trial, showed encouraging results in terms of 9 months survival, compared to the administration of bevacizumab alone, justifying a phase 3[90].

Unfortunately, also phase I/II trials for the second line treatment of recurrent or progressive GBM with Sorafenib and Sunitinib, multi-tyrosine kinase inhibitors targeting VEGF receptors (VEGFRs) and other non-endothelial receptors such as PDGFR, showed that, compared to conventional chemotherapy or bevacizumab, neither has sufficient activity in the setting of recurrent GBM[91,92].

EGFR-target therapy

EGFR is certainly one of the most prominent oncogenes in *IDH*-wildtype GBM. As described above, amplification events involving chromosomes 7 (*EGFR/MET/CDK6*) occurs in 57.4% of primary GBM patients compared to 8% of secondary GBM patients and is associated with high levels of

EGFR protein[93]. The ACT IV trial revealed that approaches targeting *EGFR* or *EGFRvIII*, as *Rindopepimut*, determined a survival increase when combined with bevacizumab in recurrent GBM (NCT01498328), but no effect were reported in newly diagnosed GBM[94]. Notably, this trial reported also that the expression of *EGFRvIII* is highly unstable, since a marked decrease was recorded in both trial groups. *Erlotinib* (Tarceva, Genentech Inc), an EGFR inhibitor, is able to reversibly bind the intracellular tyrosine kinase domain of both EGFR or EGFRvIII, thus inhibiting receptor phosphorylation and downstream signaling[95]. In preclinical studies, erlotinib showed promising antitumoral effects against GBM, reducing cell viability of GBM stem cells through the inhibition of the MAPK signaling pathway. The clinical efficacy of erlotinib was evaluated by Raizer et al. in patients with recurrent GBM and non-progressive GBM in a phase II trial[96]. Unfortunately, the study did not report any significant improvement of clinical outcome in both experimental groups. Of relevance, in the same year, Yung et al. reported a significant increase of medial OS and 6 6-months PFS in recurrent GBM patients, but the study was discontinued for insufficient number of responses[97].

TERT-target therapy

As described above, *TERT* gene encodes for the catalytic subunit of telomerase, an enzyme which adds nucleotides to telomeres[98]. The activity of telomerase is relatively low in differentiated cells, allowing cell apoptosis and senescence. Two-point mutations causing the substitution of position 228 (C228T) and 250 (C250T) of the *TERT* gene promoter (*pTERT*) are responsible for *TERT* upregulation. *pTERT* has been observed in about 90% of human tumors, being considered a key element for cancer onset and progression[99]. In *IDH*-wildtype GBM, *pTERT* mutations are the most common molecular alterations and the increase of TERT expression supports the immortalization of tumor cells[100]. Notwithstanding, *pTERT* mutations have not yet been targeted with pharmacological treatments, but *Eribulin*, an inhibitor of tubulin polymerization, has been proved to inhibit TERT activity in GBM models, prompting its clinical testing[101]. A very recent phase I/II clinical trial, actually recruiting, aims to evaluate the effect of UCPVax, a therapeutic anti-cancer vaccine based on the telomerase-derived helper peptides designed to induce strong TH1 CD4-T-cell responses in cancer patients (NCT02818426).

Mesenchymal Epithelial Transition/ Hepatocyte Growth Factor Receptor (MET/ HGF)

MET/HGF is a proto-oncogene, coding for the hepatocyte growth factor receptor, which has been reported to have a key role in the proliferation, growth, angiogenesis, invasiveness, and migration of glioma cells, also in response to hypoxia, angiogenesis inhibition and irradiation. High expression of MET has been discovered in tumor cells, peri-necrotic areas and blood vessels of glioma tissues, with

a significant correlation with OS and PFS of GBM patients[102,103]. Also, an amplification of *MET* gene has been reported in 47% of primary GBM and 44% of secondary GBM, also during the progression of low-grade gliomas to secondary GBM[104], indicating that this alteration may be important in the pathogenesis of both GBM subtypes[105]. A humanized monoclonal antibody anti-HGF, *YYB-101*, have been reported to inhibit tumor growth both *in vitro* and in orthotopic mouse models of GBM, through the downregulation of cellular signaling effectors as p-MET, p-FAK, MMP2 and Ki-67[106,107]. In another recent study the combination of YYB-101 and TMZ in GBM xenografts was reported to suppress tumor growth and increase OS, compared to single treatment. In 2005, a phase I study has started to assess the safety, tolerability, and pharmacokinetics of YYB101 in advanced solid tumor patients, refractory to standard therapy, but no results have been posted (NCT02499224). *Onartuzumab*, a single arm monoclonal anti-MET antibody, proved to inhibit GBM growth in preclinical tests[108], but in a phase II clinical trial for recurrent GBM, its combined administration with bevacizumab did not show significant clinical benefit[109]. *Cabozantinib* (XL184), a tyrosine kinase inhibitor of MET, VEGFR2 and AXL proved efficacy in xenograft models, exerting anti-proliferative, anti-invasive and anti-angiogenic activity[110], inhibiting tumor growth and invasion, and prolonging mice survival[111]. Unfortunately, in a phase II clinical trial to evaluate the response rate and the 6-months PFS of patients affected by recurrent and progressive GBM, Cabozantinib proved only modest clinical efficacy (NCT00704288).

p53 pathway

The key function of p53 is to arrest cell cycle in G0/G1 phase and to initiate apoptosis in response to genotoxic stimuli. Therefore, mutant *TP53* may decrease apoptosis, increase cell growth and survival and resistance to chemotherapy[112]. For these reasons, drugs restoring p53 functions by conformation refolding have been largely studied, but not successful results have been reported. In this context, a recruiting phase I clinical trial is actually testing the side effects and the best dose of the MDM2-inhibitor, *KRT-232,* for patients with newly diagnosed or recurrent GBM harboring unmethylated *MGMT* promoters and wild-type *TP53*, given in combination with standard radiation following surgery (NCT03107780)[113].

Immunotherapy

The CNS is traditionally considered an immune-privileged organ, due to the absence of a lymphatic drainage system and the presence of the blood-brain barrier (BBB), which guides the diffusion of molecules and cells[114]. However, it has been widely demonstrated that brain tumors are able to elicit a potent antitumor immune response, prompting the concept of immunotherapy, which in the last few

years became a column in GBM therapy. Immunotherapeutic strategies may consist in active immunotherapy, by immune stimulants, cellular vaccines or tumor vaccines, and passive immunotherapy, which transfer effector immune cells into patients, thus inducing anti-cancer effects[115].

Checkpoint inhibitors

Immune checkpoints are regulators of the immune system crucial for the self-tolerance, as they prevent the immune system from attacking cells indiscriminately. Checkpoint molecules include cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and mucin protein-3, and several others. The expression of these checkpoint molecules on T cells represents an important mechanism that the immune system uses to regulate responses to self-proteins. Recent clinical data show that these checkpoint molecules play a critical role in objective tumor responses and improved OS, so that checkpoint inhibition has revolutionized treatment of several advanced malignancies providing hope for cancer treatment. Recent studies reported that inhibition of CTLA-4 and PD-1 induce tumor regression, promoting long-term survival in glioma mouse models[116,117]. The first FDA-approved immune checkpoint inhibitor has been Ipilimumab, a humanized CTLA-4 antibody, which improved OS in a phase III clinical trial for metastatic melanoma patients, of which only 2% showed a complete response[118]. On the contrary, the inhibition of the signaling pathway PD-1/PDL-1 (Programmed Cell Death Protein Ligand-1), have shown more promising results. A completed phase II clinical trial the investigators determined the effectiveness of pembrolizumab (MK-3475), an anti-PD-1 antibody, administered alone or in combination with bevacizumab for the treatment of recurrent GBM. The results reported that pembrolizumab is well tolerated +/- bevacizumab, but it has limited monotherapy activity for recurrent GBM. Furthermore, the antitumor activity of pembrolizumab plus bevacizumab was comparable to historical bevacizumab monotherapy data (NCT02337491). Another PDL1 inhibitor is durvalumab (MEDI4736), a human high-affinity monoclonal antibody that blocks PD-L1 by binding to PD-1 and CD-80, actually used for the treatment of non-small cell lung cancer[119]. An ongoing phase II multicenter, non-randomized study of durvalumab for GBM patients aims to assess the clinical efficacy measured by the OS rate at 12 months and PFS at 6 months. The study concluded that durvalumab was well tolerated when combined with RT and seemed to have efficacy among patients with new unmethylated GBM (NCT02336165). Finally, an active randomized phase III clinical trial is comparing the efficacy and safety of nivolumab, a monoclonal antibody anti-PD1, administered alone versus bevacizumab in patients diagnosed with recurrent GBM, and to evaluate the safety and tolerability of Nivolumab administered alone or in combination with Ipilimumab in

patients with different lines of GBM therapy (NCT02017717), but actually, no results have been posted[120].

Cancer vaccines

Cancer vaccines are designed to induce an immune response against the tumor. Vaccine therapies for GBM treatment include: i) the direct exposure to tumor antigens, as glioma-associated peptides or DNA in combination with immune-stimulating molecules and ii) patient-derived antigen presenting cells (APC), as dendritic cells (DC). Among anti-tumor immunotherapies, tumor vaccines and T-cell therapies rely on the enhancement of tumor-specific T-cells to seek and destroy cancer cells. To be safe and effective, a tumor vaccine must target an antigen specifically expressed in tumor cells and not in normal cells, thus called tumor-specific antigen (TSA). A good example of this primary requirement is the epidermal growth factor receptor variant III (*EGFRvIII*). The *EGFRvIII* mutation has been reported in about 25-30% of GBM and it has been considered an independent negative prognostic factor[121]. Promising results arose from a phase II clinical trial of a 13-amino acid EGFRvIII peptide vaccine, Rindopepimut™, a conjugated EGFRvIII-specific peptide (also known as CDX-110 and PEPvIII), by Celldex therapeutics. Conjugated to adjuvant, rindopepimut gave rise to an increased OS, correlated with the extent of induced tumor immunity[122,123]. These results led to an international phase III trial, ACT IV, for newly diagnosed GBM patients with EGFRvIII mutation. Unfortunately, this trial did not give the desired results and no significant differences in OS for patients with GBM in the rindopepimut group plus TMZ versus the control group (TMZ alone) were observed[94]. Another promising single antigen vaccine is SurVaxM, a peptide mimic of survivin conjugated to Keyhole limpet hemocyanin (KLH), used as vaccination adjuvant. Survivin is a member of the inhibitor of apoptosis proteins (IAP) family, strongly expressed in a majority of tumors and absent in normal differentiated tissues[124]. Survivin inhibits apoptosis, regulates cell-cycle progression and induces chromosomal instability[125], so that its expression has also been associated with tumor grade, prognosis and chemotherapy resistance[126,127]. RNA sequencing revealed that patients with high expression of survivin had shorter OS times than those with low expression[128], so that surviving can be considered a novel prognostic factor in gliomas[129].

A multi-center phase II trial for patients with newly diagnosed GBM treated with adjuvant TMZ and survivin-targeted immunization, reported that SurVaxM is safe and that, compared to historical matched controls, the addition of SurVaxM improved 6-months PFS and 12-months OS. Interstingly enough, also patients with poor prognostic factors (unmethylated MGMT, higher survivin levels) treated with SurVaxM achieved better survival than expected[130].

Also customized vaccines represent a promising area of clinical research. A fundamental requirement for customized vaccines is the disposability of a small volume of tumor tissue, which make eligible only patients with resectable tumors. A great example is DCVax-L, developed by Northwest Biotherapeutics, which uses whole tumor lysate to pulse patient-derived DCs. A phase III trial have been designed to determine the efficacy and the impact on disease progression and survival time, as well as safety, on patient with GBM, treated with surgical resection, radiation and TMZ (NCT00045968). Over 10 years from diagnosis, some of patients recruited for the phase I trial of the vaccine were still alive, but no results of phase III study have been posted; however, recent reports described a median OS of 23.1 months for all participants (90% of whom received the DC-Vax-L treatment due to crossover design)[131].

CAR-T Cell Therapy

The genetic engineering of T cells to express chimeric antigen receptors (CARs) directed against tumor specific antigens has opened the door to a new era of personalized cancer therapy. The promising achievements of CAR-T cell therapy in hematological cancers confirmed their potential to elicit a durable remission, prompting the introduction of this technology among the therapeutic strategies for patients with solid cancers, including GBM[132]. Typically, the CAR-T therapy is based on the collection of patient-T cells or immune cells, which are genetically engineered to recognize specifically tumor antigens. Targeting with CAR-T cells has the advantages to permit the active passage of T-cells towards tumor sites in which immune cells can kill cancer cells, sparing normal cells and preventing side effects[132]. Since the limited availability of targetable TSAs in GBM, which guarantee the foster of healthy tissues, researchers are actually focused on the EGFRvIII. The treatment of 10 recurrent GBM patients with autologous EGFRvIII-CAR T-cells, in a single intravenous infusion, performed by O'Rourke and colleagues, reported that no patient manifested toxicities or cytokine release syndrome, demonstrating that systemic infusion of EGFRvIII-CAR T-cells is safe and feasible. Unfortunately, except for one patient with stable residual disease for over 18 months, no objective radiographic responses were observed. Interestingly, the scientists described a transient but significant expansion of the CAR T-cells, as well as a promising infiltration in tumor location. They ultimately observed a decrease of EGFRvIII-expressing tumor cells and an overexpression of immune inhibitory molecules, as PD-L1 and indoleamine-2,3-deoxygenase 1 (IDO1)[133]. Other trials with CAR-T cells targeting IL13Ra2, Her2/CMV have been recently conducted and results reported objective radiologic responses, however addressing essential question as tumor microenvironment remodeling and T-cell trafficking in CNS. Each study provided evidence that the selection of multiple target antigens in each patient is an important step in creating

combinatorial therapy in order to address tumor heterogeneity and achieve a greater curative potential[134]. Despite innumerable steps forward, the personalized medicine by immunotherapy for neuro-oncology patients are presently in early stages, and much effort remains to be done in order to determine their therapeutic value.

1.2.5 Glioblastoma Stem Cells

Tumors are often the result of the accumulation of genetic, epigenetic and transcriptional alterations, which confer specific properties to cells, such as high proliferation, invasiveness, metastatic potential and angiogenesis[44]. Although tumors may arise from a single mutated cell, virtually all tumors become heterogeneous in cell structure and morphology, expressing different markers and occurring at different stages of differentiation. Heterogeneity is certainly a fundamental factor for the development of the characteristics of progression and resistance to therapy and relapse[135]. GBM intratumoral heterogeneity and therapy resistance are thought to be promoted by glioblastoma stem cells (GSCs) which show two principal features of stem cells: self-renewal and differentiation[136]. GSCs recapitulate the heterogeneity of the parental tumor *in vivo*, being so called tumor initiating cells, and their biological relevance is demonstrated by their functional role in tumor growth and recurrence[137]. GSCs drive resistance to pharmacology, radiation, and surgery, and are thus a key therapeutic target[138,139]. Several markers, including CD133 (PROM1), CD15 (stage-specific embryonic antigen-1, SSEA1), L1CAM, and SOX2 are enriched in GSCs, although, similarly to normal stem cells, no marker has been identified exclusively and comprehensively marking GSCs[140,141]. Although specific pathways that contribute to the augmented aggressiveness and resilience of GSCs have been described, effective therapies remain elusive. There are two main hypotheses proposed to explain how a stem cell can originate within a tissue: the stochastic and the hierarchical model. The stochastic model argues that each cell undergoes a specific set of somatic mutations, which lead to the development of tumor stem cell characteristics that could contribute to tumor heterogeneity characteristics. On the other hand, the hierarchical model argues that mutations occur in particular in specific stem and progenitor cells, which are organized in a hierarchical manner; this results in the generation of stem and progenitor cells which produce a differentiated and heterogeneous progeny, contributing to tumor pleomorphism[142]. Several more recent studies have highlighted that the concept of stem cell is a more dynamic than rigid concept, highlighting how in the experimental context the two models are co-present due to the plasticity of the tumor cells which, spontaneously or due to particular conditions, can undergo a process of reversion and return stem cells (EMT)[143]. Starting from this concept, various speculations have been made on the role of GSCs as causative factors of several of the main tumor activities, such as metastasis and angiogenesis. For

example, it has been shown that EMT confers metastatic potential to cancer cells which, after reaching their destination, undergo the reverse process (MET, mesenchymal epithelial transition)[144]. In this context, the GSC definition does not suggest a static state. A striking plasticity exists between different cellular states of the tumor, which allows for interconversion between GSC and non-GSC states depending on several factors. Microenvironmental exposures, including hypoxia, nutrient deprivation, radiation, and others, shift the dynamics of regulation of these interconversions, bringing about changes in the GSC and non-GSC pools, along with phenotypes such as proliferation or quiescence[145]. For example, in addition to receiving growth and survival signals from the vasculature, GSCs remodel vessels by differentiating into vascular pericytes or endothelial-like cells[146]. In particular, GSC-derived endothelial-like cells rely on WNT5A signaling to promote cellular lineage infidelity and acquisition of endothelial-like phenotypes, which promotes tumor neovascularization and invasion, as described below[147].

1.3 Angiogenesis in GBM

In physiological conditions, angiogenesis is an essential and highly regulated process for the adequate supply of nutrients and oxygen to vital tissue[148]. Angiogenesis is manly mediated by endothelial cells and mural cells, through the combination of various participants as soluble growth factors, adhesion protein, proteolytic enzymes, and matrix components[149]. The low concentration of oxygen potently activates angiogenesis, by the upregulation of transcription factors which controls pro-angiogenic mediators.

In particular, hypoxia-inducible-factor (HIF-1) is known to induce transcription of more than 60 genes, including VEGF and erythropoietin, which assist in promoting and increasing oxygen delivery to hypoxic regions[150,151]. HIF-1 also induces transcription of genes involved in cell proliferation and survival, as well as glucose and iron metabolism[150], adhesion molecules and matrix component. The promotion of angiogenesis is sensibly regulated by the balance between pro- and anti-angiogenic factors. An unbalance of this delicate equilibrium, as hypoxia, lead to the release of pro-angiogenic growth factor as VEGF, transforming growth factor-β (TGF-β), fibroblast growth factors (FGFs), angiopoietin-1, and epidermal growth factor (EGF)[152]. The binding of these signalling mediators with their receptor on endothelial cell surface results in the dissolution of the vessel wall and the degradation of ECM. Consequently, matrix metalloproteinases (MMPs) remodel ECM, inducing the migration and proliferation of endothelial cells and, in turn the formation of new tube-like structure[153]. At the end of this process, the endothelial tubes are surrounded by a mature vascular basement, pericytes and smooth muscle cells, resulting in novel stable blood vessels[154].

In cancer pathogenesis, particularly in high grade tumors as GBM, aberrant neo-angiogenesis is a vital process for the mass growth: it is driven by neoplastic cells in order to respond to the tumoral hypoxic environment in the necrotic core, which increases the demand for oxygen and nutrients by neoplastic cells, and is, therefore, essential to carry out the metabolic functions on which their survival is based[155] (Figure 6).

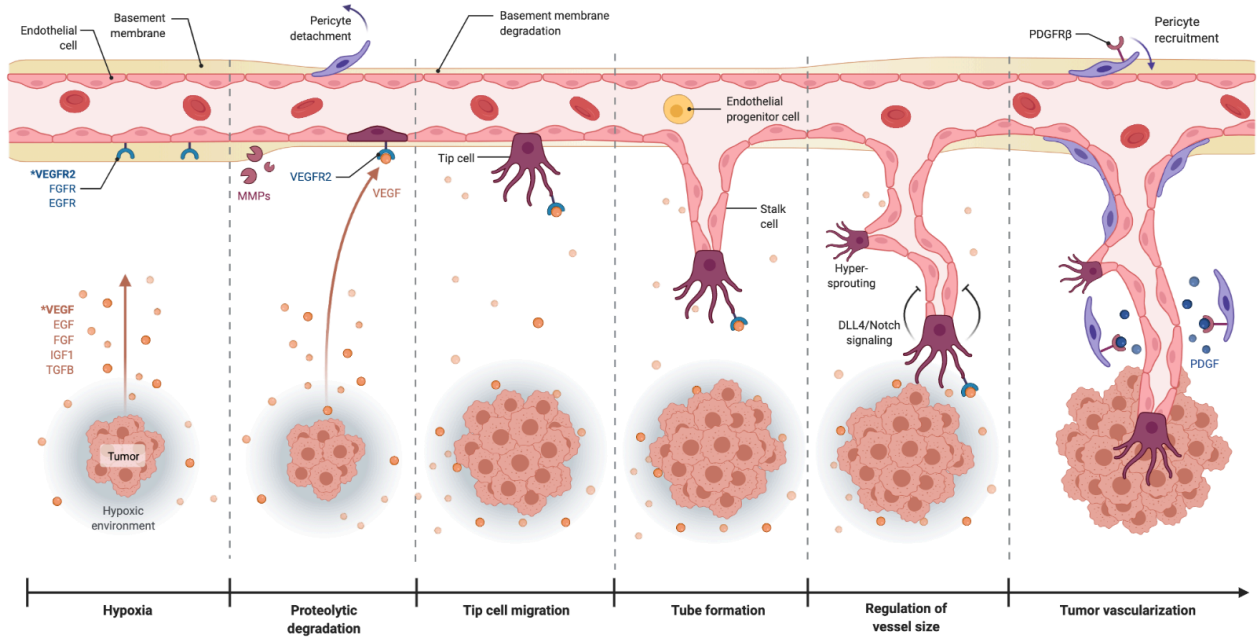

Figure 6. Tumor vascularization. Angiogenesis in GBM. The formation of new blood vessels in GBM has been explained by a variety of pathways, among which those mediated by VEGF are the best characterized. In the tissue surrounding the necrotic core, hypoxic tumor cells release VEGF, which promote vascular formation from preexisting normal endothelial cells. This process involves both endothelial-cell proliferation and increased vascular permeability. Angiogenesis occurs also thanks to the recruitment of bone-marrow derived endothelial progenitor cells and mesenchymal or hematopoietic cells, which migrate through systemic circulation into the tumor. New blood vessel formation is also influenced by GSCs, able to differentiate into endothelial cells or pericytes, which play a critical role in the regulation of blood vessel function, as blood flow, permeability of BBB and cell-to-cell communication.

On the other hand, several observations led to the knowledge that tumoral neo-angiogenesis gives rise to ultra-structurally abnormal vessels, larger than their normal counterparts, dilated, convoluted, irregularly branched and exceptionally permeable due to the presence of fenestrations and the lack of a complete basal membrane[156]. The hyperpermeability of tumor vasculature leads to local edema and extravasation of plasma, thus increasing the interstitial pressure, altering blood flow and leukocyte

flux[157,158]. The leakiness of newly formed blood vessels leaves large volumes of tumor tissue without blood flow and obstructs the delivery of blood-borne drugs, oxygen, and nutrients resulting in ischemia and necrotic regions within the tumor[159]. This, in turn, promotes a hypoxic environment that activates HIF-1-induced angiogenesis, feeding an autocrine and paracrine self-sustaining vicious cycle[160].

Moreover, it is common that the vessel walls consist of a mosaic of ECs and cancer cells. These structural anomalies reflect the pathological induction and the tumoral ability of using common physiological mechanisms with the aim of boosting the mass growth. The abnormal and aberrant growth of tumor vasculature strongly alters tumor microenvironment, inducing the ability of tumor cells to metastasize to distant sites inside the brain, the escape from immune system and tumor response to anti-cancer treatments. The key role of angiogenesis in GBM progression excuse oneself the incessant research on tumor vasculature targeting and inhibition of growth factors/signaling pathways necessary for endothelial cell growth and proliferation, which unfortunately has not yet given the desired results.

1.3.1 Cell biology of GBM angiogenesis

In 2000, Jain and Carmeliet listed six different cellular mechanisms of tumor angiogenesis in a snapshot published in Cell, including classical sprouting angiogenesis, vascular co-option, vessel intussusception, vasculogenic mimicry, bone marrow derived vasculogenesis and cancer stem-like derived vasculogenesis[161]. More recently, the existence of a seventh mechanism has been demonstrated in the process of angiogenesis driven by blood derived infiltrating myeloid cells (Figure 7). Whether and how all the above-mentioned mechanisms are involved in gliomas or GBMs angiogenesis is not yet clear. What is proved is that classical sprouting angiogenesis (the sprouting of capillaries from pre-existing vessels, known to be the most important mechanism in brain vascularization), vascular co-option (the infiltration of tumor cells into normal tissue and the adoption of pre-existing vasculature) and vasculogenic mimicry (a process where tumor cells replace ECs and form a vessel with a lumen) are involved in GBMs angiogenesis, giving the tumor its characteristic invasiveness[162]. On the other hand, experimental studies in glioma models have led to a conclusion that the importance of mechanisms like vessel intussusception (the formation of new vessels by vascular invagination, intraluminal pillar formation and splitting), bone marrow derived vasculogenesis (the recruitment of circulating endothelial precursor cells to the tumor, their integration into the vessel wall and their terminal differentiation into an ECs), cancer stem-like cell derived vasculogenesis (cancer stem-like cells that contribute to the vascular neoformation by integrating into the walls and transdifferentiating into ECs) and bone marrow derived cells driving

tumor angiogenesis (M2 polarized monocytes/macrophages, which are able to polarize into phenotypes that exerts different functions in vivo, are pro-angiogenic) in human GBM is highly controversial and, at best, they appear to be very rare events[162].

However, it is well known that GSCs and glioblastoma endothelial cells (GECs) share a symbiotic and bidirectional relationship to maintain both angiogenic process and cell stemness. In particular, the GBM hypoxic microenvironment induces the expression of HIF-1 in both cell subpopulation, generating a downstream cascade of events that promotes the synthesis and the paracrine release of some factors, as vascular endothelial growth factor (VEGF) and angiopoietin, by GSCs towards GECs, allowing the proliferation of the latter [163,164]. Mainly through this mechanism, GSCs have the ability of remodeling the perivascular niche, by actively joining the formation of new vessels and/or by getting involved in maintaining GECs phenotype[165]. On the other hand, also GECs play an active role in maintaining GSCs stemness by acting on the downstream pathway Notch (which has a vital involvement in maintaining cell stemness) through the expression of delta like ligand 4 (DLL4) or Jagged1, both inducing a sustained activity of the receptor[166,167]. Moreover, it has been proven that GECs have also the ability of producing nitric oxide (NO) through the vascular synthase eNOS/NOS3: this molecule plays a role in promoting Notch signaling, thus promoting the stem phenotype[168,169]. Therefore, it is reasonable that an effective anti-angiogenic therapy cannot prescind from having an effective action also on GSCs.

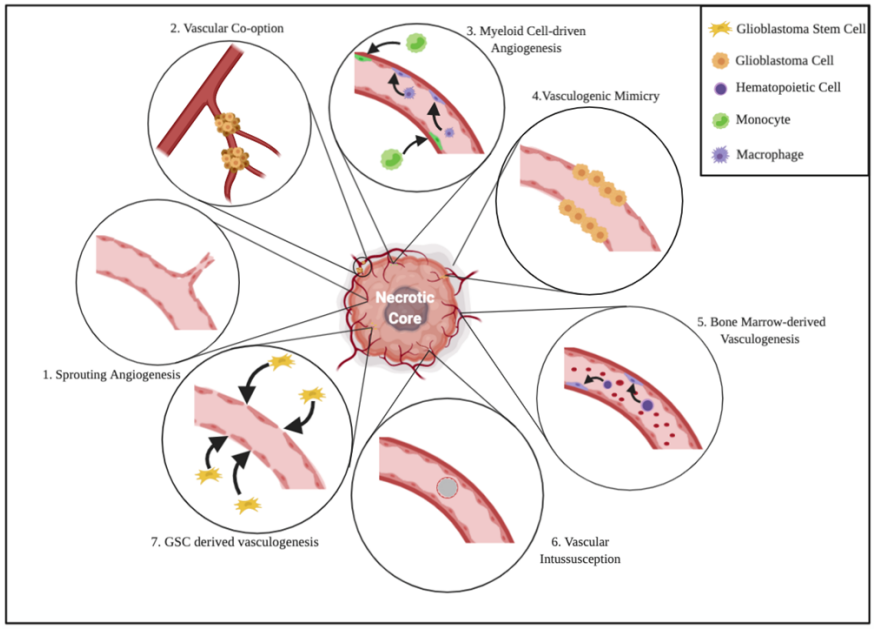

Figure 7. Cell biology of GBM angiogenesis. 1. Sprouting Angiogenesis: sprouting of capillaries from pre-existing vessels; 2. Vascular Co-option: the infiltration of tumor cells into normal tissue and the adoption of pre-existing vasculature; 3. Myeloid Cell-driven Angiogenesis: M2 polarized monocytes/macrophages, which are able to polarize into EC phenotype; 4. Vasculogenic Mimicry: tumor cells replace ECs and form a vessel

with a lumen; 5. Bone Marrow-derived Angiogenesis: the recruitment of circulating endothelial precursor cells to the tumor, their integration into the vessel wall and their terminal differentiation into ECs; 6. Vascular Intussusception: the formation of new vessels by vascular invagination, intraluminal pillar formation and splitting; 7. GSC derived Vasculogenesis: Glioblastoma Stem-like cells that contribute to the vascular neoformation by integrating into the walls and transdifferentiating into ECs.

1.3.2 Angiogenic signaling pathways in GBM

In GBM, many signaling pathways activated by the bond between growth factors and their receptors have been thoroughly studied with the aim of identifying possible targets for antiangiogenic therapies, leading to a better knowledge of their mechanisms. Among them, VEGF is the main angiogenic factor in CNS, fundamental in both embryonic development and tumor growth, together with basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and TGF-β, MMPs, and angiopoietins (Angs).

The expression levels of these mediators strongly correlate with tumor progression and often with patient's clinical outcome. Their upregulation is induced by hypoxia, loss of tumor suppressor gene function and oncogene activation. Intra-tumoral levels of VEGF in gliomas and its receptor strongly correlates with the histological grade of the tumor. In GBM, particularly in the pseudopalized necrotic region, VEGF is upregulated[170,171,172]. This condition is mainly driven by HIF family, which are overexpressed in the central necrotic core of the tumor because of its hypoxic microenvironment.

VEGF plays a fundamental role in the proliferation, survival and therapy resistance of gliomas. VEGF family consists of six isoforms (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor)[173], among which VEGF-A represent the main mediator in hypoxia-induced tumor growth. Its signalling pathways are mediated by the binding to cell surface tyrosine kinases receptors like VEGFR-1, VEGFR-2, and VEGFR-3, which induce pro-angiogenic mechanisms as endothelial cell migration, vascular permeability, pericyte recruitment and pro-survival activities[174]. Furthermore, VEGF/VEGFR axis stimulates the secretion of MMPs, responsible for ECM degradation and consequent endothelial cells proliferation and migration. VEGF is known to synergize with various growth factors[175,176], as FGF-2 or PDGF-BB, leading to the exacerbation of tumor angiogenesis[177,178]. Also, VEGF, together with granulocyte macrophage-colony stimulating factor (GM-CSF), insulin-like growth factor (IGF1) and with angiopoietins 1 and 2, are all implicated in the mobilization of circulating endothelial precursors[179]. VEGF-induced angiogenesis leads to dysfunctional and immature vessels production, associated with significant oedema and disruption of BBB[180].

The mRNA expression of VEGF was found to strictly correlate with glioma grade, with the highest levels observed in GBM, especially in the in the necrotic regions[171-172]. Therefore, VEGF overexpression was significantly associated with a dismal prognosis in high-grade lesion, suggesting that VEGF and VEGFR expression can serve as a prognostic biomarker and prompting the application of therapeutic regimens which inhibits VEGF activity[181]. Another pro-angiogenic factor is FGF, present in both the tumor cells and in the vascular basement, from which is abundantly released during angiogenic mechanisms. The two isoforms of FGF, FGF-1 or acidic FGF (aFGF) and FGF-2 or basic (bFGF), bind two tyrosine kinases receptors, FGFR-1 and FGFR-2[182]. This binding induces a signalling cascade mostly mediated by phospholipase A2[183] and protein kinase-C (PKC), which promote EC migration and capillary-like structure formation[184]. Furthermore, FGF increase the synthesis of fibronectin, collagen and proteoglycans, thus mediating ECM remodelling[184]. The confirmation of FGF implication in brain tumor progression arise from the evidence that immunohistochemical bFGF levels correlate with glioma grade, malignancy, and vascularity[185]. In addition, it has been demonstrated that FGF inhibition is effective in arresting glioma growth in in vivo models, by reducing blood vessel density[186]. Another key pro-angiogenic signalling is that induced by angiopoietins (Ang). Four types of angiopoietins have been identified (Ang-1 to Ang-4) and were shown to play a role in angiogenesis.

All the angiopoietins bind the same receptor, the tunica interna endothelial cell kinase 2 (Tie-2/TEK), but their effect on tumor vasculature may be different[187]. Ang-1 promotes interaction between ECs and peri-EC support cells to stabilize vessels, favoring structural integrity and maturation of blood vessels[188]. On the contrary, Ang-2 is an antagonist for the Tie-2 receptor that inhibits competitively binding of Ang-1, triggering endothelium activation and vascular destabilization[188]. Indeed, Ang-2 acts as a proangiogenic agent, promoting establishment of disorganized and defenestrated blood vessels with increased permeability and loss of integrity. Therefore, Ang-2 is upregulated by the both hypoxia and VEGF and enhances the VEGF-mediated endothelial cell migration and proliferation. By this way, Angs may exert both pro- and anti-angiogenic effects. Furthermore, it has been established that Tie-2 signalling can be activated only by tetrameric or higher orders of aggregation of Angs, so that monomeric or dimeric Ang may serve as Tie-2 inhibitors[189], as the case of Ang-3, a monomeric form that exerts anti-angiogenic and anti-cancer activity[190]. Ang-4 is upregulated in human GBM tissues and cells and was shown to have a more potent pro-angiogenic activity than Ang-1 and promotes intracranial growth in mouse model[191].

A clinical trial by Cam et al. reported that the administration of an orally available small inhibitor, Rebastinib (DCC-2036), succeeded in increase GBM survival. Accordingly, rebastinib is actually in clinical trial with carboplatin (NCT03717415) or paclitaxel (NCT03601897) in patients including

with GBM[192]. Due to these features, mRNA levels of Ang-2 have been detected in tumor ECs and are considered as early markers in human gliomas[193].

Another important molecule involved in the various pathways leading to angiogenesis is Notch. This protein is well-known for being intercalated on different signaling pathways leading to organ development and, more recently, some of its receptor (particularly Notch-1 and -4) have been recognized on ECs membrane. During neo-angiogenesis, the receptor Notch1 seems to be very important. Notch, together with VEGF, is vital in determining differentiative pathways of the precursor of the ECs, which can become either a tip cell or a stalk cell. VEGF-A causes an increase in VEGFR2 and 3 signals, leading to the development of tip cells; consequently, these cells cause the overexpression of the adjacent of Notch receptors, leading to the differentiation into stalk cells because of the interaction with DLL4[194]. This last molecule is present in GBM but not in glioma cells, demonstrating once again the importance of the neo-angiogenic activity particularly in these grade IV tumors[195].

1.3.3 Angiogenesis as a plausible target in GBM therapy

The dependence of tumor growth and metastasis on angiogenesis, which has been thoroughly demonstrated in murine models, has provided an important rationale to a new kind of therapeutical approach in different kinds of cancer. Even in brain tumors the strategy of targeting blood vessels has always been full of attractions; the anti-angiogenic therapy rationale in malignant brain tumor is based on the following principles: i) the high vascularity found in malignant gliomas; ii) the possibility of avoiding the issues related to the passage through the BBB, as opposed to many chemotherapy agents; iii) the normalization of the vascular network, which leads to a synergistic effect with other therapeutic agents, when applied together. Moreover, the anti-angiogenic therapy can represent an indirect way of targeting GSCs, because of their involvement in GBM resistance to radio- and chemotherapy[196]. Given this perspective, two classes of drugs have been approved for the treatment of non-CNS cancers: the monoclonal antibody Bevacizumab (Avastin®, Roche), which targets and neutralizes VEGF, and VEGF-linked tyrosine kinase inhibitors (TKIs), including Sorafenib (Nexavar®, Bayer-Onyx Pharmaceuticals), Cediranib (Recentin, AstraZeneca) and Sunitinib (Sutent®, Pfizer)[41]. While Bevacizumab is usually given in combination with other drugs (such as Irinotecan, Etoposide, Temozolomide or Fotemustine) to increase its efficacy, with a toxicity that is considered to be acceptable[89], TKIs as monotherapy show their effect both on neoplastic and stromal cells[41].

The main mechanism by which these drugs act on GBM has been thoroughly studied and characterized as vascular normalization: it consists of a focalized effect on newborn vessels, while leaving mature vessels unaltered[197]. Therefore, as observed by Batchelor et al.[180], and fully described by Jain et al.[198], vascular normalization leads to an increase in tumor perfusion and oxygenation, which breaks the vicious circle started by hypoxia. Some researchers argue that normalization followed by chemo- or radiotherapy should be the main target of any anti-angiogenic treatment, even for therapies with target other than VEGF. As a matter of fact, when combined, these drug regimen led to GECs sensibilization to cytotoxic treatment, particularly in non-metastatic brain tumors; moreover, following radiotherapy, anti-VEGF treatment causes a significant decrease in the expression of VEGF in GBM cells[170]. Finally, an important speculation around these drugs is that they could lead to the disintegration of the perivascular niche, resulting in one of GSCs ideal habitat loss and, as a consequence, the eradication of the latter[199]. While acknowledging this, it is vital to keep in mind the paradox linked with these drugs: they are designed with the aim of disrupting the vascularization while, at the same time, they need it to reach the site to perform their effects. The only way to solve this apparent problem lies in their judicious use, at the correct dose and in the correct therapeutic range, with the aim of avoiding their side effects, as demonstrated in several preclinical studies on murine models with breast cancer or GBM cellular lines[200].

However, anti-angiogenic therapies have not led to a significant improvement in OS in GBM patient, both newly diagnosed and relapsed. In 2018 Ameratunga et al. have released a meta-analysis comparing 11 multi-center and/or international studies, with the aim of acknowledging whether a difference could be found in terms of OS and PFS between GBM affected patients treated with the combination of anti-angiogenic therapy and gold standard regimen compared to the standard therapy alone. The authors concluded that various anti-angiogenic drugs did not show a significant increase in OS, while it is also evident that they increased PFS[201]. This is presumably related to both the ability of the tumor to escape the effects of therapy and to the side effects of therapy on vascularization. The problem arises from GBM localization and activity: above all, these drugs can give important side effects such as intracerebral hemorrhage, arterial thromboembolic events or, less frequently, posterior leukoencephalopathy syndrome (RPLS), that can present with headache, seizures, lethargy, confusion, blindness and other visual and neurological disturbances[41]; on the other hand, the ability of GBM of evading therapies effect is well known. Notably, the use of anti-VEGF drugs, both in preclinical and in clinical trials, seems to select more aggressive neoplastic clones, with a more aimed to invasiveness phenotype[202,203]. This confirms what it has been previously reported: targeting angiogenesis could theoretically be a good way to attack GBM; however, the implied drugs should also have an effect on GSCs, otherwise it will at least be difficult to overcome GBM resistance to

therapy. As a result, further studies should be undertaken in order to fully comprehend the eventual clinical importance of these drugs in GBM therapy.

1.4 Calpain family

The calpain story started in 1964, when a calcium-activated proteinase was identified in rat brain[204]. Further genetic and functional studied described calpains as a well-conserved family of intracellular cysteine proteinases with a complex proteolytic system [205,206]. Currently, 15 calpain genes (*CAPN*) have been described in humans, which differ by the presence of a protease domain characterizing μ-calpain, absent in m-calpains. On the basis of their domains structure, a further classification divides calpains and calpain homologs into "classical" and "non-classical"[207]. In humans, 9 out of 15 calpain genes code for classical calpains, with alternative splicing variants also generated. Based on their expression profile, at least six genes are tissue-specific, and defects of the corresponding calpains are associated with tissue-specific diseases, so that muscle Calpain-3 dysregulation was known as "calpainopathy". The precise physiological functions of the calpain isoforms and mechanisms controlling proteolytic activity remain to be fully elucidated, but several experimental studies demonstrated a clear role for calpains in many important cellular processes, including cell motility and apoptosis. Certainly, the large amount of physiological and pathological condition in which calpains are involved may be explained by the abundance of substrates cleaved by calpains, along with the ''conservative'' nature of substrate processing. Particularly, calpains have drawn a growing interest particularly in cancer research, as calpains proved to be involved in both tumorigenesis and progression of different tumor subtypes and, especially, as *in vitro* pharmacological studies demonstrated that the effect of many consolidated and novel compound is mediated by the activation or inhibition of calpains. Moreover, *in vitro* and *ex vivo* tumor models revealed a significant correlation between calpain expression, histological and clinical features[208].

1.4.1 Calpain structure and regulation

The members of calpain family are classically distinguished as μ-calpain and m-calpain, depending on the concentration of calcium ions required for their activity *in vitro.* Both μ-calpain and m-calpain are heterodimers consisting of a catalytic (80 kDa) subunit and a regulatory subunit (28 kDa). In particular, the catalytic subunits are encoded by *CAPN1* for Calpain-1 and *CAPN2* for Calpain-2, also termed conventional calpains, which share almost 60% of sequence. The common regulatory subunit is encoded by *CAPNS1*, also known as Calpain-4. Structurally, the large catalytic subunit is composed

by four domains, consisting of: i) the N-terminal anchor helix region, which is a short prodomain; ii) the conserved CysPc catalytic domain, composed of two protease core domains (PC1 and PC2); iii) the CBSW (calpain type beta-sandwich) domain involved in structural changes during calcium binding; iv) the PEF(L) (penta-EF-hand) domain, which aid the dimerization of the subunits[209]. The small regulatory subunit contains two domains: i) PEF(S) (penta-EF-hand) domain; ii) a glycine-rich hydrophobic domain at N-terminus that may enable interaction with the plasma membrane and are autolyzed during calpain activation[210]. Notably, the first four EF-hands of both large and small subunits are involved in binding calcium, while the fifth EF-hand elicits the homophilic association for active heterodimer formation[211,212]. Several calpain isoform are ubiquitously expressed, as μ-calpain and m-calpain, other ones are tissue-specific expressed, as Calpain-9, found in the digestive tract[206] (Figure 8).

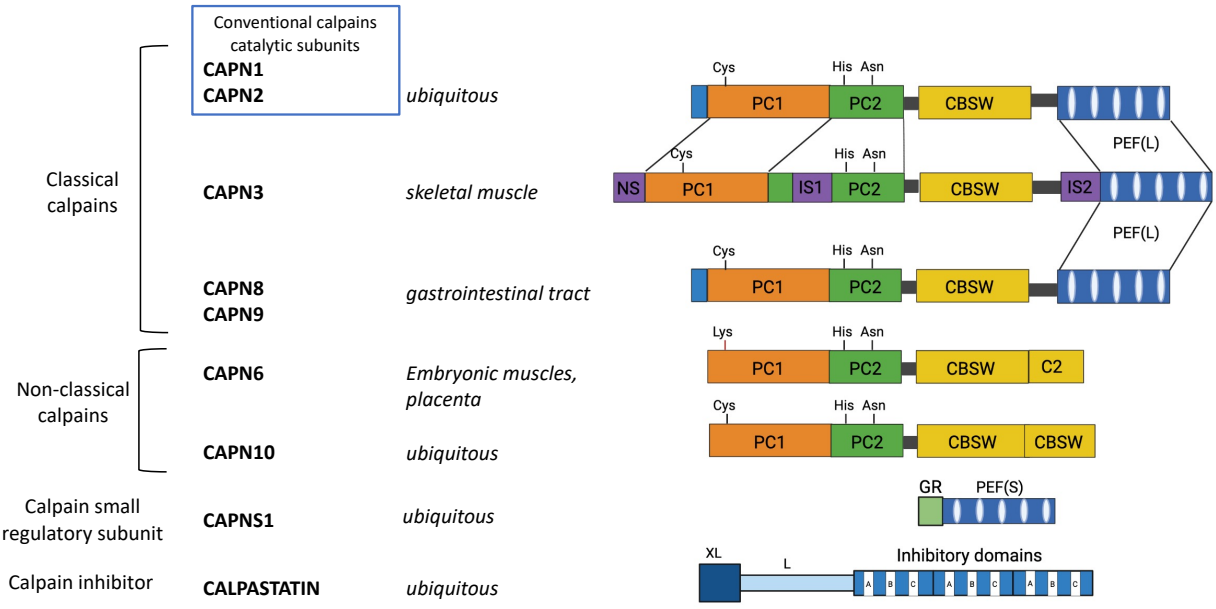

Figure 8. Schematic structure of the calpains. These include conventional calpains (the large, catalytic subunits *CAPN1* and *CAPN2* and the small regulatory subunit *CAPNS1*, *CAPN3*, *CAPN8* and *CAPN9*, *CAPN6* and *CAPN10* and their endogenous inhibitor Calpastatin (the longest isoform is reported). **PC:** protease core domains; **CBSW**: calpain type beta-sandwich; **PEF:** penta-EF-hand domain. The figure was modified from http://calpain.net/structure/human.html.

It has been reported that *CAPN2* and *CAPNS1*-knockout mice are embryonic lethal[213,214] indicating the essential role of calpains in embryogenesis, whereas Capn1- knockout mice show no pathological phenotype[215]. The activation of calpains may be promoted by the autolysis of the first domain of the

catalytic subunit[210,216] and by the interaction with membrane phospholipids, including phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP2)[217,218]. Also, alteration of calcium homeostasis causing the increase of cytosolic calcium levels may induce calpain activation and cell death. High cytosolic calcium concentrations can be achieved through diverse mechanisms in damaged cells, including the administration of several drugs as genistein[219], cisplatin and oxaliplatin[220,221,222] and resveratrol[223]. An endogenous, ubiquitous inhibitor of both Calpain-1 and -2 is Calpastatin[224,225] encoded by *CAST* gene. Calpastatin consists of a N-terminus XL domain and an L domain, subjected to alternative splicing, and four repetitive inhibitory domains. The intrinsically unstructured nature of calpastatin allows it to bind and inhibit up to four calpain heterodimers simultaneously[226,227]. Since Calpastatin binds preferentially to calcium activated calpains[228], this suggests that Calpastatin might function to attenuate calpain activity rather than prevent it.

Overall, due to the array of proteolytic substrates and their influence on cellular processes, the localization and distribution of calpain is expected to modulate cellular outcome following calpain activation[208].

1.4.2 Calpain and cancer

Numerous observations reported that calpain family members are differentially expressed in human cancers compared to normal counterparts, highlighting the key role of calpain in tumor onset and progression. Some reports demonstrated that calpain expression levels, detected by immunohistochemistry or mRNA, are directly associated to histopathological features of marked malignancy and worst clinical outcome, fueling the consideration of calpain as negative prognostic markers. However, it should be considered that high expression levels not necessarily correspond to high proteolytic activity, as high level of Calpastatin may counteract calpain activity. An altered pattern of calpain family expression, with an overexpression of *CAPN1, CAPN2* and *CAPNS1* has been reported in several cancers as breast cancer[229,230,231], colorectal cancer[232], renal cell carcinoma[233], hepatocarcinoma[234], nasopharyngeal carcinoma[235], acute myeloid leukemia[236], schwannoma and meningioma[237], and glioma[238]. Conversely, high levels of Calpastatin, the endogenous inhibitor, have been found in endometrial carcinomas[239]. Several studies have also highlighted a direct correlation between calpain expression and clinical outcome, including response to therapy, metastasic potential and patient survival[240,241,242]. Notably, interesting results regarding the potential therapeutic implications of calpains, arose from breast cancer cells positive to HER2 (Human Epithelial growth factor Receptor 2). In particular, Kulkarni et al. observed that both

Trastuzumab-resistant and sensitive cells positively responded to *CAPNS1* suppression, by decreasing cell survival and restoring Trastuzumab-response[229].

1.4.3 Calpains in cell survival/apoptosis

It has been widely documented that cancer cells have amplified survival pathways and may escape apoptotic cell death. These attributes are crucial in oncogenic transformation and tumor progression. Calpain activity has been implicated in the pro-survival activities of both the tumour-suppressor protein p53 and nuclear factor-kB (NFkB). Accumulating evidence showed that calpain is able to cleave wild-type p53, regulating protein stability and preventing p53-dependent apoptosis[243]. The same result can be obtained by the physical association between calpain and growth arrest-specific protein 2 (GAS2), a protein which is cleaved during apoptosis to allow rearrangement of the actin cytoskeleton. On the contrary, in neuronal cells calpain promotes p53 activation and cell death[244]. Furthermore, calpain can stimulate cell survival through the activation of NF-kB by the cleavage of its inhibitor IkBα, in response to tumour necrosis factor (TNF)[245] and activation of the EGFR family member ERBB2 in breast cancer[246]. The activity of Calpain influences the proteolysis of various substrates that can sensitize cells to apoptosis, such as the transcription factor MYC[247], able to increase calpain activity, suppressing the expression of Calpastatin. The inhibition of calpain in MYC-positive cells promotes the detachment-induced apoptosis, and calpastatin knockdown in Myc-negative cells promotes tumorigenicity[248]. Calpain activity can also affect the cell cycle, through mechanisms that include the progression through the G1 stage of the cell cycle in v-src-transformed cells[249], an altered cellular location of m-calpain during mitosis[250] and the cleavage of cyclin E to a more active form in breast cancer[251]. Moreover, calpain can interfere with the interaction between protein phosphatase 2A (PP2A) and AKT to prevent forkhead box O (FOXO)-mediated cell death[252].
A critical behavior of malignant tumors is represented by the escape from apoptosis, often initiated upon aberrant proliferative stimuli. Caspases are the main effectors of apoptosis and the most characterized, but their activity is combined to that of the two ubiquitous calpains. This involvement is due to the activation of caspase-3, -7 and -12 and inactivation the caspase-8 and -9 by calpains[253], with a consequent induction of apoptosis in tumor cells. The pro-apoptotic activity of calpains has been demonstrated in breast cancer cells (MCF-7) treated with genistein. In this condition, genistein was effective in increasing calcium concentration, leading to the activation of μ-calpain, which in tuns cleaved and activated caspase-12[219]. The same process was observed in hepatocellular carcinomas, in which genistein activated m-calpain[254].
Several studies demonstrated that calpains can interfere with p53 status. Since p53 is a crucial mediator for cell fate, as it balances proliferation, DNA repair and apoptosis, the calpain-mediated

processing is a key event. In particular, calpains can proteolyze wild-type or mutated p53, but their effect may leave unchanged some p53 function or mediate its degradation[244,255]. From these evidence calpain activity is crucial for the induction of apoptosis. However, the μ- and m-calpain proteases have been implicated in both pro- and anti-apoptotic functions. Partial cleavage of pro- or anti-apoptotic proteins might activate or inactivate, respectively, putative substrates including p53[256], Bcl-2[257], Bcl-xl[258], Bid[259], Bax[260], caspase-3[261,262], caspase-7, -8, and -9[253], caspase-12[258], and NFB[252]. For this point of view, calpain cleavage of p53 may protect cells from DNA damage-induced apoptosis, and calpain cleavage of caspase-9, -8, -7, and -3 may attenuate their activity during apoptosis in many cell types[261,262] (Figure 9).

Few reports suggested an anti-apoptotic function for calpain. Calpain inhibitor I blocked the TNFα-induced Nuclear Factor-B (NFB) survival pathway in the murine fibroblast cell line WEHI164[263]. Similarly, the overexpression of calpains in CHO cells was shown to protect cells from TNFα-induced apoptosis[264]. Also, ceramide-induced NFB pro-survival signaling was shown to be calpain-dependent using calpain-deficient fibroblasts[265]. In a work by Tan et al. cell death responses and apoptotic or survival signaling pathways were compared in primary mouse embryonic fibroblasts (MEFs) derived from wild type or *CAPN4* knock-out mice which lack both μ- and m-calpain activities. The study reported that capn4$^{-/-}$ MEFs were more susceptible to staurosporine (STS) and TNF-induced cell death, which provided evidence for anti-apoptotic signaling roles for calpain[266].

Overall, calpain is likely to be acting at the level of several different signaling pathways, and collectively, these contribute in a very significant way to the eventual outcome associated with different death stimuli[266].

1.4.4 Calpains and migration/invasion

The migration ability of tumor cells is crucial for the invasion of surrounding tissues, with consequent induction of metastatic potential and compromission of cognitive functions.

Differently from other biological events of tumor cells where calpains are ambiguously involved, the positive role played by calpains in tumor cells migration and invasion has been well established, both in experimental models and clinical investigations. In this contest, calpain overexpression was shown to be strictly correlated with an aggressive and more invasive phenotype. Several enzymatic and structural proteins involved in migration and invasion mechanisms have been found to a proteolytic target of calpain activity, including talin[267], focal adhesion kinas (FAK)[268], fodrin[269], vinculin[270], ezrin[230] and filamin (Figure 9). In particular, filamin cleavage is strictly involved in cytoskeleton remodeling and motility and its calpain-mediated cleavage can be activated by Wnt5A upregulation in melanoma cells[271]. Notably, as occurs in metastatic melanoma cells, this mechanism may be

increased by the loss of Kloto protein[272]. Initial explorations linked calpain to the regulation of integrin-mediated cell adhesion, since m-calpain are localized to integrin-associated focal adhesion structures and directly cleaved the focal adhesion protein talin[273]. This signalling was found to be influenced by MEKK1 through the downstream activation of calpain[274]. Studies using a combination of gene ablation, pharmacological inhibition and RNA interference to suppress calpain activity confirmed the implication of m-calpain in regulating focal adhesion turnover and cell migration in a variety of tumour-derived and oncogene-transformed cell models[275,276,277.]

E-cadherin, considered a metastatic-suppressor gene responsible of homotypic adhesion, may be proteolyzed by calpain-2, overexpressed in metastatic prostate cancer. The outcome is a disrupted fragment of E-cadherin adhesion complex, which results in tumor cells invasion[278] and lymphovascular emboli formation[279].

Another important mechanism regards the degradative remodeling of the extracellular matrix provided by MMPs. Numerous reports demonstrated that calpain are involved MMP expression and secretion[235,280,281]. For example, it has been observed that calpain activity acts upstream of membrane-type MMP1, MMP2 and urokinase plasminogen-type activators (uPAs), thus contributing to extracellular matrix remodeling and invasion[282]. Studies determining the aberrant expression of *CAPN1* mRNA in cancer have linked increased expression to lymph node metastasis and histological type in renal cancer[283]. Taken together, these observations confirm that calpain are essential for efficient tumor cell migration, invasiveness and ultimately metastatic progression[231,284,285].

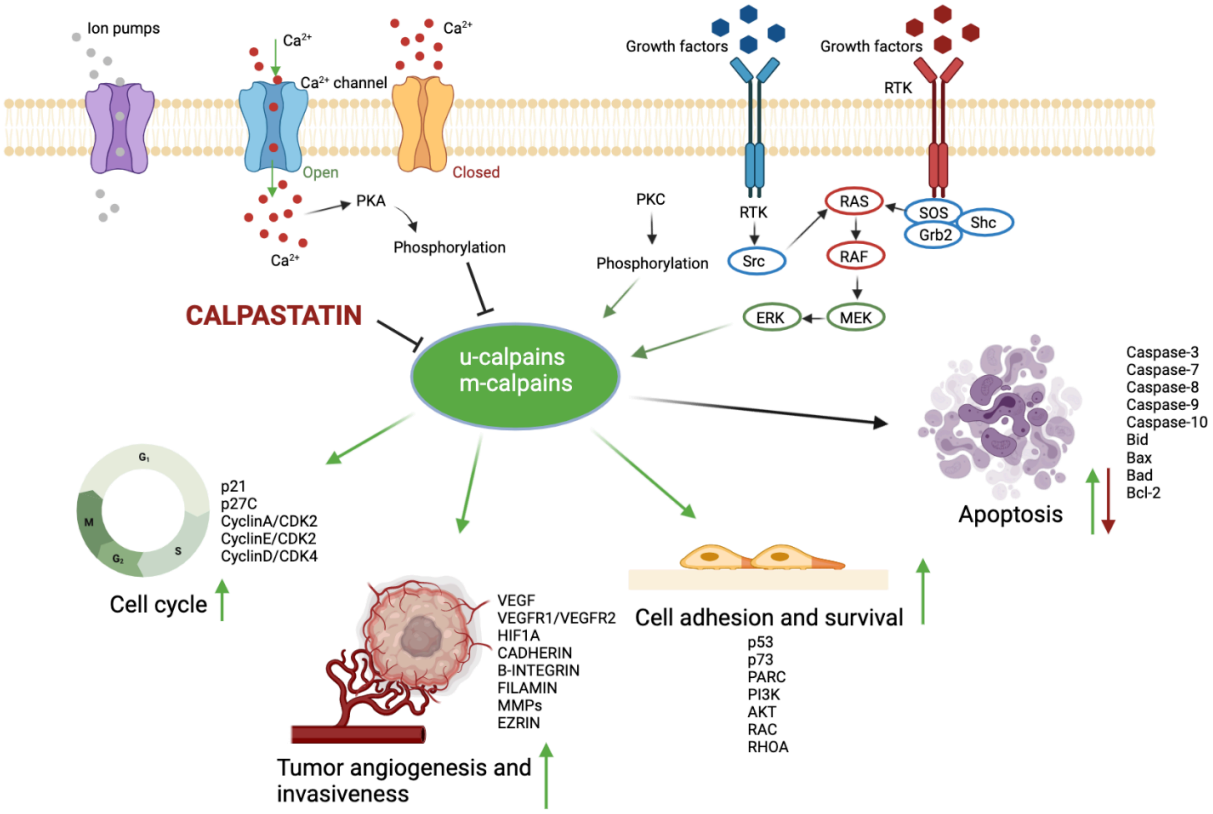

Figure 9. Schematic representation of calpain activation by Ca^{2+} concentration and growth factors-induced downstream cascade and their role in tumor mechanisms as cell cycle progression, angiogenesis and migration, survival and apoptosis.

2. MATHERIAL AND METHODS

2.1 Study population

Patients of both sexes (n=30) with newly diagnosed malignant brain tumors who underwent surgery for tumor excision at the Neurosurgery Unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico from 2014 to 2018 were eligible for this study. The Institutional Review Board approved the protocol and all patients provided informed consent. Inclusion criteria were: 1) age between 18 and 80 years; 2) Karnofsky Performance Status (KPS) >60; 3) signed consent for the study; 4) histologically proven diagnosis of GBM according to the World Health Organization classification (2016) on review by 2 independent pathologists. Exclusion criteria were: 1) previous brain surgery for other intracranial malignancies, 2) concomitant life-threatening disease, 3) history or presence of other malignancies, and 4) refusal or inability to consent to the study protocol. Demographic and clinical data covering the interval from the date of diagnosis to death, or the last follow-up visit were collected from the patient's records. Specifically, KPS, Ki-67 positivity, and *MGMT* promoter

methylation, *IDH1/2* status, loss of heterozygosis of chromosomic arm 1p/19q and immunohistochemical stains were recorded (Table 1-2). According to OS, patients were subclassified in long-term survivors (LTS) with an OS > 30 months and short-term survivors (STS), with an OS < 12 months.

2.2 Tumor sample processing

Tissue samples deriving from tumor excision were transported from the operating room to the Laboratory of Experimental Neurosurgery and Cell Therapy under sterile conditions. Tumor specimens were washed in D-PBS (Euroclone, Milan, Italy) and suspended in DMEM/F12 (Thermo Fisher, Waltham, Massachusetts) containing 1% penicillin/streptomycin at 4 °C, for up to 24 h after surgery. For each sample, an aliquot of tissue was frozen dry at −80 °C for successive DNA isolation, whereas another aliquot was processed by finely mincing with surgical scalpel in D-PBS and antibiotics. After shredding, samples were enzymatically digested with 0.625 Wu/ml Liberase Blendzyme 2 (Roche, Mannheim, Germany) for 1 h at 37 °C. Cells were plated into 25 cm^2-flask coated with bovine type I collagen (BD Biosciences, Milan, Italy) and cultured in endothelial proliferation medium (EndoPM) at 37 °C, 5% CO_2, 5% O_2, according to published protocols to isolate glioblastoma endothelial cells (GECs)[170,171]. The media were changed 1-2 times a week and passaged at a split ratio of 1:4 every 14 days.

2.3. Molecular characterization

2.3.1 DNA isolation

DNA from the tumour samples was obtained using DNeasy Blood & Tissue Kits Isolation kit (Qiagen, USA), following manufacturer's instructions. DNA quantification and integrity assessment were performed with NanoDrop 3000 (Thermo Fisher).

2.3.2 IDH1 and IDH2 Mutation Analysis

The assessment of hotspot mutations in *IDH1*, *IDH2*, and *TERT* promoter was performed as previously described[286]. In brief, the molecular test investigates the following mutations in *IDH1* and *IDH2* genes, using MassARRAY Analyzer 4 system (Agena Bioscience, CA, USA): - *IDH1*: c.394C> T p.R132C, c.394C> G p.R132G, c. 394C> A p.R132S, c.395G> A p.R132H, c.395G> T p.R132L, c.395G> C p.R132P; - *IDH2*: c.514A> C p.R172R, c.514A> G p.R172G, c.514A> T p.R172W, c.515G> A p.R172K, c.515G> C p.R172T, c. 515G> T p.R172M, c.516G> A p.R172R, c.516G> C p.R172S, c.516G> T p.R172S[38]. PCR, SAP, and IPLEX reactions were conducted as described in

manufacturer's protocol (Agena Bioscience, San Diego, CA, USA). Samples were transferred to a SpectroCHIP (Agena Bioscience, San Diego, CA, USA) and analyzed by mass spectrometry. The spectral profiles generated by MALDI-TOF mass spectrometry were evaluated using Typer v.4.0 software (Agena Bioscience, San Diego, CA, USA).

2.3.3 MGMT Promoter Methylation Evaluation

The molecular test investigates the methylation of the *MGMT* gene promoter (GRCh37 / hg19 chr10: 131.265.471-131.265.581), by pyrosequencing, with the "Pyromark Q96 ID System". The protocol used is reported in Fontana et al.[287]. DNA was extracted from FFPE tumor sample using QIAamp DNA FFPE kit (Qiagen, USA) and treated with sodium bisulfite using EZ DNA Methylation-GOLD kit (Euroclone, Italy). The test detects the methylation levels of 10 CpG sites located in the promoter region of the MGMT gene with a sensitivity of 95%. The methylation analysis was implemented as previously described[287]. PCR was performed on at least 20 ng of bisulfite-treated DNA and about 10 pmol primers. Quantitative DNA methylation analysis was carried out on the Pyro Mark ID instrument using Pyro Gold Reagents (Qiagen) and 1 pmol of sequencing primer. Methylation data were analyzed by the Q-CpG software v1.9 (Qiagen) and the levels of methylation of each sample are represented by the mean of the methylation percentages at each CpG site of the investigated region. The analysis of the methylation levels of the *MGMT* gene promoter represents a prognostic and predictive marker of response to standard treatment with alkylating agents (e.g., TMZ). The presence of *MGMT* promoter methylation, with an average methylation rate greater than 9%, is associated with a better response to chemotherapy treatment.

2.3.4 CGH Analysis

A 60K oligonucleotide SurePrint CGH microarray (Agilent Technologies, Palo Alto, CA) was used for the detection of copy number changes in DNA purified from tumors tissue samples. Normal male/female reference DNA and genomic tissue DNA were labeled with different fluorochromes (cyanine 3-deoxyuridine triphosphate-dUTP and cyanine 5-dUTP) and hybridized following the manufacturer's recommendations. Arrays were scanned at 3 µm resolution using an Agilent SureScan, and image data were processed using Feature Extraction version v5.0.0.1 and Genomic Workbench version 7.0. The ADM-2 algorithm was applied to define CNVs using a "three probes minimum" filter, with a threshold of 6 and without a fuzzy zero correction. Copy number amplifications and losses were defined as a log 2 ratio of 0.25. For statistical analysis, samples were divided in two groups, short-term and long-term survivors. To identify common aberrations between the two groups, was applied the T-Test Common Aberration algorithm with overlap threshold of 0.9

and p-value <0.05, while to identify genomic intervals that have an overabundance of gains or losses in STS group of samples compared to the LTS group in each genomic region, we used a Differential Aberration filter. Interval penetrance was estimated to identify the most strongly tumor-associated CNVs; in particular, was taking into consideration the CNVs present in at least 70% of samples. A functional and pathway enrichment analyses were performed using DAVID and MetaCore tools. PPI network relationship was evaluated using Network Analyst.

2.4 Cellular characterization

2.4.1 Immunofluorescence analysis

GECs (1×10^4/well) were seeded into µ-Slide 8 Well, ibiTreat (Ibidi, Martinsried, Germany) collagen-coated. When cells reached the desired confluence and were fixed in paraformaldehyde 4% for 20 min at RT, washed twice with D-PBS and incubated with 0.1 M glycine to quench auto-fluorescence. Then, the coverslip was incubated with PBS + 0.25%Triton x100 to permeabilize cell membranes and then blocked in PBS + 5%BSA for 30 min at RT. Incubation with primary antibodies (AB-I) diluted in blocking buffer was performed overnight at 4 °C. The following AB-I were used: anti-vascular endothelial growth factor (VEGF), anti-Von Willebrand Factor (VWF), anti-VEGF receptor-1 (VEGFR1), anti-VEGF receptor-2 (VEGFR2) anti-VE-Cadherin, as specific markers for GECs.

2.4.2 Flowcytometric analysis (FACS)

GECs (1 × 105/tube) were resuspended in 200 µl of D-PBS and stained with phycoerythrin (PE)- or Fluorescein isothiocyanate (FITC)-conjugated anti-human CD31, CD15, CD133, CD105, CD90, CD56, CD309, CD144 and CD34 (all purchased by BD Biosciences), for 20 min at RT in the dark. In order to exclude dead cells from the analysis, 7-aminoactinomycin D (7-AAD, BD) was added to each tube. Flow cytometric analysis were performed using FACScalibur flow cytometer Cell Quest software (FACS, BD Bioscience, version 8.0).

2.4.3 Estimation of proliferation rate

GECs (5×10^4) were seeded into 25 cm^2 collagen-coated flask, cultured in EndoPM at 37 °C, 5%CO_2, 5%O_2 and passaged at a split ratio of 1:2, as they were in confluence. At each passage time point, ECs detached by TrypLE Select were stained with Trypan Blue (ThermoFisher) and counted in a Fuchs Rosenthal counting chamber, to evaluate growth rate.

2.4.4 Vascular permeability assay

Transwell insert (0.4 µm pore size, Corning, Corning, USA) were coated with bovine type I collagen for 45 min at 37 °C. After complete drying, GECs (2×10^4) were seeded on the insert and cultured with 150 µl and 650 µl of EndoPM in the upper chamber and in the lower chamber, respectively. GECs were cultured for 3 days at 37 °C, 5%CO_2, 5%O_2, until they reached the appropriate confluence, and then FITC-70 kDa dextran (Sigma Aldrich) was added to the insert (4 µl, 25 mg/ml initial concentration). Every 30 min, 10 µl of EndoPM were collected from the lower chamber, for a total of 7 samples (30 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 6 h). After the latest time point (6 h), each sample was diluted to 200 µl with 1 × D-PBS and 100 µl of each diluted sample were transferred in duplicate into 96-well black plates. Fluorescent content of the samples was measured at 492/520 nm absorption/emission wavelengths for FITC-dextran, with Tecan Infinite 200 PRO. Notably, the assessment of GECs permeability was conducted using endothelial cells isolated from low grade gliomas (LGG-ECs) and meningiomas (MNG-ECs).

2.4.5 Tube Formation Assay

µ-Plate Angiogenesis 96 Well (Ibidi) were coated with 12.5 mg/mL Matrigel (BD Bioscience), 10 µL/well, at 4 °C. After gentle agitation to ensure complete coating, plates were incubated for 30 minutes at 37 °C to allow solidification of Matrigel. ECs ($1x10^4$/well) from GBM, LGG and MNG were seeded in triplicate in EndoPM and incubated at 37 °C, 5%CO_2, 5%O_2. Cord formation was monitored with an inverted Eclipse Ti-E microscope (Nikon Instruments, Florence, Italy), equipped with a high resolution cSMOS camera (Andor Zyla, Andor Technology, Belfast, UK) and NIS_Elements 4.51 software, using differential interference contrast (DIC). After 24 h of incubation, five random images were acquired and analysed with “Angiogenesis Analyzer” plugin in Image48.

2.4.6 Migration Assay

GBM-ECs were seeded ($1x10^4$ cells, each side) into Ibidi Culture-Inserts (Ibidi) and cultured until 95% confluence was reached. After that, the inserts were removed, and cells were stained with 1 mg/mL Calcein AM (Thermo Fisher Scientific) for 30 minutes at 37 °C. Then, fresh EndoPM was added in the presence of different molecules, as described for MTT assay. After 24 hours, images of GECs migrated into cells-free gap were acquired with an inverted Leica DMI6000B widefield microscope at 20x magnifications in 5 random fields. Cells migrated into the gap were than counted using “Analyze Particles” in ImageJ.

2.4.7 Drug treatment

Based on CGH analysis results, it was decided to assess the effectiveness of Calpain inhibitors against GECs. Calpain Inhibitor 1 and Calpain Inhibitor 2 were administered at 25 μM, 50 μM and 100 μM, whereas AC-Calpastatin was administered at 10 μM and 20 μM, to evaluate dose-dependent efficacy and toxicity. All compounds were purchased by Calbiochem. TMZ (Schering-Plough, Milano, Italy) 200 μM was administered to evaluate the synergistic effect of tested compounds.

2.4.8 MTT assay

3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide (MTT) assay was used to assess cell viability as a function of redox potential. GECs (5×10^3/well) were seeded and cultured in 96-well plate for 24 h. Then, culture media were replaced with fresh media containing the specific treatments. Tests were performed in triplicate following 24 h and 48 h of treatment. At each time point, culture media were replaced with 100 μl of fresh media + 10 μl of MTT 5 mg/ml in D-PBS. After 4 h of incubation, media were removed and cells were lysed with 100 μl of 2-propanol/formic acid (95:5, by vol) for 10 min. Then, absorbance was read at 570 nm in microplate reader.

2.4.9 Live and Dead Assay

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit provides a two-color fluorescence (Calcein AM and EthD-1) cell viability assay, which measure intracellular esterase activity and plasma membrane integrity, thus determining live and dead cells. GECs were seeded into 24-well plates ($2x10^4$/well) until confluence, and then treated with the specific treatments for 72h. Then, the mixture of Calcein AM and EthD-1 was prepared following manufacturer's instruction and administer to cell cultures. Fluorescence images were acquired with Eclipse Ti-E microscope (Nikon Instruments, Italy).

2.4.10 Caspase 3/7 Activity

The Caspase-Glo® 3/7 Assay is a luminescent assay added as a "add-mix-measure" format resulting in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal, produced by luciferase. GECs (5×10^3/well) were seeded and cultured in 96-well plate for 24h and then treated with the specific treatments. After 72h, Caspase-Glo® 3/7 Reagent was added in equal volume and luminescence, proportional to the amount of caspase activity, was read with a microplate reader.

2.4.11 Quantitative real-time PCR analysis (qRT-PCR)

GECs (1×10^5/well), as well as LGG-ECs and MNG-ECs used as control, were seeded into 25 cm^2 collagen-coated culture flasks. When 90% confluence was reached or at the end of the above listed treatment, total RNA was extracted following TRI-Reagent protocol and quantified with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Reverse transcriptase reaction was executed using TranScriba Kit (A&A Biotechnology), loading 1 µg of RNA (A260/A280 > 1.8), according to manufacturer's instructions. qRT-PCR was performed using StepOnePlus™ (Thermo Fisher Scientific) using 1 µg of cDNA, forward and reverse primers (250 nM each one) and PowerUp SYBR Green Master Mix (ThermoFisher). Data were normalized to 18S expression, used as endogenous control. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are provided in Table 1.

Table 1. Primer Sequences for qRT-PCR

Gene	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Tm (°C)
18 S	ACTTTCGATGGTAGTCGCCGT	CCTTGGATGTGGTAGCCGTTT	61
AKT	TCT ATG GCG CTG AGA TTG TG	CTT AAT GTG CCC GTC CTT GT	58
ANG-1	GGGCACACTCATGCATTCCT	GGTTGCACATCCAAGCCAAG	60
ANG-2	CCTGTTGAACCAAACAGCGG	GTGGGGTCCTTAGCTGAGTT	60
BAX	AGC AAA CTG GTG CTC AAG G	TCT TGG ATC CAG CCC AAC	57
BCL-2	AGT ACC TGA ACC GGC ACC T	GCC GTA CAG TTC CAC AAA GG	60
BID	ACC GTG GTC TTT CCA GCA CC	TCT GCG GAA GCT GTT GTC AG	61
CAPN1	CCG GCC CCT CCT CAG A	GGT CCT TGT AAC CCA GGC TC	60
CAPN2	CCC CGA CCT TTC TCT GCG	TCT CCC CAG GGA TTT CGG AT	60
CAPN3	CGA TGA CCC TGA TGA CTC GG	CCG AAA CGA AGA TGA TGG GC	61
CAPNS1	TGC GGC GCA GTG AGT C	ATT GGG CCC TGG ATG TTG AG	58
CAPNS2	GAT TGT CCG CCG GTA TGC TA	TTG AAG GCA CGA AAC ATG GC	59
CASPASE-3	ATG GTT TGA GCC TGA GCA GA	GGC AGC ATC ATC CAC ACA TAC	60
CASPASE-7	GAG CAG GGG GTT GAG GAT TC	GTC TTT TCC GTG CTC CTC CA	61
CAST	ATC GCC TTC CAA ACC AGG AG	TGG AGC AGC ACT TCT GAC TG	60
ERK-1	ACTCCAAAGCCCTTGACCTG	CTTCAGCCGCTCCTTAGGTA	60
FGF-2	TCCACCTATAATTGGTCAAAGTGGT	CATCAGTTACCAGCTCCCCC	63
HIF-1α	GTCTGAGGGGACAGGAGGAT	GCACCAAGCAGGTCATAGGT	61
MEK-1	CTTCGCAGAGCGGCTAGG	AGCTCTAGCTCCTCCAGCTT	61
NCAM	GCAGCGAAGAAAAGACTCTGG	ATCCTCTCCCATCTGCCCTT	60
p21	AGT ACC CTC TCA GCT CCA GG	TGT CTG ACT CCT TGT TCC GC	61
p27	TGG CTT GTC AGG AAC TCG AC	CTA GTC TCC AGG GAG GTG CT	63
p53	AGG CCT TGG AAC TCA AGG AT	CCC TTT TTG GAC TTC AGG TG	58
RAF	GGT TTT GGC GTA GAT TCC CC	ACC TGA AGC AAA GAT GGC GT	59

RAS	AGCAGGTGGTCATTGATGGG	CCGTTTGATCTGCTCCCTGT	60
TIE-2	GGAAGGTGCCATGGACTTGA	GTCATCCTCTGTATGCCTTGCT	61
VEGF	TACCTCCACCATGCCAAGTG	ATGATTCTGCCCTCCTCCTTC	60
VEGFR1	GCAAAGCCACCAACCAGAAG	ACGTTCAGATGGTGGCCAAT	60
VEGFR2	CGGTCAACAAAGTCGGGAGA	CGGTCAACAAAGTCGGGAGA	60
VWF	ACACCTGCATTTGCCGAAAC	ATGCGGAGGTCACCTTTCAG	60

Tm: Melting temperature

2.4.12 Western Blot Analyses

GECs ($2x10^5$) were seeded into a 25 cm^2 collagen-coated culture flasks precoated with Collagen Bovine Type I and cultured until they reached the appropriate confluence (about 80%-90%). Then, cells were lysed with M-PER Protein Extraction Reagent (Thermo Fisher Scientific) in presence of Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Proteins were quantified by the Pierce Detergent Compatible Bradford Assay Kit (Thermo Fisher Scientific). Protein lysates (30 mg) were separated in Bolt 10% Bis-Tris Plus Gels (Thermo Fisher Scientific) in Mini Gel Tank (Thermo Fisher Scientific) and transferred onto nitrocellulose iBlot 2 Transfer Stacks using iBlot 2 Dry Blotting System (Thermo Fisher Scientific). After transfer, the membrane was blocked in Tris-buffered saline/Tween 20 þ 5% milk solution and incubated separately with anti-GAPDH, anti-Calpain-1, anti-Calpain-2, anti-Calpain-3, anti-Calpain small subunit 1, anti-Calpain small subunit 2 and anti-Calpastatin (all purchased from Santa Cruz Biotechnology), overnight at 4°C. After incubation with HRP-labeled secondary antibody (Invitrogen, Carlsbad, California, USA), protein bands were scanned with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and detected by ChemiDoc XRSþ (Bio-Rad, Hercules, California, USA). Densitometric analysis were performed using ImageJ.

2.4.13 Statistical analysis

All analyses were done with GraphPad Prism (GraphPad Software, Inc., La Jolla, California, USA). Continuous variables are presented as median and interquartile range (IQR). Categorical variables are presented as counts and percentages. Parameters were compared and analyzed by a one-way analysis of variance. When significant differences were detected, Dunnet post hoc comparisons versus control group were made. The Pearson correlation test was used to assess the univariate association between variables and was made using IBM SPSS Statistics 26.0 software. Differences were considered statistically significant for $P<0.05$. Values are expressed as mean±SD of at least 3 independent experiments.

3. RESULTS

Study population

The demographic, clinical and molecular features of patients and their respective GBM tissues used for array-CGH are provided in Table 2. The median age of patients at diagnosis was 61 years (IQR: 52-71) 53% were males. The median value of *MGMT* promoter methylation was 17 % (IQR: 4-31) and all GBMs were *IDH*-wildtype. Notably, a value of *MGMT* promoter methylation >9% is considered a favorable prognostic indicator, associated with a better response to treatment[287]. Four patients showed a loss of heterozygosity of 1p or 19q.

Table 2. Demographic, clinic and molecular features of enrolled patients.

ID	WHO Grade	Sex	Age (years)	Tumor location	KPS	Ki-67 (MIB-1)	OS (months)	MGMT	IDH	1p/19q LOH	IHC
Poli 19	IV	M	67	Right TI	80	25%	62	4%	wt	wt	GFAP-1 (+); WT-1 (+)
Poli 48	IV	M	72	Right F	90	20%	38	8%	wt	wt	p53 (+); GFAP-1 (+)
Poli 49	IV	M	51	Left F	90	50%	46	32%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+)
Poli 78	IV	F	67	Right T	80	17%	31	22%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+); CD34 (+)
Poli 93	IV	M	62	Right F	70	35%	41	44%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+); CD34 (+)
Poli 41	IV	F	45	Right TI	100	37%	72	23%	wt	wt	GFAP-1 (-/+);WT-1 (+); CD34 (+)
Poli 79	IV	F	71	Right P	70	20%	59	28%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+); CD34 (+)
Poli 66	IV	M	47	Right T	80	30%	29	63%	wt	wt	GFAP-1 (+)
Poli 117	IV	F	56	Right FTP	70	30%	29	67%	wt	wt	-
SC15	IV	M	36	Left Tr	80	60%	58	32%	wt	wt	-
SC21	IV	M	46	Right P	90	60%	34	41%	wt	LOH 1p	-
Poli 121	IV	M	50	Right P	90	60%	23	26%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+); CD34 (+)
Poli 169	IV	M	52	Right F	70	67%	31	13%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+)
Poli 60	IV	M	49	Right F	90	70%	16	2%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+)
Poli 90	IV	F	58	Left T	80	30%	16	21%	wt	wt	GFAP-1 (+)
Poli 143	IV	M	61	Right F	70	30%	17	23%	wt	wt	GFAP-1 (+)
Poli 09	IV	F	80	Left FP	70	15%	4	4%	wt	LOH 1p	GFAP-1 (+)
Poli 10	IV	F	63	Right FI	70	35%	4	9%	wt	LOH 19q	p53 (+); GFAP-1 (+);WT-1 (+)
Poli 33	IV	F	65	Right T	80	70%	11	14%	wt	wt	GFAP-1 (-/+);WT-1 (+)
Poli 56	IV	M	75	Left T	70	40%	2	3%	wt	wt	GFAP-1 (+)
Poli 85	IV	M	59	Left P	80	30%	12	4%	wt	LOH19q	GFAP-1 (+)
SC02	IV	M	59	Right T	60	15%	4	5%	wt	wt	-
Poli 31	IV	M	75	Left FP	70	25%	3	4%	wt	wt	p53 (-/+); GFAP-1 (+);WT-1 (+);
Poli 38	IV	M	54	Right PO	70	20%	6	5%	wt	wt	GFAP-1 (+); WT-1 (+)
Poli 30	IV	F	76	Left PO	60	25%	4	41%	wt	wt	-
Poli 21	IV	F	54	Right P	80	80%	2	49%	wt	wt	-
Poli 164	IV	F	63	Right F	70	40%	4	2%	wt	wt	p53 (+); GFAP-1 (+);WT-1 (+)
Poli 104	IV	F	70	Right F	70	85%	5	3%	wt	wt	p53 (+); GFAP-1 (+)
Poli 40	IV	F	76	Right T	70	35%	3	27%	wt	wt	p53 (-/+); GFAP-1 (+);WT-1 (+);
Poli 166	IV	F	73	Left T	70	30%	13	4%	wt	wt	GFAP-1 (+)

Molecular test of *IDH1* (codon 132) and *IDH2* (codon 172) genes performed by MassARRAY Analyzer 4 system (SEQUENOM). Methylation analysis of the *MGMT* gene promoter performed by pyrosequencing with the "Pyromark Q96 ID System" after treatment of the DNA with sodium bisulfite. Analysis of loss of heterozygosity of chromosomal arms 1p and 19q in the tumor, performed by MassARRAY Analyzer 4 system (SEQUENOM), using a panel of polymorphic markers (SNPs) located on chromosomes 1 and 19.

T: temporal lobe; **I:** insular lobe; **F:** frontal lobe; **P:** parietal lobe; **O:** occipital lobe; **Tr:** trigone; **KPS:** Karnofsky performance status; **OS:** overall survival; **MGMT:** O6-methylguanine-DNA methyltransferase; **IDH:** isocitrate dehydrogenase; **LOH:** loss of heterozygosis; **IHC:** immunohistochemistry.

According to OS, patients were subclassified into LTS and STS GBM patients. A summary of demographic, clinical and molecular parameters are provided in Table 3. Notably, age (p=0.015), KPS (p=0.007) and *MGMT* promoter methylation (p=0.006), and obviously OS (p=0.000) resulted statistically significant, confirming their prognostic value.

Table 3. Summary of demographic, clinical and molecular features of LTS and STS GBM patients.

	STS (n=17)	LTS (n=13)	p-value
Sex (Male, n° (%)	7 (41)	9 (69)	0.135
Age (years)	63 (59-75)	52 (47-67)	**0.015**
KPS	70 (70-80)	80 (70-90)	**0.007**
Ki-67 (%)	30 (25-40)	35 (25-60)	0.958
OS (months)	4 (4-12)	38 (31-58)	**0.000**
MGMT (%)	5 (4-21)	28 (22-41)	**0.006**
IDH	100 (100)	100 (100)	-
LOH 1p/19q	3 (18)	1 (8)	0.444

Data are listed as median (IQR). **STS:** short-term survivors; **LTS**: long-term survivors; **KPS**: Karnofsky performance status; **OS**: overall survival; **MGMT**: O6-methylguanine-DNA methyltransferase; **IDH**: isocitrate dehydrogenase; **LOH:** loss of heterozygosis. P-value calculated with t student test, two-sided, for independent samples.

CGH analysis

Copy number variation (CNVs) events were used to assess the differential profile in GBM short-term and long-term survivors. A total of 1111 (639 in 13 LTS, 472 in 16 STS) CNVs were identified in 29 GBM genomes, including 43 copy number deletions (mean size, 349.1 kb) and 76 copy number amplification (mean size, 419.0 kb). CNVs were scattered across chromosomes 1 to 22. We observed that prototypical GBM lesions such as CNVs on chromosome 7 and chromosome 10 were present in both groups at similar frequencies. Specifically, the analysis of penetrance rate showed a significantly enriched deletion in Chr10p15.3-11.21 and amplificated CNV regions in Chr7p22.1-11.2/q11.22-36.6 and 14q32.33, which a much higher interval penetrance of 70%. The analysis of common aberrations, at the same percentage of penetrance, revealed recurrent aberration in Chr7, 10, and 14 for STS and recurrent aberration regions in Chr7/9/10/14/19/20 for LTS. Differential aberration analysis was used to identify genomic intervals that have an overabundance of gains or losses in STS group of samples compared to the LTS group in each genomic region. This approach did not result in a clear separation of the groups. However, we found a major number of LTS patients (77%; 23 amplificated regions) with enriched aberrations on Chr19 respect to STS (18%; 9 deleted and 8

amplificated regions) and a major number of LTS patients (69%; 30 amplificated regions) with enriched aberrations on Chr20 respect to STS (25%; 9 amplificated regions). Furthermore, we assessed the allelic status of chromosomes 1p, 19q, and 10q to determine whether these chromosomes are predictors of tumor aggressiveness and poor clinical outcome in STS patients. We found, a small percentage of STS patients with loss in 1p19q (25%) and a high percentage of STS patients with Chr10q loss (94%). In LTS groups, we observed the same percentage of Chr10q losses regions, no codeletion 1p19q but 61% with the lost regions on Chr1.

Analysis of recurrent CNVs in LTS and STS patients

Table 4 shows the analysis of recurrent CNV present in almost 70% of patients. In LTS, deletions on Chr9p21.3 and amplifications on Chr7p11.2 and Chr14q32.33 were most frequently represented, while in STS we found an enriched amplified regions in Chr14q32.33 and Chr22q11.22 and no recurrent deletion regions. The analysis was focused on genes mapping on Chr9, Chr19 and Chr20 that are enriched in LTS, to define their implication on better prognosis.

Table 4. Analysis of recurrent CNV present in almost 70% of patients

	Recurrent CNV >70%	**Chromosomic region**
LTS	Deletion	**Chr9** p21.3
	Amplification	**Chr7**p11.2; **chr14**q32.33
STS	Deletion	No recurrent deletion
	Amplification	**Chr14**q32.33; **Chr22**q11.22
LTS	Gain	**Chr2; Chr7; Chr14; Chr19; Chr20**; **ChrX**
	Loss	**Chr1; Chr4; Chr9; Chr10**;
STS	Gain	**Chr3**p26.1; **Chr3**p25.1; **Chr3**q13.31 - q27.3; **Chr3**q13.31-q13.33; **Chr3**q21.3 - q26.1; **Chr3**q22.3; **Chr3**p21.31-p21.2; **Chr3**q27.3-q28; **Chr3**q25.33-q26.1; **Chr3**q26.1-q28; **Chr3**q22.1-q29; **Chr3**p14.2-p14.1; **Chr7**; **Chr14**;
	Loss	**Chr10; Chr15**

Deletion of Chr9p21.3

The PPI analysis performed on deleted genes on Chr9p21.3 with Network Analyst tool highlighted *TEK* gene as a hub (Figure 10). Specifically, among pathways and biological processes, the involvement of "*Tie2* signaling pathway", "Angiogenesis" and "Apoptotic process" may be considered the most relevant in term of neoplastic progression.

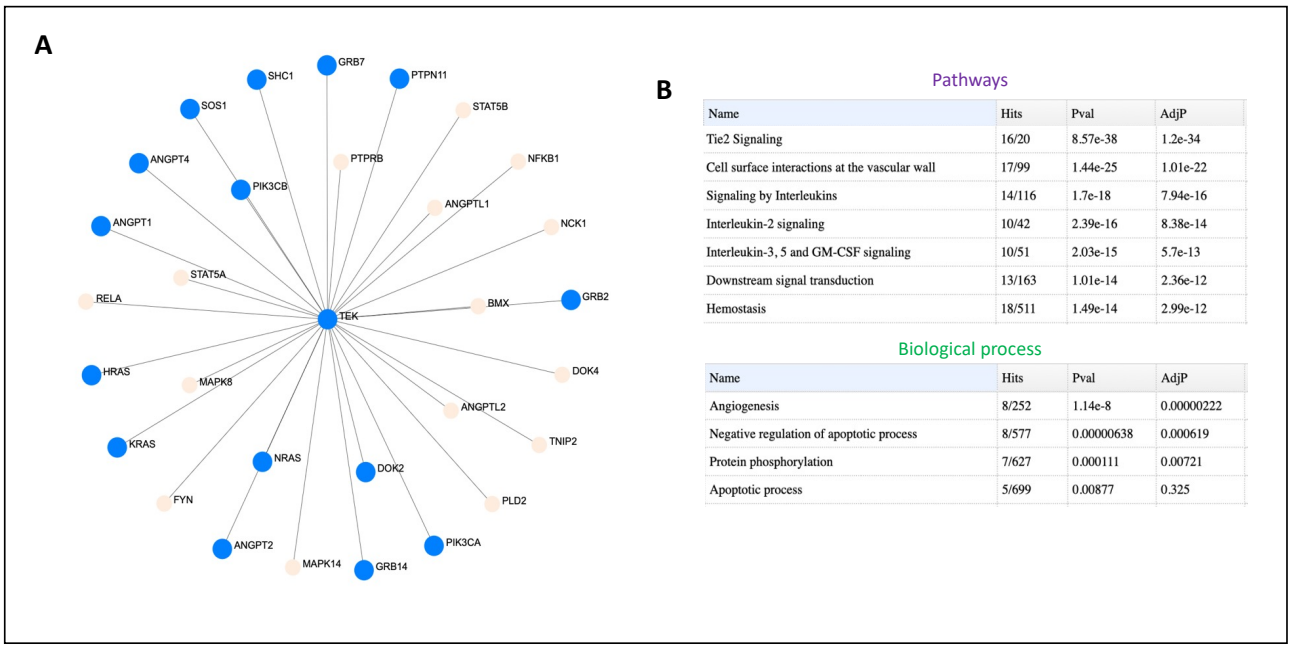

Figure 10. (A) Extended protein-protein interaction (PPI) network of encoding products genes mapping on Chr9p21.3, predicted using the STRING database and visualized with Network Analyst 3.0 software and (B) the relative pathways and biological process involved.

Gain of Chr19 and Chr20

Notably, we here reported that simultaneous gains of chromosomes 19 and 20 is a highly significant prognostic factor and were associated with a better prognosis[288].

To evaluate the relation of chromosomal aberrations to disease, we focused on genes mapping the chromosomal regions identified with specific attention for chr19 and 20 in LTS group. Gene enrichment for Chr19 and Chr20 was performed using MetaCore software to evaluate pathway and Network Analyst tool for network evaluation. This approach highlighted the involvement of several pathway among theme "Thromboxane A2 signaling pathway" (Figure11) and "Signal transduction_PKA signaling" (Figure 12).

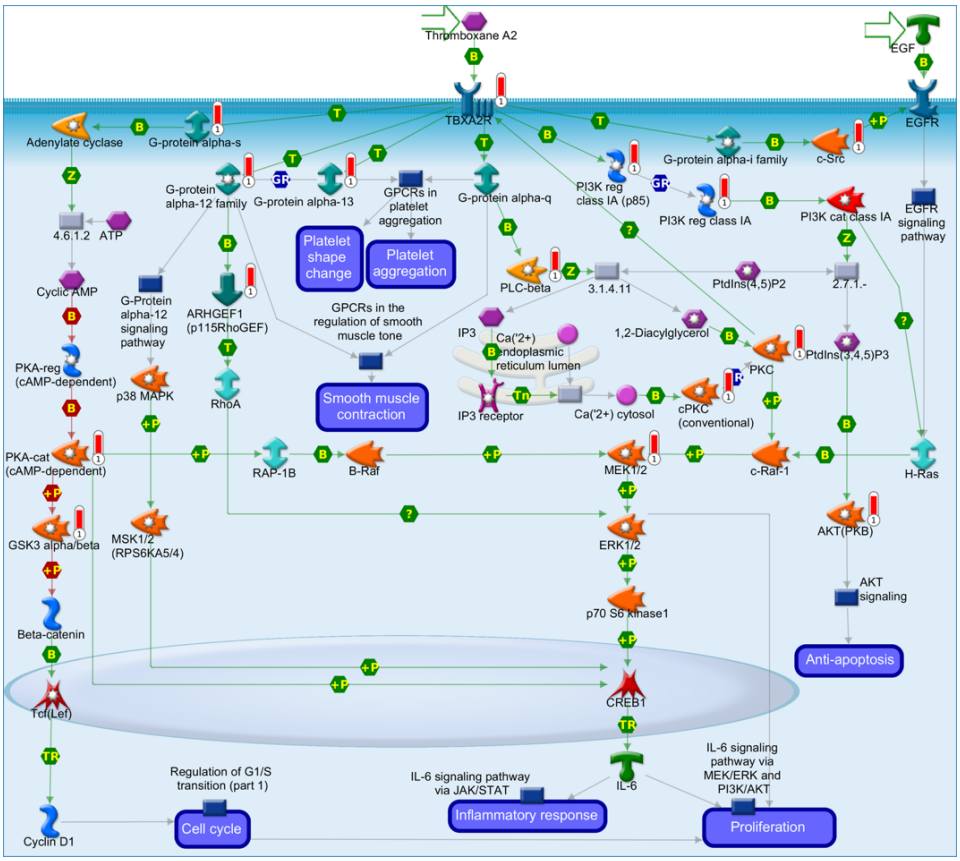

Figure 11. Thromboxane A2 signaling pathway. The top scored map based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

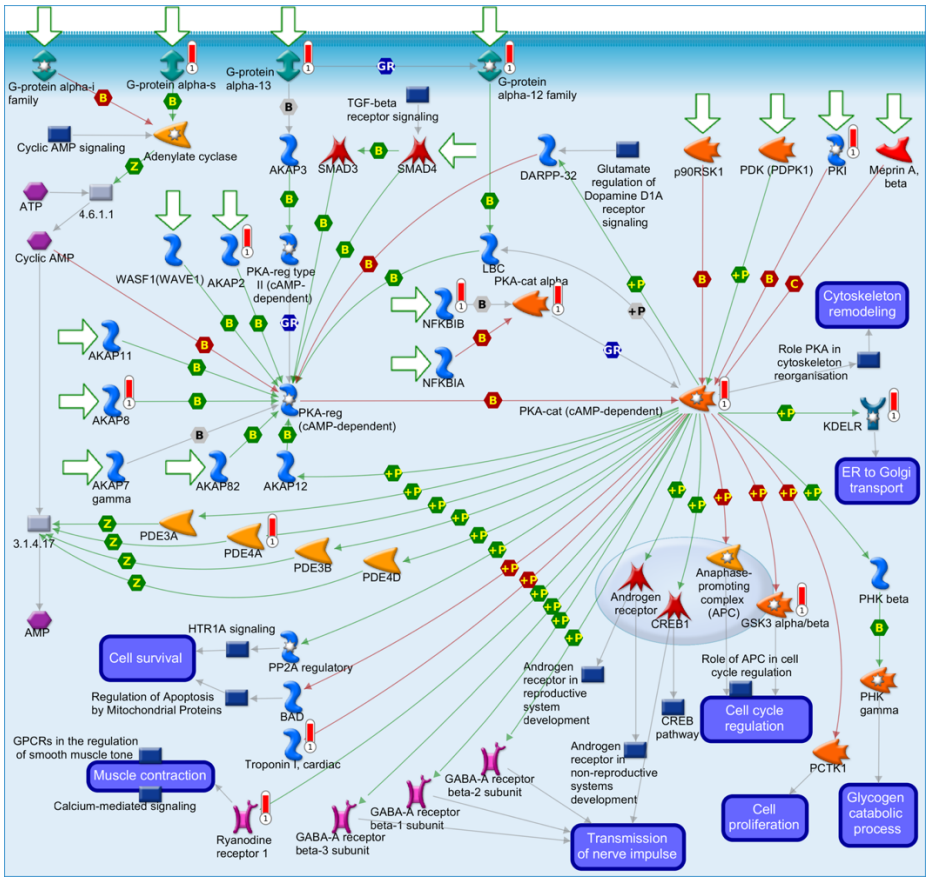

Figure 12. Signal transduction/PKA signaling. The top scored map based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

The PPI analysis performed with Network Analyst tool on gain genes encompassing on Chr19, highlighted as hubs *Uba52* gene which overexpression has been associated with better OS [289] and RPS5 and RPS11. Low expression of ribosomal protein genes has been associated with worse OS but notably, in our LTS group these proteins were found overexpressed[290] (Figure 12).

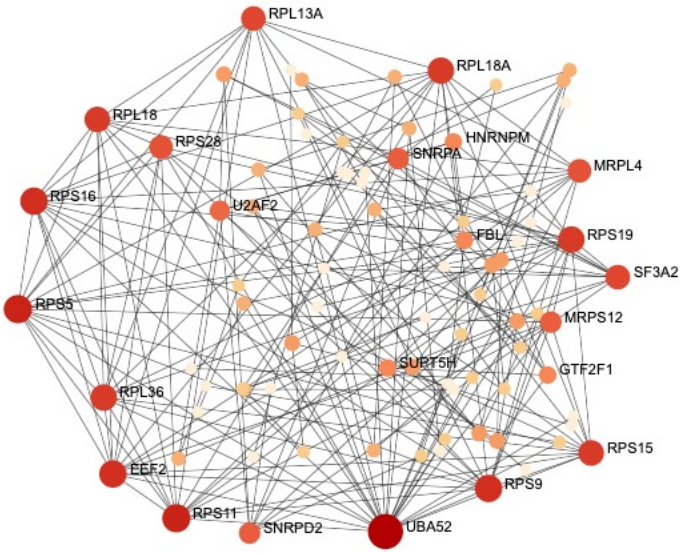

Figure 12. Extended PPI network of encoding products of genes mapping on Chr19, predicted using the STRING database and visualized with Network Analyst 3.0 software.

In parallel, the PPI analysis performed with Network Analyst tool on gain genes encompassing on Chr20, highlighted as hubs *SRC* and *PCNA* (Figure 14). Although *SRC* is an oncogene directly involved in the development of cancer, *PCNA* is involved in cell survival, and possibly in pathways of energy metabolism, such as glycolysis.

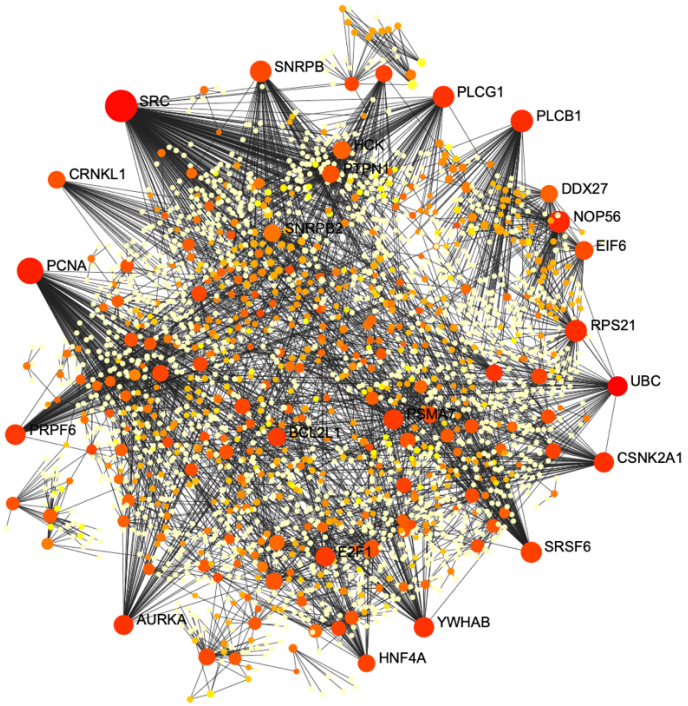

Figure 14. Extended PPI network of encoding products of genes mapping on Chr20, predicted using the STRING database and visualized with Network Analyst 3.0 software.

Gene Enrichment analysis of deregulated genes in LTS and STS patients

To identify a set of genes associated with better or poor prognosis in LTS and STS, we performed a comparison between deregulated genes in the two groups of samples at 70% of penetrance. With this approach, we identified 250 gains and 12 losses genes that are deregulated only in LTS group respect to STS samples (Figure 15).

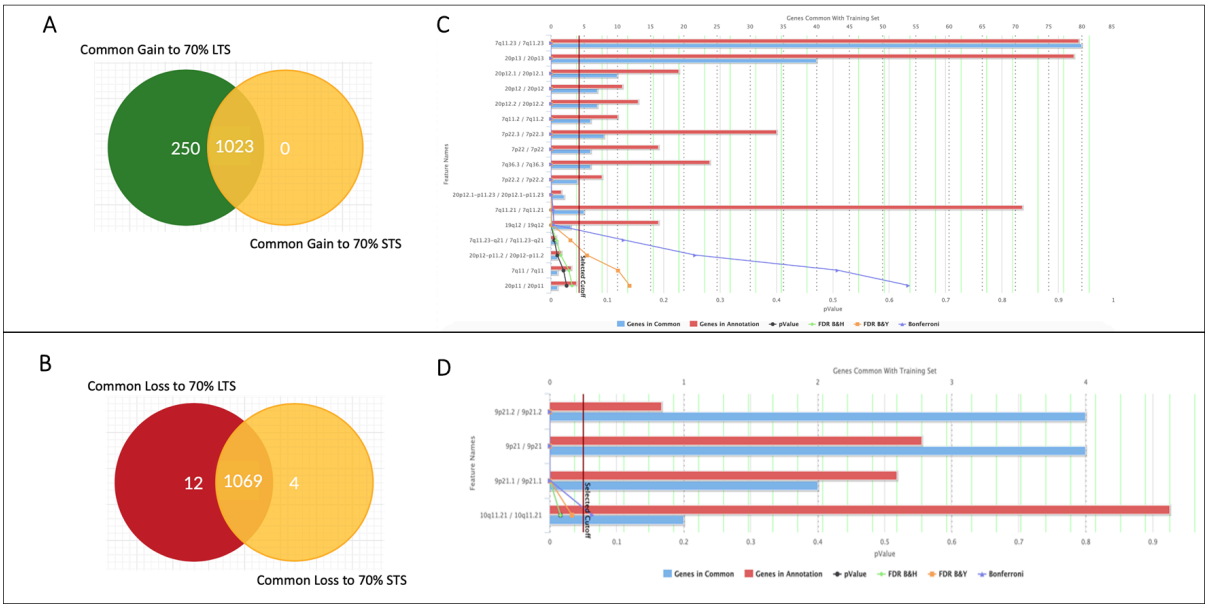

Figure 15. **(A-B)** Venn Diagram showing the intersection of two data sets as lists of genes in gain **(A)** or loss **(B)** in 70% of LTS and 70 % STS. Results show n= 250 genes in gain exclusively in LTS group, and n=12 genes in loss exclusively in LTS group. **(C-D)** Cytoband-level gains and losses with relative p-values of n= 250 genes in gain **(C)** and n=12 genes in loss **(D)** exclusively in LTS group.

Eliminating the orphan genes, the list of genes in gain and loss condition exclusively in LTS cohort is provided in Table 5

Table 5. List of genes found in gain or loss condition in LTS patient cohort

GAIN
ABHD11, ABHD11-AS1, AMZ1, ANKEF1, APTR, AVP, BAZ1B, BCL7B, BMP2, BRAT1, BTBD3, BUD23, CALN1, CARD11, CASC20, CASTOR2, CCDC146, CCL24, CCL26, CCT6P3, CHST12, CLDN3, CLDN4, CLIP2, CPXM1, DNAJC30, DTX2, DTX2P1, EBF4, EIF3B, EIF4H, ELFN1, ELFN1-AS1, ELN, ERV3-1, ERV3-1-ZNF117, ESF1, ESYT2, FAM185BP, FDPSP2, FGL2, FKBP1A, FKBP1A, FKBP6, FLRT3, FZD9, GALNT17, GNA12, GNRH2, GRIFIN, GSAP, GTF2I, GTF2IP1, GTF2IP4, GTF2IP7, GTF2IRD1, GTF2IRD2, GTF2IRD2B, HAO1, HIP1, HSPB1, IDH3B, INTS4P1, IQCE, ISM1, ISM1-AS1, JAG1, KIF16B, LAMP5, LAMP5-AS1, LAT2, LFNG, LIMK1, MACROD2, MACROD2-AS1, MACROD2-IT1, MAD1L1, MDH2, METTL27, MIR1292, MIR3914-1, MIR3914-2, MIR4284, MIR4648, MIR4650-1, MIR4650-2, MIR4651, MIR4655, MIR5707, MIR590, MIR595, MIR6836, MIR6869, MIR6870, MIR8062, MKKS, MLXIPL, MRM2, MRPS26, NCAPG2, NCF1, NCF1B, NCF1C, NDUFAF5, NOP56, NSFL1C, NSUN5, NSUN5P1, NSUN5P2, NUDT1, OTOR, OXT, PAK5, PARAL1, PCED1A, PCSK2, PDYN, PDYN-AS1, PHTF2, PLCB1, PLCB1-IT1, PLCB4, PMS2P2, PMS2P3, PMS2P5, PMS2P7, PMS2P9, POM121, POM121C, POMZP3, POR, PSMF1, PTPN12, PTPRA, RAD21L1, RCC1L, RFC2, RHBDD2, RPL13AP1, RSBN1L, SBDSP1, SDCBP2, SDCBP2-AS1, SDK1, SEL1L2, SIRPA, SIRPB1, SIRPB2, SIRPD, SIRPG, SIRPG-AS1, SLX4IP, SNAP25, SNAP25-AS1, SNORA114, SNORA14A, SNORA15B-1, SNORA15B-2, SNORA22C, SNORA51, SNORD110, SNORD119, SNORD56, SNORD57, SNORD86, SNPH, SNRPB, SNRPB2, SNX8, SPDYE10P, SPDYE11, SPDYE13P, SPDYE14P, SPDYE15P, SPDYE16, SPDYE17, SPDYE18, SPDYE5, SPDYE7P, SPDYE8P, SPTLC3, SRRM3, SSC4D, STAG3L1, STAG3L2, STAG3L3, STK35, STX1A, STYXL1, TASP1, TBL2, TFAMP1, TGM3, TGM6, THEG5, TMC2, TMEM120A, TMEM239, TMEM270, TMEM60, TMEM74B, TMX4, TRIM50, TRIM73, TRIM74, TSHZ3, TTYH3, TYW1B, UPK3B, VIPR2, VPS16, VPS37D, WDR60, YWHAG, ZNF107, ZNF117, ZNF138, ZNF273, ZNF343, ZNF536, ZP3
LOSS
TEK, EQTN, PLAA, LINC00032, IFNK, LINGO2, MIR876, MIR873, CAAP1, CCNYL2, MOB3B

The Gene Ontology analysis performed by DAVID GO using the list of 250 gain genes, reported 24 clusters, of which the most enriched is that containing *GTF2I* gene (score: 4.58, p-value: 1.38E-3). The enrichment analysis performed on pathways maps reported the “Protein folding and maturation” as the top statistically significant map (Figure 16).

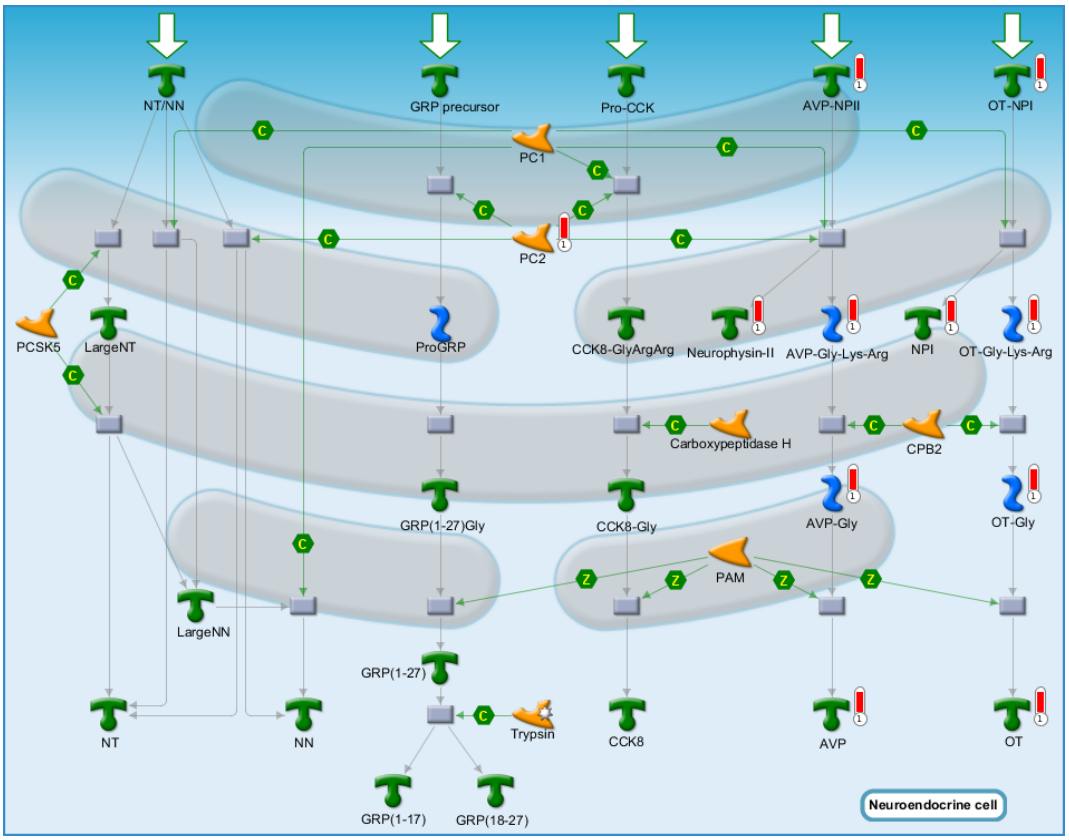

Figure 16. Protein folding and maturation. The top scored map based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Up-ward thermometers have red color and indicate up-regulated signals and down-ward (blue) ones indicate down-regulated expression levels of the genes.

To translate the role of deregulated genes in a biological context, and, in particular, to link them with the disease, we used MetaCore Tool and Network Analist to perform pathway and network analysis. Specifically, the designed pathway shows the most relevant genes that can be involved in a better prognosis of LTS patients (Figure 17).

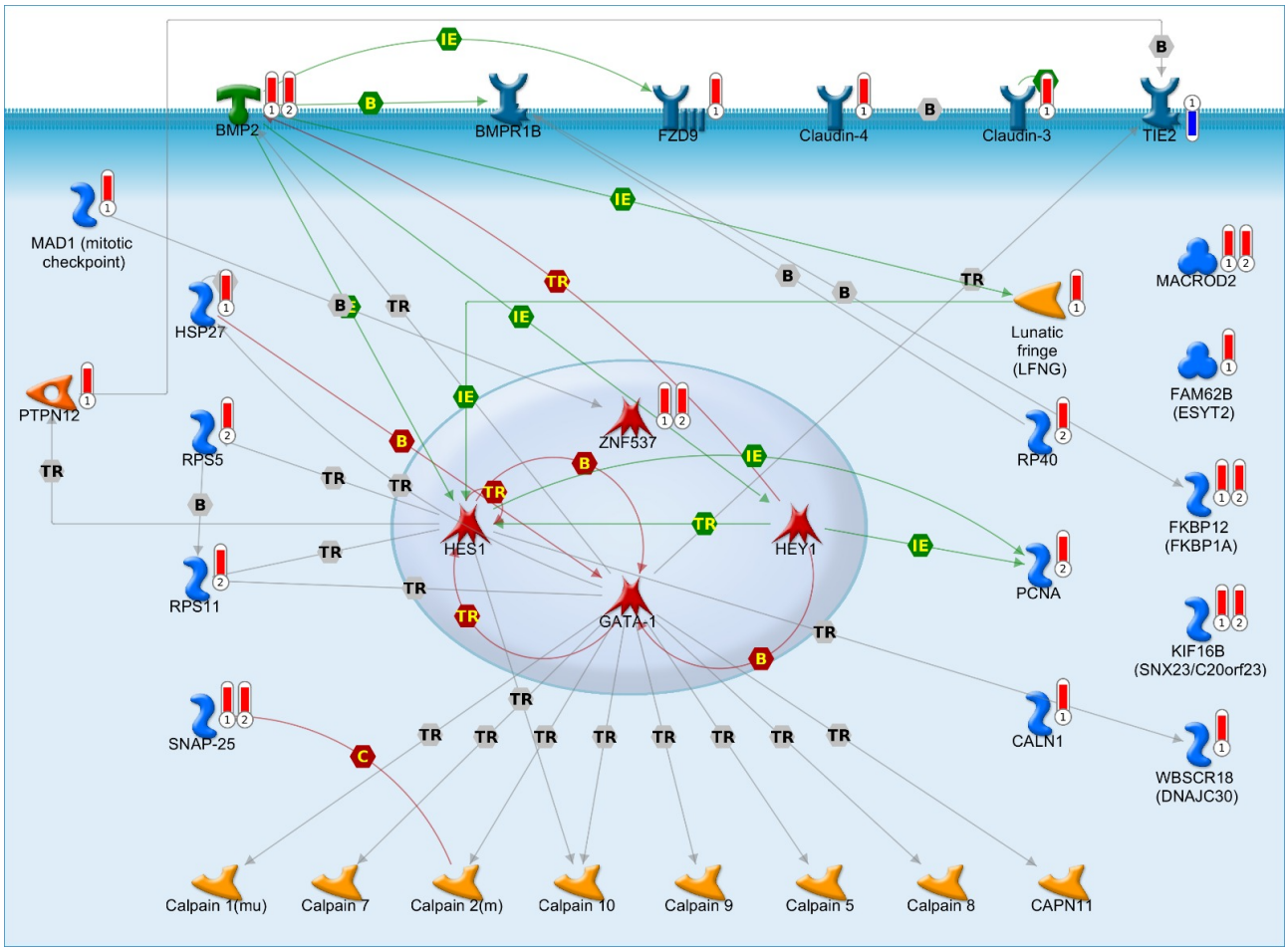

Figure 17. The proposed scheme is a general summary of CNVs detected by aCGH. The map was created using the MetaCore Pathway Map Creator tool (GeneGo). CNV values are presented on the map as 'thermometer-like' figures with loss and gain data of LTS represented as thermometer #1 and deregulated gene encompassing Chr19 and Chr20 as #2.

The PPI analysis was performed to investigate the relationship between deregulated genes and identify potential candidate hub genes in LTS samples. Network construction was performed using Network analyst tool (Figure 18).

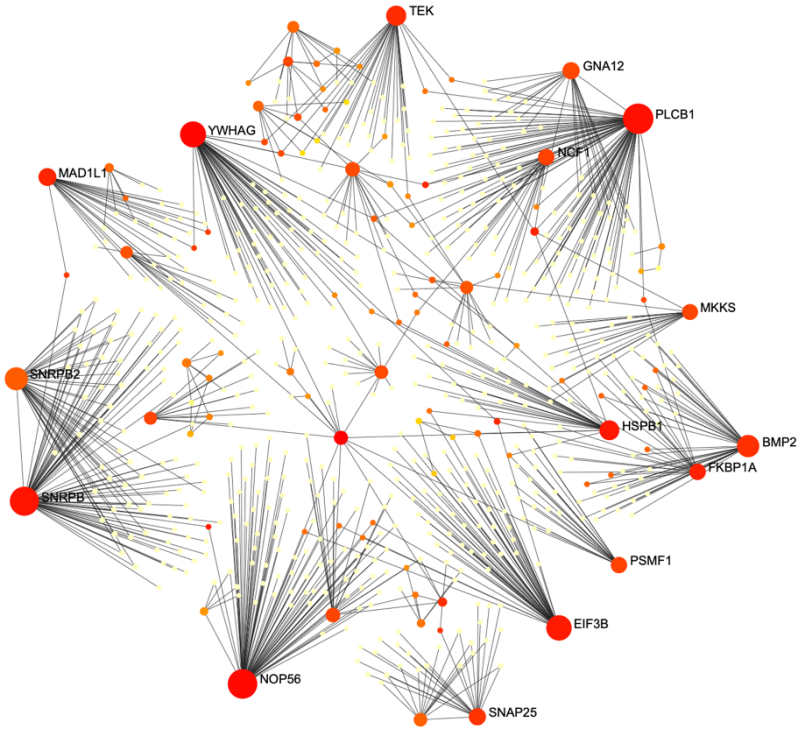

Figure 18. Extended PPI network of encoding products of gain and loss genes in LTS cohort, predicted using the STRING database and visualized with Network Analyst 3.0 software

The deeper investigation of gene ontology results, reported the enrichment of pathways related to cell activation, cell cycle process, proteolysis, calcium-dependent cysteine-type endopeptidase activity, negative regulation of apoptotic process, positive regulation of cell survival, revealing an altered pattern of Calpain-family genes. Array-CGH revealed an altered pattern of calpain family genes in 75% of LTS and 70% of STS. Calpain-1 and Calpain-2 genes (*CAPN1* and *CAPN2*) were found in gain in 60% of GBMs, in gain condition in STS and loss in LTS, whereas Calpain-3 (*CAPN3*), which exerts a proapoptotic action was found in loss in 50% of GBMs. Calpain Small Subunit 1 and 2 (*CAPNS1/2*), known as regulator subunits, are altered in 43% of GBMs, of which 70% were in gain condition, especially in LTS (Figure 19).

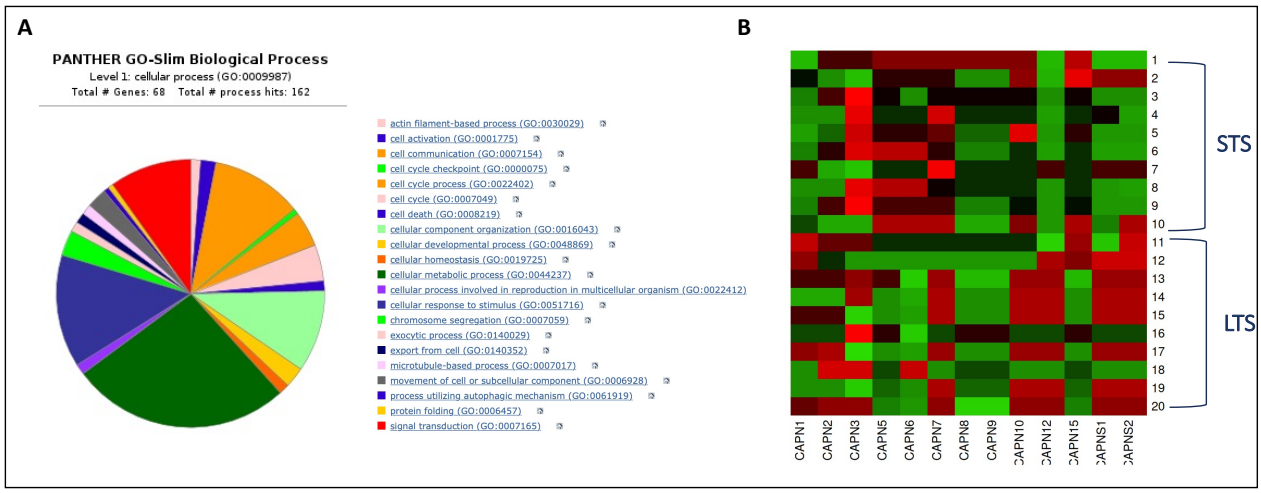

Figure 19. (A) Gene ontology analysis of n=250 genes in gain and n=12 genes in loss in LTS group; **(B).** Heatmap of chromosomic alterations related to calpain-family genes in STS and LTS. In green are displayed gain regions, while in red those in loss condition.

Cellular characterization

The demographic, clinical and molecular characteristics of patients, whose GBM biopsies were used to isolate GECs are listed in Table 6. The median age of patients at diagnosis was 60 years (IQR: 47-76) and 67% were males. Five patients suffered from a tumor located in the temporal lobe. The median value of *MGMT* promoter methylation was 14 (IQR: 6.5-31.5) and all GBMs were wildtype for *IDH*. Notably, a value of *MGMT* promoter methylation >9% is considered a favorable prognostic indicator, associated with a better response to treatment[287].

Table 6. Clinical and molecular data of GBM patients enrolled for GEC isolation

	Age	Sex	Tumor location	KPS	IDH	MGMT	MIB-1
Poli 09	81	F	FP left	60	wt	4%	15%
Poli 182	41	F	T left	70	wt	49%	55%
Poli 183	41	M	T right	80	wt	14%	40%
Poli 187	45	M	F right	100	wt	37%	20%
Poli 208	60	M	P right	80	wt	14%	30%
Poli 210	52	M	P left	70	wt	15%	55%
Poli 214	66	M	T left	80	wt	60%	65%
Poli 231	82	F	T left	70	wt	3%	27%
Poli 231	80	M	T right	80	wt	2%	40%

EC and phenotypic description

After tumor processing, GECs expanded in EndoPM, grown as a typical cobblestone monolayer (Figure 20A), typical of endothelial cells. Compared to LGG- and MNG-ECs, GECs showed a higher proliferation rate. Indeed, the growth curve of 18 days shows that GEC, LGG- and MNG-ECs maintained the same proliferation rate in the first 96h, after which, GECs started growing with an exponential rate, whereas LGG-ECs and MNG-ECs reached a plateau stage (Figure 20B).

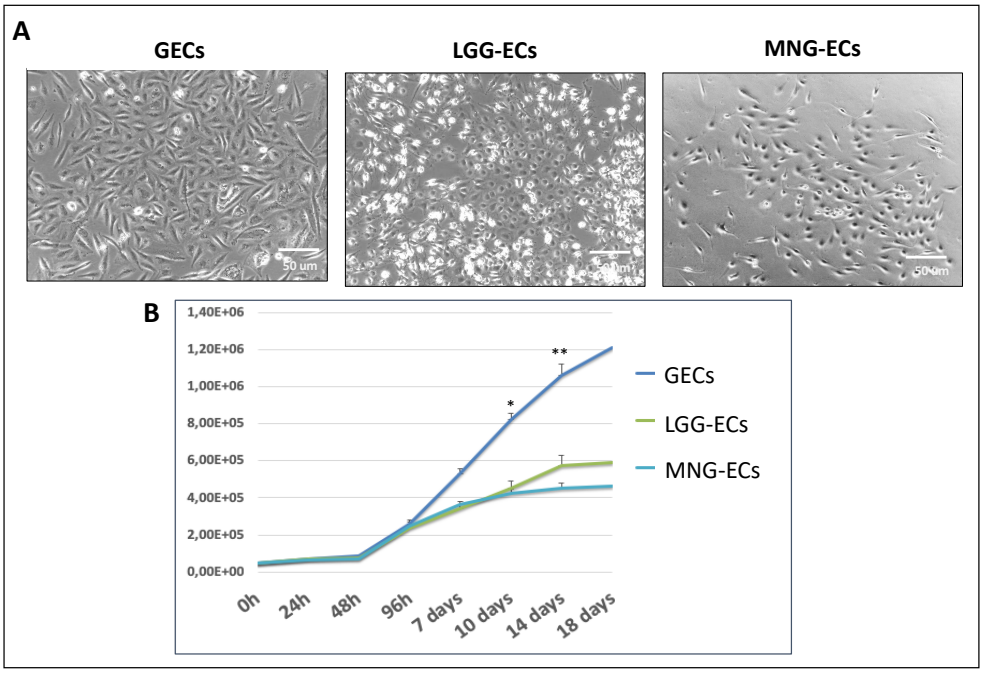

Figure 20. (A) Isolation and expansion of primary GECs, LGG- and MNG-ECs. As shown, ECs form a "cobblestone" monolayer. Scale bar 50 um; **(B)** Estimation of proliferation rate of GECs, LGG-ECs, MNG-ECs for 18 days after seeding. Data are the means of ±SD of three independent experiments. *GECs vs LGG-ECs and MNG-ECs, *P < 0.05, **P < 0.01

Immunofluorescence analysis revealed that GECs show a higher positivity for VEGF and its receptors VEGFR-1/2, suggesting an overexpression of pro-angiogenic signaling axis (Figure 21). Similarly, GECs were able to form VWF fibers, whereas LGG- and MNG-ECs showed a slight positivity to VWF. On the contrary, a lower intensity in GECs, compared to LGG- and MNG-ECs, was observed for VE-Cadherin labelling. Notably, VEGF is the best characterized pro-angiogenic factor and its overexpression leads to the formation of a high density of fragile microvessels, with a disrupted structure and increased permeability. Likewise, VWF fibers are a peculiar feature of highly activated ECs and microvascular proliferation. Several reports have described a direct interaction between the staining intensity of VWF in ECs and the different grade of brain tumors. For VE-Cadherin, the

different trend may be explained as it is a tight junction protein essential for the integrity of the BBB. In GBM, the decreased expression of VE-Cadherin may contribute to the leakiness of tumor blood vessels. In addition, flow citometry reported that GECs were highly positive to CD105, a transmembrane glycoprotein expressed on activated vascular ECs. CD105 expression has been found on intratumoral vessels and its overexpression correlates positively with patient outcome. Moreover, a high percentage of GECs (about 70%), showed positivity to CD56, or NCAM, a cell-surface glycoprotein that regulates intercellular adhesion and cell migration. Previous studies evidenced a positive association between NCAM expression and tumor aggressiveness. A similar trend was observed for CD34, whose expression has been correlated with a higher density of blood vessels.

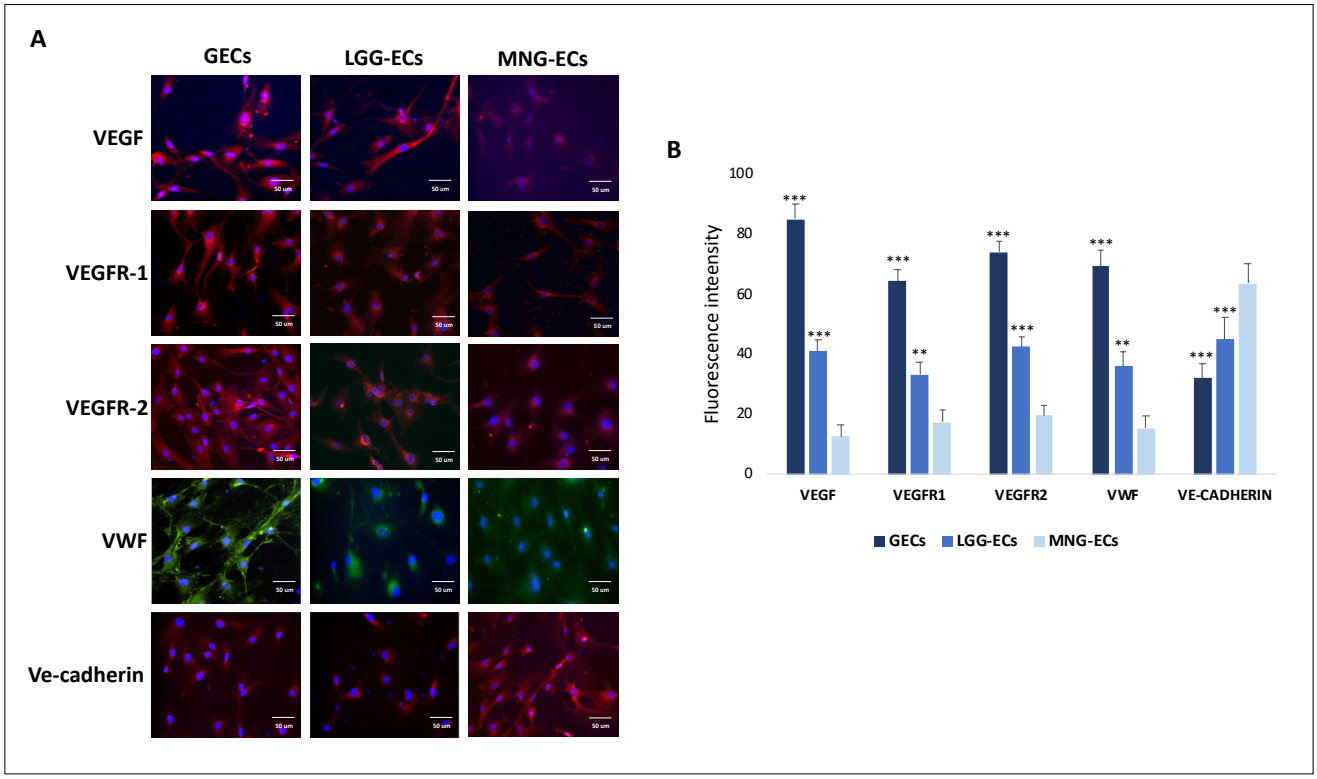

Figure 21. **(A)** Immunofluorescence staining of GECs, LGG- and MNG-ECs for VEGF (red, fluorescent labelling), VEGFR-1 (red fluorescent labelling), VEGFR-2 (red fluorescent labelling), VWF (green, fluorescent labelling), and VE-Cadherin (red fluorescent labelling). Scale bar 50 µm. (**B**) Estimation of fluorescence intensity after immunolabeling. Measurement performed by ImageJ (arbitrary unit).

Flow cytometric analysis reported that HGG-ECs were highly positive to CD105, a transmembrane glycoprotein expressed on activated vascular ECs (Figure 22). CD105 expression has been found on intratumoral vessels and its overexpression correlates positively with patient outcome[291]. Moreover, a high percentage of HGG-ECs showed positivity to CD56 (NCAM), a cell-surface glycoprotein that regulates intercellular adhesion and cell migration. Previous studies evidenced a positive association between NCAM expression and tumor aggressiveness [292].

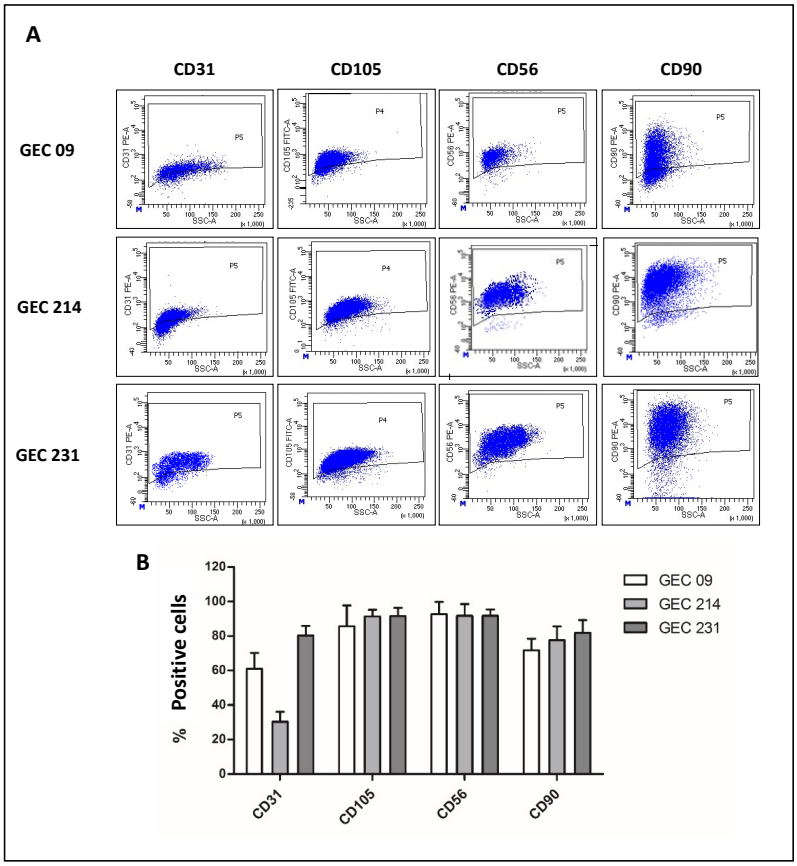

Figure 22. (A) Representative plots of flow cytometric analyzes conducted GECs, for CD105, CD31, CD56 and CD90, considered markers of endothelial activation and tumor malignancy. **(B)** Quantification of the percentages of positivity of the three cell lines to the investigated markers. The data represent the mean ± standard deviation of three independent analyses.

At functional level, the analysis of tube-like structures formation assay (Figure 23), revealed that GECs were able to form a complex and branched architecture, composed by cords, junctions and meshes, showing the ability to create an intense vasculature. On the contrary, LGG-ECs and MNG-ECs failed to form tube-like structures, lacking an organized network. Similarly, migration assay revealed a greater ability of GECs to repair a scratch, migrating into the gap (Fig. 4A-B). Notably,

the higher number of migrated cells does not depend on cell proliferation, since GEC growth rate was the same in the first 24h of culture.

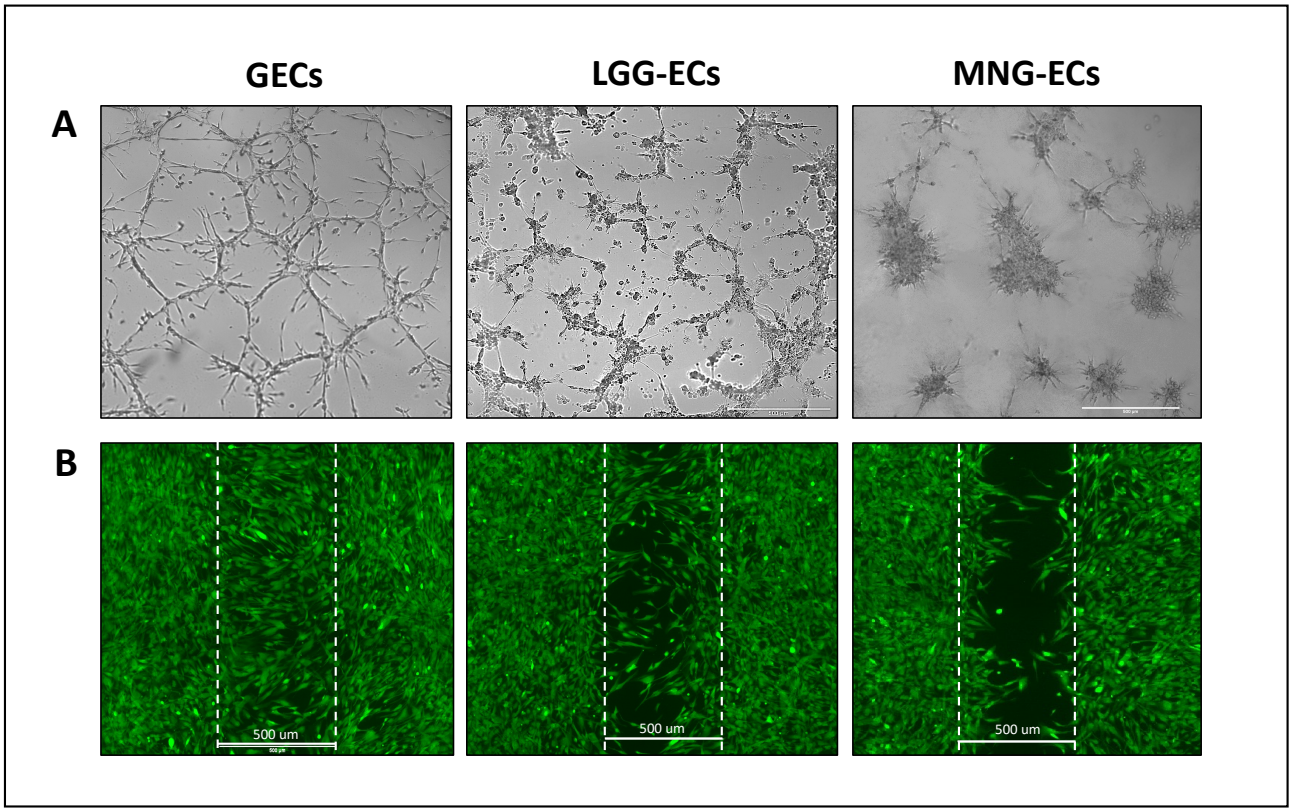

Figure 23. Representative images of **(A)** tube-like structure formation assay performed on GEC, LGG- and MNG-ECs seeded on Matrigel, and **(B)** migration assay performed on GECs, LGG- and MNG-ECs stained with CalceinAM for 24h. Scale bar, 500 µm.

Furthermore, vascular permeability assay demonstrated that GECs were not able to form a closed barrier, allowing the passage of a great quantity of FITC-dextran in a time-dependent manner (Figure 24). This increased permeability may reflect the impairment of BBB. Indeed, BBB leakage allows plasma proteins extravasation, leading to alteration of extracellular matrix and promotion of angiogenesis and it is an important parameter for drug therapy, because it might indicate lower drug availability in the tumor region[293].

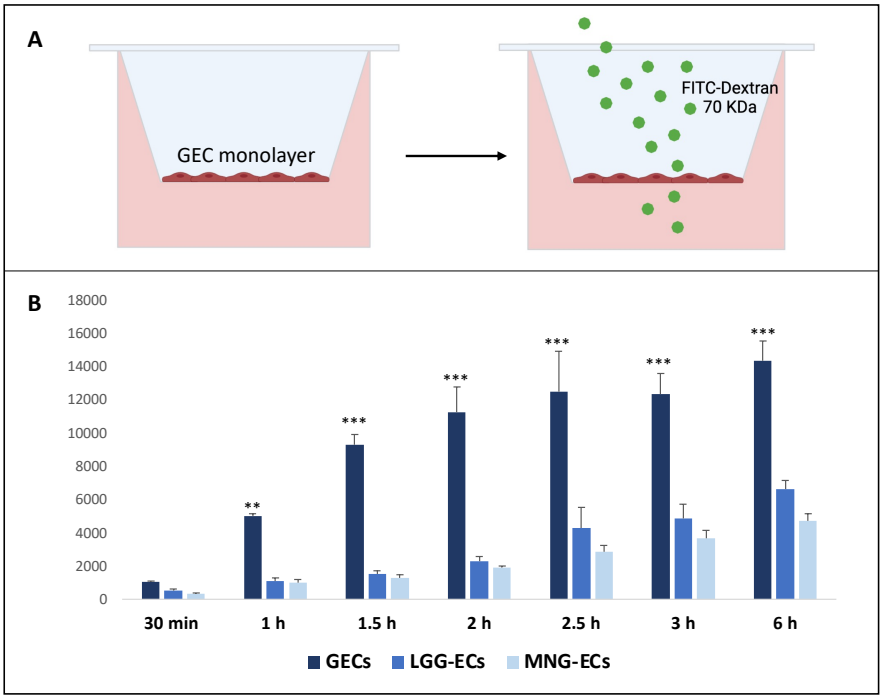

Figure 24. **(A)** Schematic representation of vascular permeability assay performed administrating FITC-70 kDa on GECs, LGG-ECs and MNG-ECs confluent monolayer cultured in Transwell insert. **(B)** Quantification of permeated FITC-dextran in a time-dependent trend across GEC, LGG-EC and MNG-EC monolayer. Data are the mean ± SD of three experiments in triplicate. *GECs vs MNG-ECs, *** P<0.001.

To investigate the transcriptomic profile of brain tumor ECs, qRT-PCR was performed GECs, LGG-ECs and MNG-ECs. Results were expressed as fold change relative to human brain microvascular endothelial cells, used as control. Data reported an upregulation of pro-angiogenic mediators, represented by VEGF and its receptors VEGFR1/VEGFR2, VWF and HIF-1α. Notably, also Ang-2, neural cell adhesion molecule (NCAM), fibroblastic growth factor-2 (FGF-2), and angiopoietin receptor Tie-2 mRNA levels followed a similar trend, showing a significant up-regulation in GECs, compared to LGG-ECs and MNG-ECs. On the contrary, mRNA expression of Ang-1 was significantly lower in GBM and LGG, whereas was quite similar in MNG (Figure 25A). The overproduction of VEGF and its receptors was also confirmed also by protein expression (Figure 25B).

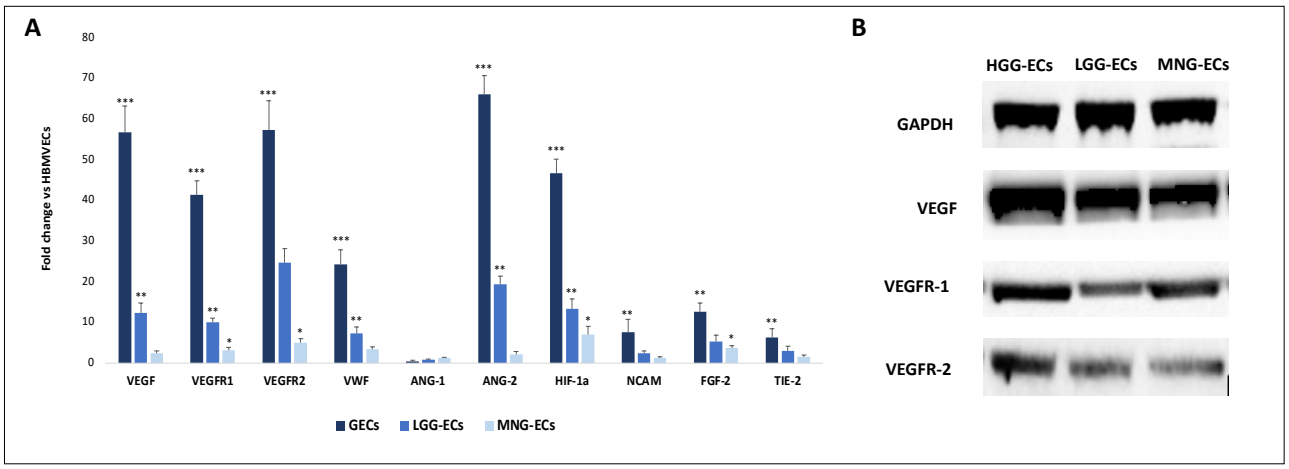

Figure 25. **(A)** Gene expression and **(B)** protein expression of pro-angiogenic factors in GECs, LGG- and MNG-ECs. In (A), fold change is expressed respect to hBMVEC mRNA levels. Statical significance was calculated against hBMVEC. *P<0.05, **P<P.01, ***P<0.001

The expression screening of calpains in GECs revealed a significant overexpression of *CAPN1-2-3*, and their small regulator subunits *CAPNS*1-2, in GECs compared to ECs from lower grade glioma and human brain microvascular ECs. An opposite trend was observed for *CAST* gene, encoding foe the endogenous inhibitor of calpains (Figure 26).

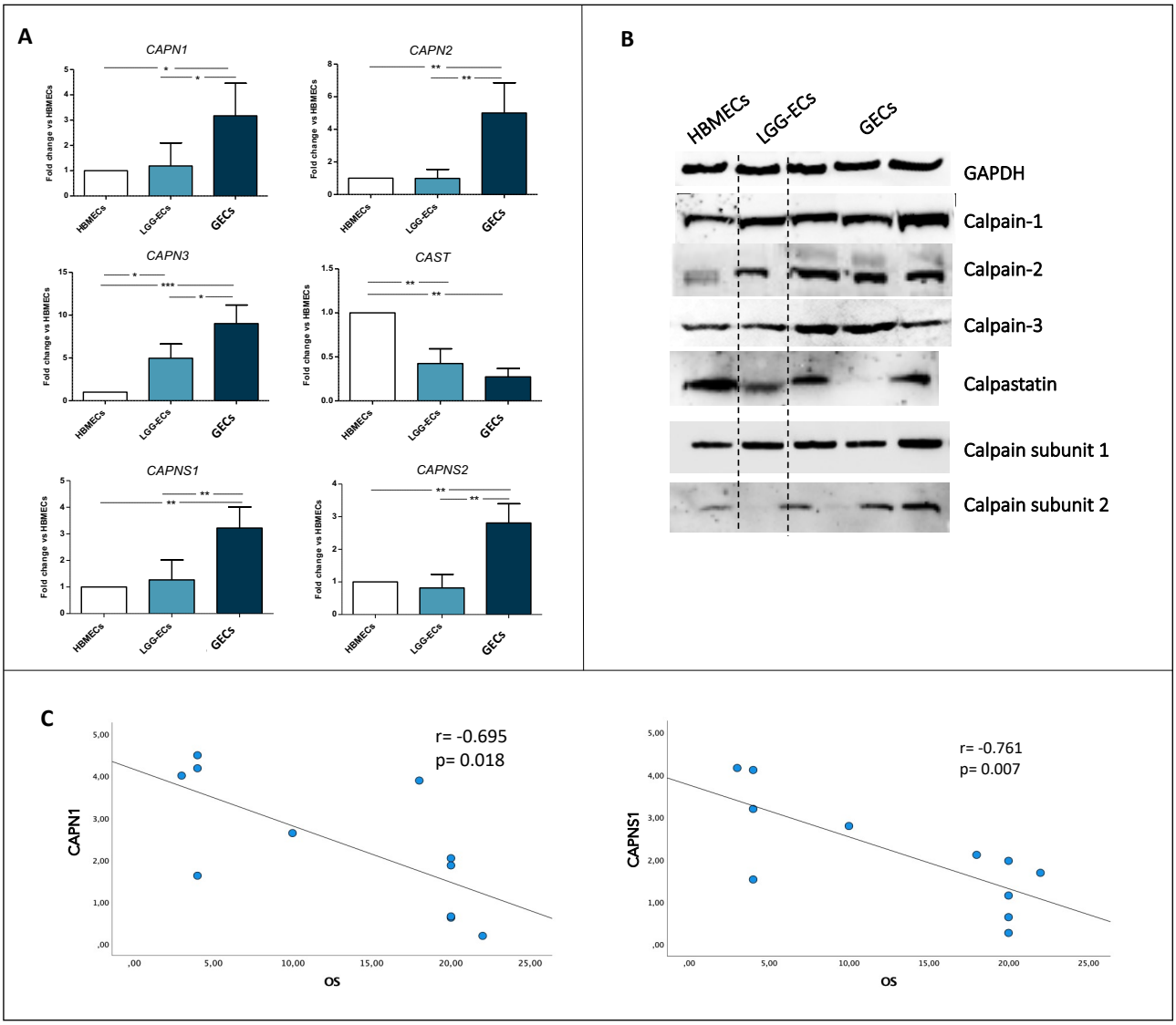

Figure 26. Gene **(A)** and protein **(B)** expression analysis of Calpain 1,2,3 (*CAPN1, CAPN2, CAPN3*), Calpastatin (*CAST*) and Calpain small subunit 1,2 (*CAPNS1, CAPNS2*) on human brain microvascular endothelial cells (HBMECs), low-grade glioma-derived endothelial cells (LGG-ECs) and glioblastoma endothelial cells (GECs). **(C)** Pearson correlation between patient overall survival (OS) and gene expression levels of *CAPN1* and *CAPNS1*. *P<0.05, **P<0.01, ***P<0.001

These results prompted the idea to test inhibitors of calpain signaling, in order to restore the physiological condition. To this aim, the effect of Calpain-1 and -2 inhibitors and Calpastatin, the natural inhibitor of both Calpain-1 and -2 was assessed on GEC viability, proliferation, apoptosis and functionality. Toxicity test was performed by MTT and Live and Dead assays with a dose escalation. These tests revealed a dose-response trend, especially for Calpain-1 inhibitor, whose maximum effect was observed at the concentration of 100 µM (Figure 27, A). Contrary, Live and Dead assay revealed a toxic effect at this concentration, with a high percentage of necrotic cells (Figure 27, B), suggesting

carrying out further experiments using the optimal concentration of 50 µM. The doses of the other compounds were chosen accordingly.

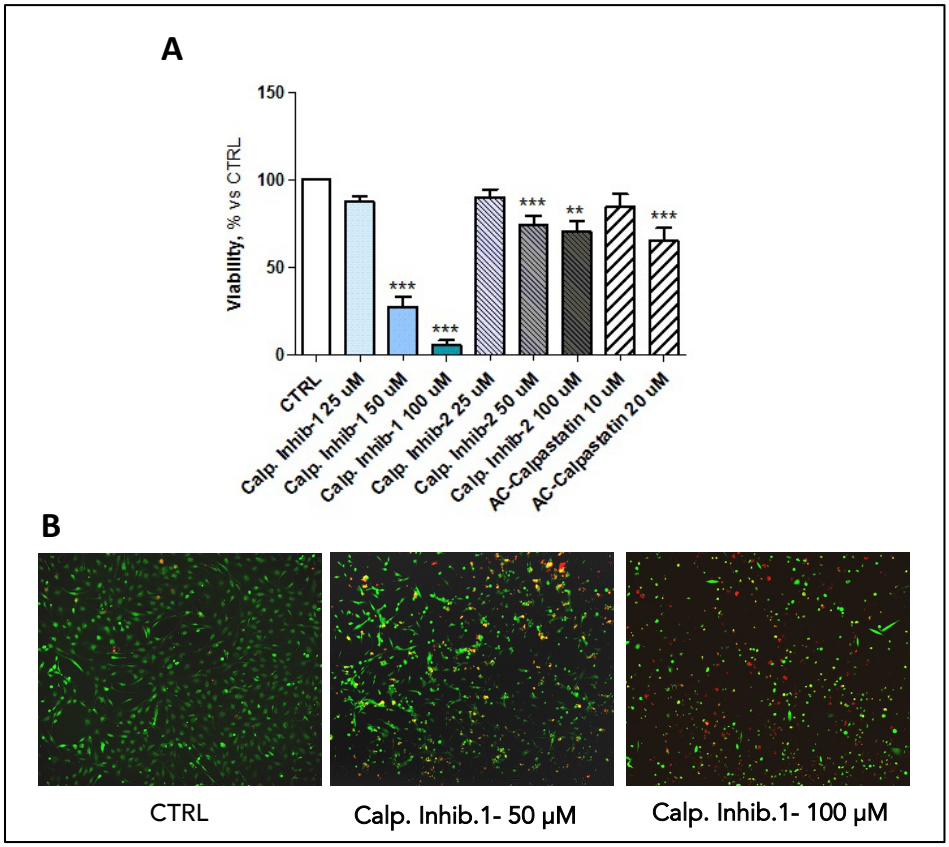

Figure 27. (A) MTT assay performed after the administration of different doses of Calpain Inhibitor 1-2 and Calpastatin. (B) Representative images of Live and Dead assay conducted to assess the toxicity of tested compounds. **P<0.01; ***P<0.001.

The viability test on GECs showed that Calpain Inhibitor 1 had the strongest effect in decreasing GEC viability and promoting cell death (Figure 28, A), as the viability reached the 20% and the mortality reached the 400% compared to untreated control. These data were confirmed by the statistically significant increased activity of Caspase-3 and -7, considered as effector caspases of apoptotic events (Figure 28, B). Interestingly, viability test also revealed a synergic activity of Calpain inhibitors and TMZ, suggesting the potential efficacy of a combined treatment.

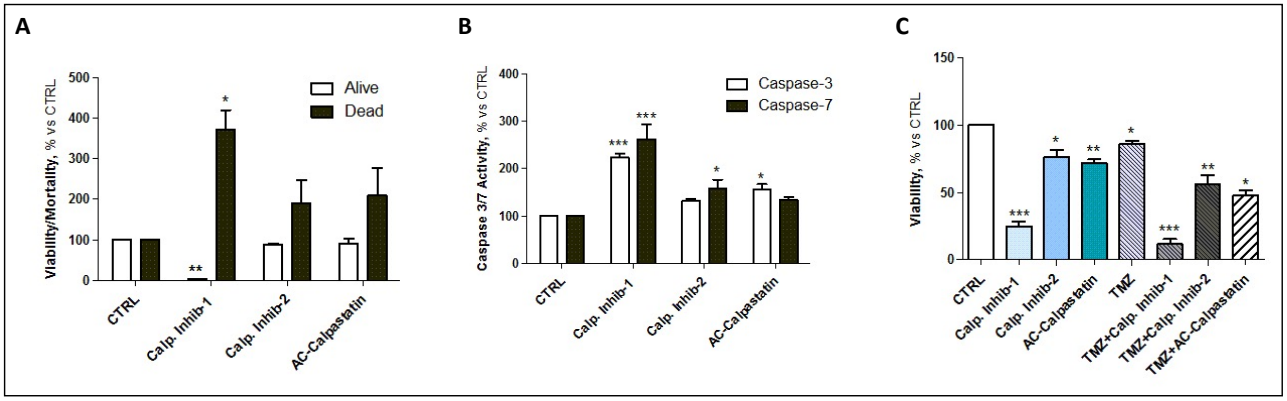

Figure 28. (A) Estimation of viable and dead GECs after treatment with Calpain inhibitors 1-2 and Calpastatin. (B) Caspase-3 and -7 activity assay performed after GEC treatment. **(C)** Viability test conducted by MTT on GECs treated with Calpain inhibitors 1-2 and Calpastatin, alone or in combination with TMZ. *P<0.05 **P<0.01; ***P<0.001.

Then, in order to test the effect of Calpain inhibitors on angiogenic potential, tube-like structure assay on primary GECs confirmed the inhibition of vascular network formation (Figure 29A), with a statistically significant reduction of total tube length (Figure 29B). Moreover, the same effect was observed in migration assay, as the administration of Calpain inhibitors resulted in a significant decrease of the number of cells migrated into the gap (Figure 29, C-D).

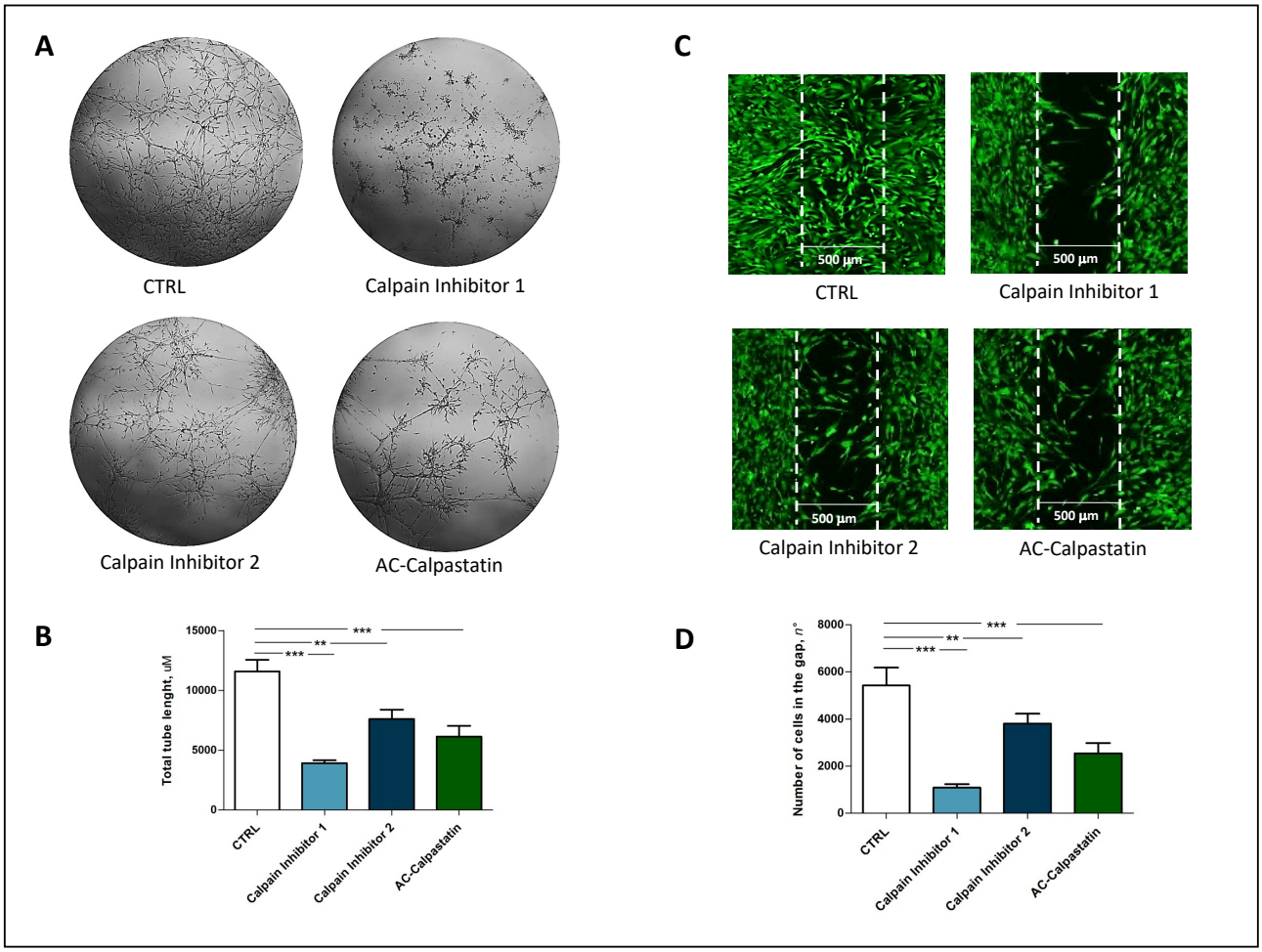

Figure 29. (A) Tube-like structure assay and **(C)** migration assay performed on GEC after treatment with Calpain Inhibitors. The estimation of treatment efficacy was performed by calculating the total tube length **(B)** and the number of cells migrated into the gap (D). *P<0.05 **P<0.01; ***P<0.001.

Finally, with the aim of investigating a potential molecular mechanism underlying these effects, a gene expression screening was performed, reporting again that Calpain inhibitor 1 is particularly effective in inhibiting proliferative signalling by the downregulation of proliferative signalling as MAPK, as RAF/RAS/MERK/ERK, proangiogenic pathways mediated by VEGF and its receptors and anti-apoptotic regulators as Bcl-2, and by upregulating proapoptotic mediators, as Caspases and Bax-family (Figure 30).

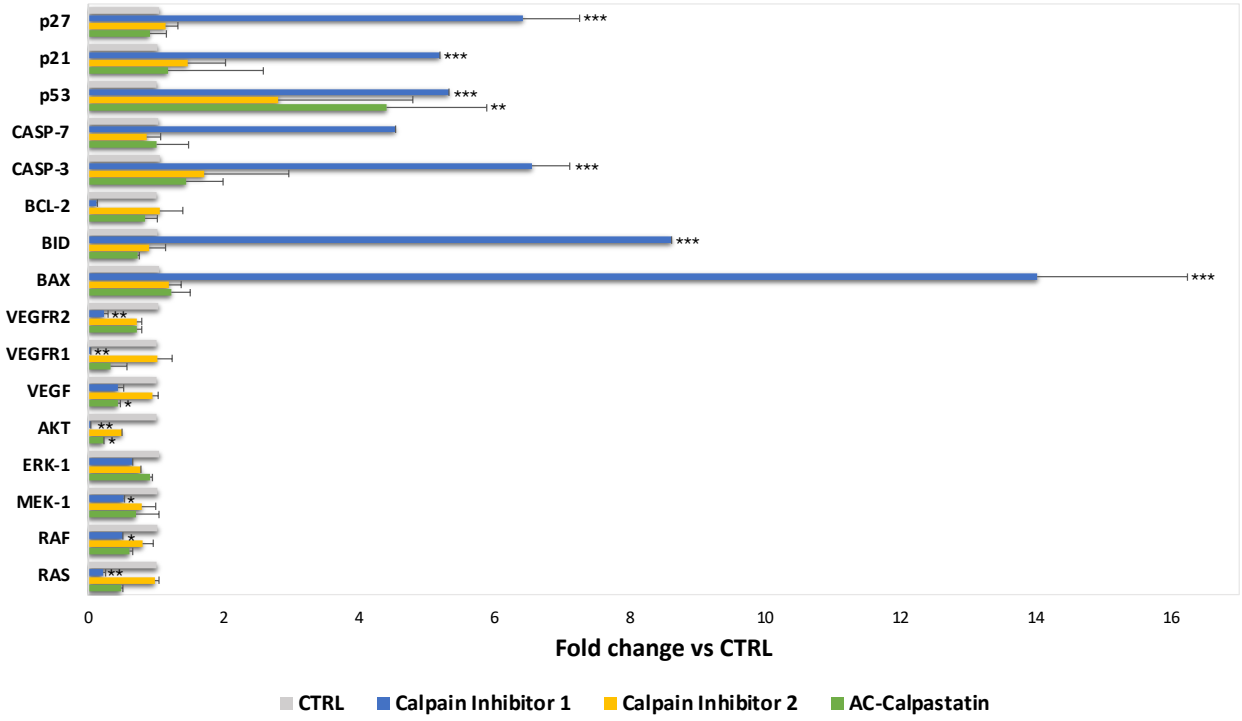

Figure 30. Gene expression analysis conducted on GECs after treatment with Calpain Inhibitors by qRT-PCR. *P<0.05 **P<0.01; ***P<0.001.

4. DISCUSSION

Glioblastoma is the most aggressive primary brain tumor in adults, resulting in the assignment of the highest grade (grade IV) in the WHO classification. With an incidence of 16% of all primary brain tumors, it is the most common brain malignancy, and it is almost always lethal[294]. Despite the aggressive therapeutic regimens, consisting in gross total resection, when possible, followed by radiotherapy with concomitant and adjuvant therapies with TMZ, most patients suffer for a recurrence in less than one year. Due to the presence of different cell populations, with different response to signals from the microenvironment, GBM are very heterogeneous, presenting various genetic changes. In addition to nucleotide sequence variants as single nucleotide polymorphisms (SNPs), genomic structural variants include many different types of chromosomal rearrangement encompassing both inversions and balanced translocations as well as genomic imbalances commonly referred to copy number variation (CNVs). CNVs are increasingly recognized as significant disease susceptibility markers in many complex disorders, including GBM. Many studies have catalogued CNVs by computational methodologies[286], identifying frequently amplified genes such as *EGFR*, *MET*, *PDGFRA*, *MDM2*, *PIK3CA*, and *CDK6* and deleted genes including *CDKN2A/B*, *PTEN*, and *RB1*. Nowadays, several molecular markers are commonly tested as part of the routine clinical interrogation of GBM patients including: *MGMT* promoter methylation, *IDH* mutation, loss of heterozygosity (LOH) of chromosomes 1p and 19q, loss of heterozygosity 10q, *EGFR* amplification, or mutation in *EGFRvIII*[295]. In particular, studies about the association between genetic mutations and survival show that GBMs with *IDH1/2* mutations and *MGMT* promoter methylation are more responsive to surgical resection and chemotherapy and have better prognosis[296]. High-throughput genomic technologies have been widely applied to facilitate to understand the mechanisms involved in the genesis of disease processes. For example, comparative genomic hybridization (array-CGH) has successfully contributed to improve the detection rate of genomic unbalances and alterations in cancer and to correlate recurrent CNVs to cancer pathomechanisms and prognosis[297]. Similarly, DNA microarray is recognized as very important and powerful tool for identifying the diversity of functional genes and identifying in-depth characterization of changes in gene expression. Despite many efforts have been made to profile GBMs, the genetic characterization of GBMs is still failing to be translated to clinical practice, suggesting that other discovery paradigms should be considered. In an our recently published study, we conducted array-CGH on 10 GBM biopsies and the matched glioblastoma stem cells (GSCs), which are self-renewing, pluripotent and highly proliferative and can hierarchically drive onset, proliferation and tumor recurrence[298]. Interestingly, our data reported a highly intra- and intertumoral heterogeneity, with numerous amplificated and deleted regions involved in cancer-related pathways[286]. In both GBM and matched GSCs, we identified recurrent

CNVs, as Chr 7 polysomy, Chr 10 monosomy, and Chr 9p21deletions, which are typical features of primary GBM. These observations suggest a condition of strong genomic instability both in GBM and GSCs but also showed the robust similarity between GBM mass and GSCs, with a peculiar signature associated with survival. Indeed, the K-means algorithm has identified an impairment of pathways related to the development and progression of cancer, such as angiogenesis and immune system regulation. Furthermore, our data confirmed the preservation of the genomic landscape from tumor tissue to GSCs, supporting the relevance of this cellular model to test *in vitro* new target therapies for GBM[286]. The extensive molecular testing reported a great number of genetic and transcriptional differences associated with patient's OS. Indeed, although GBMs from short- and long-term survivors (STS and LTS) are histologically the same, their biological and molecular characteristics are remarkably different [299,300], suggesting that factors that contribute to patients' longevity are important for precise diagnosis and correct clinical management of the disease. Notably, due to short life expectancy, GBM-LTS, defined as patients who live longer than two years post-diagnosis, comprise <15% of all cases, thus comparative studies on molecular differences between LTS and STS are challenging and promising, whit a potential great impact in clinical practice. The discovery of novel potential prognostic and predictive biomarkers, by the "omics" approach, will allow to optimize patient's management, as early prediction of those patients who are likely to be STS or LTS, and a full understanding of their clinical course can thus assist clinicians in providing tailored treatments and support patients and their families. However, beyond prognosis, what will really impact clinical management of GBM is if genomic analyses can lead to customized treatment and ultimately improved survival, following the concept of precision medicine. To the aim of investigating the specific signature which characterize "sensitive" and "resistant" GBMs and identifying novel potential prognostic and targetable genetic biomarkers, we performed array-CGH on two cohort of GBM patients, LTS and STS.

The investigation of GBM chromosomic alterations revealed a high number of CNVs across chromosomes 1 to 22. Both LTS and STS at similar frequencies, showed the polysomy of chromosome 7 and the monosomy of chromosome 10, which are typical features of primary GBM, essential for gliomagenesis and usually conserved in the whole GBM mass[301,302,303]. The polysomy of chromosome 7 has been found to be strongly associated with *EGFR* amplification. *EGFR* amplification has been identified as a genetic hallmark of primary GBM and occurs in approximately 40–60% of primary. Generally, 70–90% primary GBM with *EGFR* overexpression present also *EGFR* amplification[304]. Due to its pro-oncogenic effects, it is not surprising that chromosomic amplification and increased *EGFR* expression is associated with the malignancy of glioma[305]. The polysomy of chr7 may also involve the *MET* locus, encoding a member of the receptor tyrosine kinase

family and the product of the proto-oncogene *MET*. The activity of *MET* regulates many physiological processes including proliferation, scattering, morphogenesis and survival. Recruitment of downstream effectors by *MET* leads to the activation of several signaling cascades including the RAS-ERK, PI3 kinase-AKT, or PLCgamma-PKC. The RAS-ERK activation is associated with the morphogenetic effects while PI3K/AKT coordinates prosurvival effects. For these reasons it is possible to hypothesize its overexpression and involvement in GBM malignancy. In parallel, the deletion of 10q is commonly reported in GBM[306,307], and may be a mechanism for the inactivation of the tumor suppressor phosphatase and tensin homolog (*PTEN*), associated with GBM[308]. The LOH resulting from 10q deletion may result in haploinsufficient expression, or exposure of inactivating PTEN mutations within the retained allele. However, this large-scale loss may indicate that additional tumor suppressor genes across chromosome 10 may also play a role in retaining cell-cycle equilibrium. Two putative tumor suppressor genes potentially impacted by chromosome 10 LOH are *ANXA7* and *PFKFB3*. *ANXA7* acts as a positive regulator of *EGFR*, and haploinsufficiency of *ANXA7* results in stabilized *EGFR* protein[309]. Furthermore, Fleischer and colleagues showed that LOH of the *PFKFB3* gene results in the reduction of *UB12K4* expression, a growth-inhibiting splice variant of *PFKFB3*, concluding that this shift in *UB12K4* expression tends toward more aggressive tumor growth[310].

The further exploration of recurrent CNVs was focused on genes mapping on Chr9, Chr19 and Chr20, enriched exclusively in LTS, with the aim to define their implication on better prognosis. The protein-protein interaction analysis on deleted genes mapping on Chr9p21.3 highlighted the enrichment of several pathways and biological process involved in tumor progression as angiogenesis, protein phosphorylation and negative regulation of apoptotic process. Noteworthy, this analysis highlighted *TEK* gene as a hub. As described in the introduction section, *TEK* encodes for a tyrosine kinase receptor with a unique extracellular region that contains two immunoglobulin-like domains, three epidermal growth factor (EGF)-like domains and three fibronectin type III repeats. Tek act as cell-surface receptor for ANGPT1, ANGPT2 and ANGPT4 and regulates angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading, reorganization of the actin cytoskeleton, but also maintenance of vascular quiescence. Considerable evidence suggests that Tek signaling may regulate the crosstalk between glioma cells and vascular endothelial cells of the tumor microenvironment[311]. In migrating endothelial cells that lack cell-cell adhesions, ANGT1 recruits Tek to contacts with the extracellular matrix, leading to the formation of focal adhesion complexes, activation of PTK2/FAK and of the downstream kinases MAPK1/ERK2 and MAPK3/ERK1, and ultimately to the stimulation of sprouting angiogenesis. Signaling is modulated by Ang-2 that has lower affinity for Tek but inhibits Ang1-mediated signaling by competing for the same binding site,

thus inducing the GBM typical aberrant angiogenesis. Moreover, Ang-2 has also been shown to act as a chemoattractant for Tek-expressing monocytes/macrophages (TEMs), a subset of pro-angiogenic cells appearing in response to anti-angiogenesis therapy[312] and associated with an invasive phenotype in gliomas[313,314]. Since the overexpression of Ang-2 functions as a chemoattractant for TEMs that drive tumor vessel formation and metastasis, targeting the Ang-2/Tek signaling pathway has been shown to inhibit tumor growth and invasion in GBM[315]. In GBM, selective Tek inhibitors such as Rebastinib, was able to successfully inhibit tumor growth and prolong survival by targeting an N-terminal truncated form of p75 (ΔNp73), which transcriptionally regulates both Tie2 and Ang-2[192]. Similarly, rebastinib has been demonstrated to inhibit the growth, invasion, and metastasis of breast cancer cells by an "allosteric switch control" mechanism[316]. Overall, the discover of *Tek* gene deletion in our LTS group may underlie the downregulation or suppression of Tek-mediated signalling pathways, with a consequent inhibition of angiogenesis, proliferation and cell survival that may explain the better prognosis of LTS.

Regarding the simultaneous gain of chromosomes 19 and 20 in LTS, the gene enrichment analysis reported the involvement of several pathway among theme Thromboxane A2 signaling pathway and Signal transduction/PKA signaling. Thromboxane A2 (TXA2) is produced by activated platelets during hemostasis and has prothrombotic properties: it stimulates activation of new platelets as well as increases platelet aggregation. Its signalling is mediated by TXA2 receptors (TP) that are G-protein–coupled receptors expressed on many cell types. TXA2 acting through TP is a potent modulator of vascular responses[317]. Both TXA2 levels and TP expression are increased in multiple disease states, including ischemia. TP exists as 2 isoforms in humans, TPα22 and TPβ, which arise from alternate splicing of a single transcript with TPα[318]. It has been reported TP stimulation is a modulating factor for VEGF-induced migration and angiogenesis. In particular, TP stimulation abrogates the angiogenic and chemotactic properties of VEGF on EC, with a mechanism involving the suppression of PI3K/NO and FAK/Src activation by TPβ. These pathways culminate in reduced focal adhesion formation on αvβ3, which inhibits VEGF-induced angiogenesis and migration[319]. Accordingly, the potential overexpression of TXA2, the ligand for TP in our LTS cohort, may have the effect of inhibiting the downstream cascade, thus inhibiting VEGF-induced angiogenesis and mitigating GBM malignancy. In parallel, Cyclic AMP (cAMP) and cAMP-dependent protein kinases, also named protein kinases A (PKAs) play various roles in tumor cell proliferation[320]. The cAMP-mediated pathway is linked to Ras protein activation through multiple steps: PKA activation inhibits cell proliferation, with negative feedback through the phosphorylation of phosphodiesterases that ultimately lowers cAMP concentration[321]. Low cAMP levels are detected at mitosis, while higher levels are present in G1 and early S; on the other hand, PKA activation influences the cell cycle,

because PKA phosphorylates the macromolecular complexes responsible for the destruction of mitotic cyclins and separation of the sister chromatids at anaphase–metaphase transition[322]. Also, it has been demonstrated that cAMP inhibits p44/42 MAPK activity and proliferation in PTEN-depleted human glioblastoma cells *in vitro* through PKA activation[323]. From these premises, the gain of PKA signaling in LTS may explain the lower progression rate of GBM and the better response to treatment.
Furthermore, the PPI analysis on genes mapping on chromosome 19 highlighted the *Uba52*, *RPS* and *RPL* genes as hubs. *Uba52* encodes for Ubiquitin, which regulates the function of a large number of proteins in various physiological and/or pathological conditions. A gene expression analysis on GBMs revealed that *Uba52* belongs to the group of downregulated genes associated with a worst prognosis, so that its overexpression was associated with a longer overall survival, according with our data[289]. RPS and RPL, encoding ribosomal proteins that are components of the 40S and 60S subunits respectively, were found significantly mutated in different cancer types. RPSA, RPS5, RPS20, RPL5, RPL11 and RPL23A were identified as six interesting cancer driver candidates and patients with low RPL5 expression displayed worse OS in GBM[290], confirming our association between chromosomic gain and increased OS in LTS group.
The same analysis was conducted on genes mapping on chromosome 20, which identified *SRC* and *PCNA* as hubs. *SRC* is an oncogene directly involved in the development of cancer, *PCNA* is involved in cell survival, and possibly in pathways of energy metabolism, such as glycolysis.
SRC is a central downstream intermediate of many RTKs, which triggers the phosphorylation of many substrates, therefore, promoting the regulation of a wide range of different pathways involved in cell survival, adhesion, proliferation, motility, and angiogenesis, sustaining tumor growth[324]. In this case, its identification from PPI analysis of gain genes in LTS group may be not associated with prognosis, but it can be considered as an upregulated pathway driving GBM malignancy, since it should not be neglected even when analyzing patients with a better prognosis. The same concept applies to *PCNA,* an important nuclear DNA replication and repair protein, often referred to as the "ring-master of the genome", which regulates a myriad of proteins via the interdomain connector loop and whose upregulation has been associated with shorted OS[325].
The comparison of altered gene lists from LTS and STS led to the identification of 250 genes in gain condition and 12 gene in loss condition exclusively in LTS. The debate of the role of each gene on GBM aggressiveness would require an entire dedicated book chapter, therefore we here discuss the most relevant and better characterized.
Among the genes in gain in LTS group, *Fzd9, Bmp2, Claudin-3 and Claudin-4* has been associated with antitumoral effect, differentiation and apoptosis, restraint of tumor growth[326,327]. *Fzd9* gene was found to be the most frequently hypermethylated gene in the 80% of a GBM series was *Fzd9*[328]. This

gene is one of the Frizzled (FZD) family receptors that transduces canonical WNT signals to the β-catenin signaling cascade for cell-fate determination. Hypermethylation of *Fzd9* was reported recently in more than 70% of patients with acute myeloid leukemia and was significantly associated with a decreased 12-month survival rate[329]. These data may suggest the correlation of the genomic gain with longer survival.

Bone Morphogenetic Protein (BMP) is a member of the transforming growth factor-β (TGF-β) family. BMP ligands and receptors mediate multiple processes throughout neural development, including the survival, proliferation, morphogenesis, lineage commitment, differentiation and apoptosis of neural stem cells in the CNS[330,331]. Among them, BMP2 plays an important role in the development of many kinds of tumors, and it can induce differentiation and apoptosis of tumor cells[332,333]. This mechanism is mainly attributed to its BMPR1B receptor dependence and its own concentration dependence. If both are highly expressed, the prognosis of the patient is good, as potentially occurs in our LTS cohort. If either is decreased, the prognosis of patient will be worse.

Claudins are transmembrane proteins and important components of tight junctions (TJs), which are central for the regulation of paracellular permeability and the maintenance of epithelial cell polarity. Epithelial tumor cells lose TJ function, leading to the loss of cell polarity and impairment of epithelial integrity during tumorigenesis[334,335]. Accordingly, loss of claudin expression was assumed to contribute to tumor progression in association with the loss of cell adhesion and promotion of cell migration and angiogenesis[334,336]. This would be the case of our LTS cohort in which a potential overexpression of *Claudin-3 and Claudin-4* may stabilize TJs, thus inhibiting tumor invasiveness and progression. However, a number of studies have shown that also increased expression of claudins may promote tumor progression through its positive effect on cell migration, invasion and metastasis, especially in metastatic tumors as breast and lung cancer and melanoma[337].

As described before, *Tie2/Tek* is associated with increased malignancy and glioma progression from lower to higher grade. In the current study, we reported a loss of *Tie2* in LTS samples. Impairment of *Tie2* could have significant effect in other signaling pathway that mediate its pivotal role in vascular and hematopoietic development. The overexpression of *Caln1* has been associated with better OS in GMB patients[338]. The *Ism1* encodes for Isthimin 1 that acts as an angiogenesis inhibitor. It has been demonstrated that isthmin can inhibit VEGF-stimulated endothelial cell proliferation and significantly suppress glioma growth[339]; furthermore, human protein atlas reports the correlation between high expression and better OS. The Gene Ontology analysis performed on 250 gain genes, reported 24 clusters, of which the most enriched is that containing *GTF2I* gene. *GTF2I* encodes a phosphoprotein that interacts with the basal transcription machinery by coordinating the formation of a multiprotein complex at the *c-FOS* promoter and linking specific signal responsive activator

complexes. *GTF2I* mutations have been reported to be recurrent in thymic epithelial tumors and are rare in other malignancies[340]. *GTF2I* alterations were present in 39% cases of The Cancer Genome Atlas (TCGA) cohort. These mutations are generally detected in type A and B thymomas which are relatively less aggressive, and hence plausibly associated with a better survival. The most common mutation augments the *GTF2I* expression post-transcriptionally by preventing its degradation. This eventually culminates in cell proliferation which is activated by binding of *GTF2I* to the *FOS* promoter. The frequency of recurrent *GTF2I* mutations and its expression appears to be very high specially in the case of indolent tumors, as it represents a marker of favorable prognosis, accordingly to our results.

The enrichment analysis performed on pathways maps reported the "Protein folding and maturation" as the top statistically significant map. Protein folding is the process by which the newly synthesized protein molecule folds into its unique, 3D structure in order to acquire its functionally active, native state. Protein misfolding occurs during different biochemical processes and may lead to the development of diseases such as cancer, which is characterized by genetic instability. Tumour cells undergo both oncogenic and environmental stresses during cancer progression, as they need to meet increased demands for protein and lipid production needed for rapid proliferation and must adapt to growth in an oxygen- and nutrient-deprived environment. To overcome such challenges, cancer cells exploit intrinsic adaptive mechanisms such as the unfolded protein response (UPR). The UPR is a pro-survival mechanism triggered by the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER). Upon induction of ER stress, tumor cell work to re-establish ER homoeostasis, thus aiding cell survival, proliferation, migration, angiogenesis and chemoresistance[341]. Over the past decade, evidence has emerged supporting a role for the UPR in the establishment and progression of several cancers, including breast cancer, prostate cancer and GBM. In our GBM cohort, the upregulation of protein folding mechanism mediators may overcome UPR, stabilizing protein functionality and thus inhibiting the downstream ER stress-induced cascade.

Taken together, our analysis on chromosomic unbalances exclusive of LTS group showed the upregulation of oncosuppressor pathways, regulating cell proliferation, survival, angiogenesis and response to treatment. The CNV investigation together with the gene ontology analysis led to the identification of several biological processes, among which that mediated by Calpain family appeared to be potentially interesting at therapeutic level. The calpains are a conserved family of cysteine proteinases that catalyze the controlled proteolysis of many specific substrates. Calpain activity is implicated in several fundamental physiological processes, including cytoskeletal remodeling, cellular signalling, apoptosis and cell survival. Due to their implication in numerous oncopromoter

pathways, the second phase of this project was focused on the investigation of calpain involvement in GBM malignancy, and their potential to be targeted by anti-cancer compounds.

In particular, their potential role in mediating angiogenic process, suggested the benefit to examine their role in GECs, in term of expression and functionality.

To accomplish this task, the project starts with the isolation and characterization of primary ECs from glioma biopsies, from MNGs and LGGs to GBMs, in order to evaluate ECs functionality, pro-angiogenic factor expression, and their contribution on drug resistance/sensitivity of GBM tumor cells[170]. Starting with the phenotypic characterization, our results reported a significant increased positivity for endothelial marker in GECs compared to LGG-ECs and MNG-ECs[342]. One of the most critical pro-angiogenic factor found was Von Willebrand Factor (VWF), a multimeric plasma glycoprotein that mediates platelet adhesion to both the subendothelial matrix and endothelial surfaces and acts as a circulating carrier for coagulation factor VIII[343]. VWF plays an essential role in hemostasis: its dysfunction or deficiency causes von Willebrand disease (VWD), the most common congenital bleeding disorder in humans[344] and increased levels of VWF are involved in acute coronary thrombosis and are a clinical marker of risk associated with atherosclerosis[345]. Endothelial VWF is also involved in the regulation of inflammation by modulating leukocyte adhesion through direct and indirect mechanisms[342,346]. VWF is synthesized by ECs and stored within Weibel-Palade bodies, from which it can be rapidly released into circulation upon EC activation[347], which in turn, leads to platelet recruitment, aggregation and activation. These aggregates may protect cancer cells from immune cells or may directly influence tumor extravasation of activating-ECs factors. At the site of injury indeed, platelets degranulate, releasing pro-angiogenic factors such as VWF, VEGF, FGF, PDGF, thus acting as functional circulating carriers and feeding an autocrine and paracrine self-sustaining cycle[348]. In line with our results, numerous evidence reported a direct correlation between VWF intensity in ECs and brain tumor grade[347]. Previous studies by our research team demonstrated that GBM patients had supraphysiological plasma VWF antigen level that correlates with poor prognosis and shorter survival[349]. Our data confirmed the overexpression of VWF also by mRNA levels, compared not only to normal brain, but also to LGG and MNG, confirming its correlation with tumor malignancy and its potential use as prognostic factor.

VEGF is certainly the best characterized pro-angiogenic mediator and as previously discussed, its overexpression leads to the formation of fragile microvessels, with a disrupted structure and increased permeability[350,351]. VEGF exerts its many effects promoting ECs proliferation, migration and survival and several studies reported a strictly correlation between VEGF, microvascular density and tumor grade and, consequently, with clinical outcome and prognosis[352,353]. The results of this study showed that mRNA expression of VEGF, and its receptor VEGFR-1, was significantly increased in GBM,

compared to both healthy brains, LGG, and MNG. These data suggest that angiogenesis is not equally enhanced in GBM, LGG and MNG, confirming the importance of the signalling pathway VEGF/VEGFR in tumor angiogenesis and tumor malignancy grade. Moreover, the overexpression of HIF-1α is strictly related to glioma malignancy and several studies reported its association with poor prognosis in different types of cancer, including gliomas[354]. Also, Ang and their receptor, Tie-2, play a critical role in angiogenesis and vascular stability, as previously discussed[355]. In particular, the upregulation of Ang-2 triggers endothelium activation and vascular destabilization[188], promoting the establishment of disorganized and defenestrated blood vessels with increased permeability and loss of integrity. These results were further confirmed by our blood brain barrier (BBB) model, in which GECs were not able to form a closed and functional barrier, allowing the passage of a great quantity of dextran in a time-dependent manner. This increased permeability may reflect the impairment of BBB. The leakage of BBB allows plasma proteins extravasation in extravascular space, leading to alteration of extracellular matrix and promotion of angiogenesis and it is an important parameter for drug therapy, because it might indicate lower drug availability in the tumor region[356]. The increased permeability of GECs monolayer could be due to the lack of functional adherent and tight junction. This hypothesis may be supported by the lower fluorescence intensity of VE-Cadherin labelling in GECs, as VE-Cadherin is a tight junction protein that plays an important role in the integrity of the BBB. Several investigations reported that GBM tumor microvessels present a downregulation of tight junction proteins[293], including VE-Cadherin[357]. A further confirmation arose from tube-like structures formation assay, as GECs were able to form a complex network, characterized by cords, junctions and meshes, better organized and in a greater number respect to LGG-ECs and MNG-ECs, which, in the same time frame, did not succeed to form an *in vitro* vascular architecture. Phenotypically, GECs proved to be highly positive to endoglin (CD105), a transmembrane glycoprotein expressed on activated ECs, which acts as accessory protein of the TGFR[358]. In several cancer types, endoglin overexpression on peritumoral and intratumoral vessels, positively correlates with patient outcome and survival rates[358]. Similarly, CD90 is highly expressed on activated ECs, serving as a marker for tumorigenesis and angiogenesis[359], and reflecting the propensity of LGGs to undergo malignant transformation, developing secondary GBMs. Moreover, a high percentage of GECs (about 70%), showed positivity to NCAM (CD56), a cell-surface glycoprotein widely characterized in CNS, when it regulates intercellular adhesion and cell migration, being positively correlated with tumor grade and aggressiveness[360].

Once isolated and characterized, the next step of the project consisted in the investigation of calpain family role on GECs and in the screening of anti-Calpains efficacy. Firstly, the examination of calpain expression in GECs revealed an upregulation of the most active calpains, *CAPN1* and *CAPN2* and

their small regulator subunits (*CAPNS1* and *CAPNS2*) and a downregulation of *CAST*, their endogenous inhibitor. Notably, the overexpression of *CAPN1* and *CAPNS2* was found to significantly correlate with patient OS, suggesting their potential to serve as novel prognostic biomarkers. Calpains residing in the endothelial cells play important roles in angiogenesis. It has been shown that calpain activity can be increased in endothelial cells by growth factors, primarily VEGF. VEGF/VEGFR2 induces calpain-2 dependent activation of PI3K/AMPK/Akt/eNOS pathway, and consequent nitric oxide production and physiological angiogenesis[361]. Under pathological conditions such as tumor angiogenesis, endothelial calpains can be activated by hypoxia, triggering a self-sustaining vicious cycle.

The rapid progression of GBM requires large quantity of oxygen and nutrients, supplied by angiogenic processes and pro-angiogenic factor secretion. The abundance and the key role of VEGF in this mechanism has been widely discussed, but here VEGF in ECs comes into play in activating calpain-2, so that the administration of calpain inhibitors or siRNA proved to abolish VEGF-induced endothelial NO production and angiogenesis[362,363,364,365]. Furthermore, the faster growth of tumor cells due to hypoxic condition leads to the upregulation of calpain expression and activity in ECs[366,367,368]. Interestingly, calpain in tumor cells serves as a newly identified regulator of the HIF-la/VEGF pathway[369]. For example, Zheng et al. have shown that hypoxia promotes calpain-induced filamin-A proteolysis in melanoma cells, which in turn facilitates HIF-1α nuclear translocation. In a tumor xenograft model, it has been observed that, since VEGF is transcriptionally activated by HIF-1α, the overexpression of filamin-A is able to increase HIF-1α recruitment to VEGF promoter, thus promoting tumor angiogenesis. However, calpeptin inhibition of calpain attenuates HIF-1α nuclear accumulation and transactivation[369].

In order to investigate the involvement of endothelial calpains in tumor angiogenesis, Miyazaki et al. examinate tumors and nearby normal tissues from patients with malignant astrocytoma, colon and lung adenocarcinomas. Immunostaining of calpastatin showed that its expression was significantly reduced in ECs of tumor vessels compared to nearby normal vessels[370]. Furthermore, they generated transgenic mice harboring EC specific transgene of calpastatin, observing that in these animals, tumor angiogenesis was attenuated in a Lewis lung carcinoma allograft transplantation model. It emerged that calpastatin inhibits VEGF-C production through calpain/ SOCS3/STAT3[370]. Following these premises about the potential pro-angiogenic role of calpains, we decided to test calpain inhibitors, in order to restore the physiological condition and abolish GBM angiogenesis. To this aim, calpain inhibitor 1-2 and Calpastatin were administered to GECs (Figure 31).

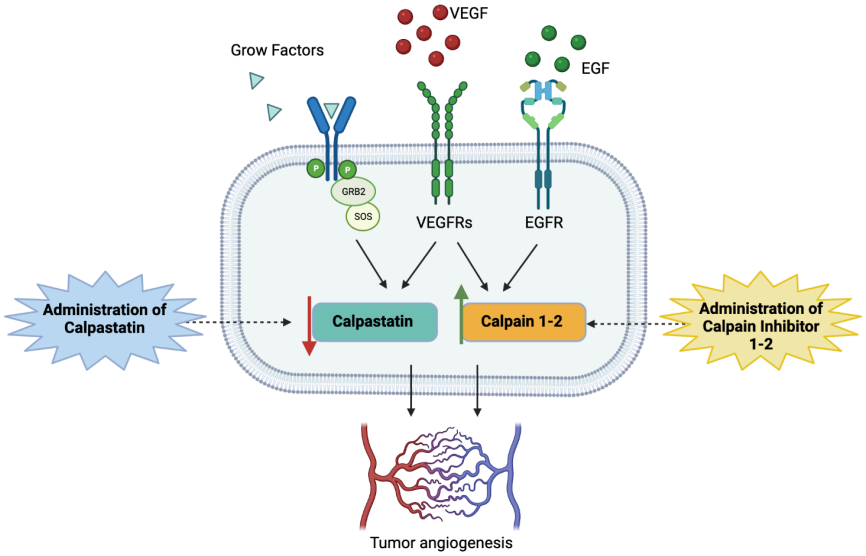

Figure 31. Schematic representation of calpain restoring in ECs, by the administration of calpain inhibitors or calpastatin, their natural endogenous inhibitor.

Our data demonstrated a great ability of calpain inhibitors and calpastatin to slow down GEC proliferation and survival, by inducing also apoptotic mechanism mediated by caspase-3 and caspase-7 activation. Notably, calpain inhibitors succeeded to inhibit GEC functionality, as migration and angiogenesis *in vitro.* The higher effect was observed after the administration of Calpain inhibitor 1, which was able to strongly arrest cell proliferation and viability and counteract tube-like structure formation. Of relevance, the investigation of the potential molecular mechanism underlying these effects revealed a downregulation of MAPK and an upregulation of pro-apoptotic mediators as BAX-family. The Ras/RAF/MEK/ERK (MAPK) signaling represent one of the best-characterized pathways in cancer biology, and its hyperactivation is responsible for over 40% human cancer cases. The signaling by MAPK promotes cellular overgrowth by turning on proliferative genes, and simultaneously enables cells to overcome metabolic stress by inhibiting AMPK signaling. Mechanistically, upon binding of RTKs or other stimulations, Ras small GTPases are activated by GTP/GDP exchange factors (GEFs), which in turn recruit RAF/MEK complexes to the plasma membrane and trigger the RAF/MEK/ERK kinase cascade through facilitating RAF/RAF (or KSR), RAF/MEK, and MEK/MEK interactions as well as subsequent phosphorylations[371]. Active ERKs are further translocated into the nuclei or in the cytoplasm, where they phosphorylate a number of substrates that regulate cell functions, as proliferation and survival[372,373,374]. An aberrant activation of MAPK signaling frequently induces human cancers or developmental disorders, as in GBM[375,376]. In our data, the downregulation of MAPK is accompanied by the Bcl-2 decreased expression. Bcl-2-family proteins regulate all major types of cell death, including necrosis, autophagy and apoptosis,

thus operating as nodal points at the convergence of multiple pathways with broad relevance to oncology. Overexpression of the Bcl-2 and related antiapoptotic proteins has been demonstrated to inhibit cell death induced by many stimuli, including growth factor deprivation, hypoxia and oxidative stress, thus the effect of calpain inhibitors of decreasing Bcl-2 may explain cell death[377]. This result is further confirmed by the overexpression of Bax and Bid, pro-apoptotic members of Bcl-2 family and caspase-3 and -7, the major executioner caspases of apoptosis mechanisms. Caspase-3 and caspase-7 are both universally activated during apoptosis, irrespective of the specific death-initiating stimulus, and both proteases are widely considered to coordinate the demolition phase of apoptosis by cleaving a diverse array of protein substrates[378]. Interesting data arise also from the upregulation of p53, p21 and p27 by calpain inhibitors. The *TP53* gene encodes a transcription factor that is a critical barrier to carcinogenesis. Inactivation of *TP53* is the most common mutation in sporadic human cancers, suggesting a strong selection against p53 function during tumorigenesis. p53 is thought to act as a tumor suppressor by serving as a cellular stress sensor. The inheritance of a mutant *TP53* allele is observed in Li-Fraumeni syndrome, predisposing patients to early onset cancer development, further underscoring the role of p53 in tumor suppression[379]. Similarly, p21 functions as a cell cycle inhibitor and anti-proliferative effector in normal cells and is dysregulated in some cancers. P27 is considered a tumor suppressor because of its function as a regulator of the cell cycle. In cancers it is often inactivated via impaired synthesis, accelerated degradation, or mislocalization[380]. Finally, calpain inhibitors were able to interfere with VEGF signalling, downregulating its expression and that of its two major receptors, VEGFR1 and VEGFR2, suggesting the disruption of the most active pro-angiogenic axis.

Most of the scientific literature on the relation between calpains and cancer, are focused on the correlation between their expression and patient prognosis. The limiter number of reports investigating their blockade are still controversial, as although the numerous protumoral pathways induced by calpains, they are able to sensitize cancer cell to chemotherapy[229]. According to our results, it was observed that calpain inhibition with calpeptin, as well as with a synthetic calpain inhibitor (ALLN), was able to suppress cell cycle progression and proliferation of cancer transformed cells, as well as their anchorage-independent growth. Similarly, the inhibition of calpain activity by different inhibitors was effective in repressing the effects of the transformation induced by other oncoproteins such as v-Jun, v-Myc, v12k-Ras and v-Fos[277]. Furthermore, it has been reported that the simultaneous inhibition of calpains and ERK/MAPK pathway coupled with an activation of p38 MAPK was sufficient to restore ability of v-Src-transformed myoblasts to differentiate[381]. Interestingly, calpain inhibition was also shown to induce apoptosis of transformed cells, thanks to an accumulation of c-Myc, previously identified as a calpain substrate[382].

Furthermore, the inhibition of calpains is known to reduce the invasiveness of tumor cells, as in lung cancer cells, where the addition of calpain inhibitors, such as calpeptin, blocks calpain activation and reduces the invasiveness by blocking cancer cell migration[383]. The treatment of the same cells with C2-ceramide, an activator of the phosphatase PP2A, limits tumor invasion by inducing the dephosphorylation of calpains and thus their inactivation[384]. Likewise, the inhibition of m-calpain using synthetic inhibitors, as calpain inhibitor I, reduces the invasiveness of prostate carcinoma cells[275]. Very similar results were obtained with rhabdomyosarcoma treated with calpeptin, Indeed, the invasiveness of these cells was dramatically reduced in the presence of calpeptin, restoring a condition closed to normal myoblasts[385]. Of relevance, inhibition of calpains can also reduce the expression of MMPs. Indeed, the treatment of leukemic cells with CP1B, a specific inhibitor of calpains derived from calpastatin, decreases the expression as well as the secretion of MMP-2 and MMP-9, thus reducing matrix degradation and tumor invasion[386].

Overall, the activity of the two ubiquitous calpains could be targeted to inhibit cell transformation by suppressing the enhanced motility, adhesion disassembly and the cell cycle progression and by inducing tumor cell death. Interestingly, it has been reported that the inhibition of calpain activity could be useful also to improve the sensitivity of lung cancer cells to the proteasome inhibitor bortezomib[387]. It is possible that while calpain inhibition may decrease apoptosis, the cells may be redirected to other modes of death. Still the sum of the published data suggests an interesting approach would be to target calpains to improve chemotherapy efficiency.

Targeting calpain activity with specific inhibitors could be a novel approach to limiting the development of primary tumors and the formation of metastases, by inhibiting tumor cell migration and invasion, which allows dissemination as well as tumor neovascularization, which in turn allows tumor progression.

Taken together, the results of this research project led to the awareness that molecular mechanisms underlying GBM malignancy and aggressiveness need to be deeper investigated. The development of target therapies for patients with brain cancer, through the modulation of angiogenesis, invasiveness, and pharmacological sensitivity/resistance, is urgently need in the era of precision medicine. Furthermore, the discovery of novel molecular mediators, from genetics to epigenetics and proteomics, as potential prognostic and predictive biomarkers may be handy and recognizable by an "omic" approach and may impact clinical practice, in terms of patient management. Personalized medicine aims to optimize patient management, considering the individual traits of each patient. The main purpose of personalized therapy is to obtain the best response, improving health care and lowering costs. Extending traditional approaches, personalized medicine introduces novel patient-specific paradigms from diagnosis to treatment, with greater precision. Personalized medicine should

rely on a multidisciplinary and multimodal approach, through the coordination of surgeons, oncologists, radiation oncologists, geneticists and biologists, to optimize cancer outcomes by the combination of tailored therapeutic strategies as surgery, radiotherapy, chemotherapy, growth factor inhibitors, immunotherapy, and cell/gene therapies[388].

Thus, the main pillar of personalized medicine is tumor classification, stratification, and patient clustering, in parallel with the identification of additional indicators, for strict prediction of the brain tumor status and prescription of the most effective therapeutic options.

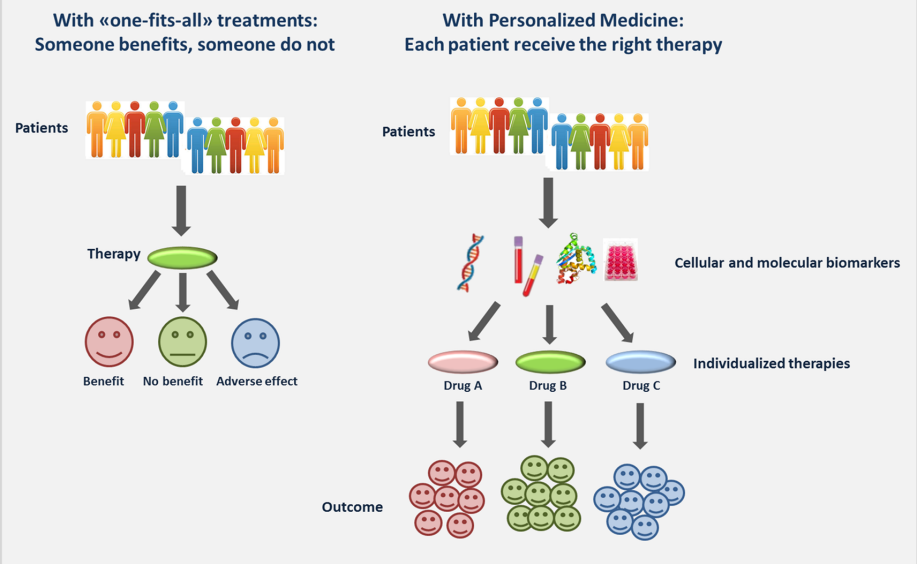

Figure 32. Advantages of PM in cancer treatment. The "one-fits-all" treatment approach do not consider individual's susceptibility, eliciting different therapy response, from benefit to adverse effects. On the contrary, PM allows to examine patient's specific disease-related features, leading to favorable outcome.

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