From the Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

ASTROCYTES IN GLIOBLASTOMA: ENHANCERS OF TUMOR GROWTH, PREDICTORS OF PATIENT SURVIVAL AND POTENTIAL THERAPEUTIC TARGETS

Alessandro Mega

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Astrocytes in glioblastoma: enhancers of tumor growth, predictors of patient survival and potential therapeutic targets

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By

Alessandro Mega

Principal Supervisor:
Arne Östman, PhD
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisors:
Linda Sleire, PhD
Karolinska Institutet
Department of Oncology-Pathology

Monica Nistér, MD, PhD
Karolinska Institutet
Department of Oncology-Pathology

Daniel Hägerstrand, PhD
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Dolores Hambardzumyan, PhD
Icahn School of Medicine at Mount Sinai
Department of Oncological Sciences

Examination Board:
Sonia Lain, PhD
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Stefan Bergström, MD, PhD
Uppsala University
Center for Research & Development, Gävleborg

Fredrik Johansson Swartling, PhD
Uppsala University
Department of Immunology, Genetics and Pathology

To my family

ABSTRACT

The tumor microenvironment plays an important role in glioblastoma, the most malignant primary brain tumor in adults. Astrocytes are a major component of the glioblastoma tumor microenvironment; therefore, their influence on glioblastoma biology needs to be clarified.

In this thesis, the role of astrocytes was explored with regard to glioblastoma growth, patient survival and their potential as a therapeutic target through a set of *in vitro* and *in vivo* studies and analyses of clinical samples.

Co-culture experiments identified astrocytes as enhancers of glioblastoma cell growth in cell lines and in a patient-derived culture. Furthermore, orthotopic co-injection of astrocytes with glioblastoma cells reduced survival of NOD scid mice, compared to mice that received mono-injection of glioblastoma cells. A gene signature reflecting glioblastoma-activated astrocytes was associated with poor prognosis in two glioblastoma datasets. Through this set of experiments, astrocytes were thus shown to enhance glioblastoma growth.

In a glioblastoma tissue collection, a subset of peritumoral astrocytes co-expressing PDGFRα and GFAP was examined for biomarker significance; experiments showed that such astrocytes did not carry tumor markers, supporting their non-malignant nature. Inter-case variability was observed, both with regard to the presence of such a subset and the general astrocyte density. High density in the peritumoral areas of the PDGFRα and GFAP co-expressing astrocytes, but not total astrocyte density, was identified as an independent poor prognostic factor. This observation suggests the presence of differentially functional astrocyte subsets in glioblastoma holding clinical relevance.

A high-throughput screening assay was designed to screen a library of compounds in a novel glioblastoma/astrocyte co-culture system. The assay was implemented to identify compounds that specifically blocked the astrocyte-driven enhancement of glioblastoma growth. Three such compounds were identified and one of them was further validated in an additional cell line. Results from the high-throughput screen suggested the crosstalk between glioblastoma cells and astrocytes as a potential therapeutic target.

In conclusion, these studies suggest clinically and biologically relevant roles of astrocytes, as validated in patient datasets and peritumoral tissue. Co-culture specific drug response implies the crosstalk between malignant cells and astrocytes as a candidate target for novel therapies. Further studies will lead to better characterization of the mechanisms behind the glioblastoma-astrocyte crosstalk, while the clinical association of the novel $PDGFR\alpha^{+}/GFAP^{+}$ peritumoral astrocyte subset should be further investigated and validated in larger cohorts.

LIST OF SCIENTIFIC PAPERS

I. **Astrocytes enhance glioblastoma growth**
Mega A, Hartmark Nilsen M, Leiss LW, Tobin NP, Miletic H, Sleire L, Strell C, Nelander S, Krona C, Hägerstrand D, Enger PØ, Nistér M, Östman A.
Glia. 2019 Sep 11. doi: 10.1002/glia.23718

II. **Platelet-derived growth factor receptor α/glial fibrillary acidic protein expressing peritumoral astrocytes associate with shorter median overall survival in glioblastoma patients**
Leiss LW, **Mega A**, Olsson Bontell T, Nistér M, Smits A, Corvigno S, Rahman MA, Enger PØ, Miletic H, Östman A.
Glia. 2019 Nov 26. doi: 10.1002/glia.23756

III. **A high-throughput screen to explore the astrocyte-driven enhancement of glioblastoma growth as a candidate therapeutic target**
Mega A, Leiss LW, Liu J, Nekhotiaeva N, Eriksson A, Haraldsson M, Otrocka M, Hägerstrand D, Nistér M, Östman A.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

5-ALA	5-aminolevulinic acid
ADA	Adenosine deaminase
AIF1	Allograft inflammatory factor 1
AKT	Protein kinase B
AKT3	AKT serine/threonine kinase 3
ALK	Anaplastic lymphoma receptor tyrosine kinase
ANKRD1	Ankyrin repeat domain-containing protein 1
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BRAF	Serine/threonine-protein kinase B-raf
BTSC	Brain tumor stem cell
CAIX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
CCLx	Chemokine (C-C motif) ligand x
CCND2	G1/S-specific cyclin-D2
CCRx	Chemokine receptor x
CD	Cluster of differentiation
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin-dependent kinase inhibitor 2B
CDKN2C	Cyclin-dependent kinase inhibitor 2C
CHI3L1	Chitinase-3-like protein 1
CNS	Central nervous system
CX3CL1	C-X3-C motif chemokine ligand 1
CX3CR1	C-X3-C motif chemokine receptor 1
Cx43	Connexin43
D2HG	D-2-hydroxyglutarate
DRD2	Dopamine receptor D_2
ECM	Extracellular matrix

EGF	Epidermal growth factor
EGFR	EGF receptor
EGFRvIII	EGFR variant III
ERK	Extracellular signal-regulated kinase
ETAR	Endothelin receptor type A
ETBR	Endothelin receptor type B
FACS	Fluorescence-activated cell sorting
GBM	Glioblastoma
G-CIMP	Glioma-CpG island methylator phenotype
GDF-15	Growth/differentiation factor-15
GFAP	Glial fibrillary acidic protein
GSC	Glioblastoma stem-like cell
H&E	Hematoxylin and eosin
HIF-1α	Hypoxia-inducible factor 1-alpha
HTS	High-throughput screen
IDH	Isocitrate dehydrogenase
IL-13Rα2	IL-13 receptor alpha 2
IL-x	Interleukin x
JAK	Janus kinase
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
MDM2	Mouse double minute 2
MDM4	Mouse double minute 4
MERTK	Tyrosine-protein kinase Mer
MET	MET proto-oncogene, receptor tyrosine Kinase
MGMT	O-6-methylguanine-DNA methyltransferase
MMP-x	Matrix metallopeptidase x
MRI	Magnetic resonance imaging
MVP	Microvascular proliferation
MYC	MYC proto-oncogene, basic helix-loop-helix transcription factor

NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Nestin
NF1	Neurofibromatosis-1
NFKBIA	NF-κB inhibitor α
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Neural/glial antigen 2
OS	Overall survival
PARK2	Parkinson protein 2 E3 ubiquitin protein ligase isoform 3
PDGFRA/PDGFRα	Platelet-derived growth factor receptor alpha
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1
PLA2R1	Phospholipase A2 receptor 1
POSTN	Periostin
pSTATx	Phosphorylated STATx
PTA	Peritumoral area
PTEN	Phosphatase and tensin homolog
PTN	Pleiotrophin
PVN	Perivascular niche
RANKL	Receptor activator of NF-κB ligand
RB	Retinoblastoma
RB1	Retinoblastoma-associated protein
RET	Rearranged during transfection
RTK	Receptor tyrosine kinase
SDF-1α	Stromal cell derived factor-1 alpha
SHH	Sonic Hedgehog
SRGN	Serglycin
STAT3	Signal transducer and activator of transcription 3

STATx	Signal transducer and activator of transcription x
TAA	Tumor-associated astrocyte
TAM	Tumor-associated microglia/macrophage
TGF-β	Transforming growth factor beta
TK	Thymidine kinase
TLR4	Toll-like receptor 4
TMZ	Temozolomide
TTFields	Tumor-treating fields
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
α-KG	α-ketoglutarate

1 GLIOBLASTOMA: GENERAL INTRODUCTION

Gliomas are central nervous system (CNS) tumors that display characteristics of glial cells. They are mainly classified as astrocytic, oligodendroglial or ependymal tumors, encompassing benign, low-grade and high-grade malignancies.

In particular, diffuse astrocytic and oligodendroglial tumors range from World Health Organization (WHO) grade II to IV. Glioblastoma (GBM) is a grade IV astrocytic malignancy that belongs to this category and represents the most common malignant brain tumor in adults [1].

1.1 EPIDEMIOLOGY

According to the Swedish Cancer Registry, 1400 people are diagnosed with brain tumor every year in Sweden [2]. Among primary CNS tumors, GBMs represent 16% of all cases and 56.6% of all gliomas, with a median age at diagnosis of 65 years and incidence 1.6 times higher in males compared to females [3]. The prognosis of GBM has remained extremely poor during the last decades, with a median survival of 15 months despite intensive treatment [4]. Risk factors for GBM are largely investigated; currently, ionizing radiations are the most established environmental risk factor for glioma [5].

1.2 PATHOGENESIS

GBMs can be divided into primary (more than 90% of the cases) and secondary. Primary GBMs arise *de novo*, without detectable signs of a preexistent pathology [6]. Secondary GBMs are rare and show a better prognosis [7]. They result from the evolution of lower grade diffuse or anaplastic astrocytomas (WHO grade II or III), and commonly affect patients of younger age (below 45 years). Primary and secondary GBMs cannot practically be distinguished based on morphology from a histopathological point of view; nevertheless they show genetic differences suggesting differential dependency of driver oncogenes and, possibly, sensitivity to novel therapies [6]. Distinct cells of origin are in fact thought to develop into primary and secondary GBMs [8]. However, the cell of origin of GBM remains largely unknown; it is generally thought that neural stem cells, glial progenitors or more differentiated cells such as astrocytes may play this role [9].

1.2.1 Genetic landscape of glioblastoma

GBMs are characterized by great genetic instability at multiple levels, from wide chromosomal alterations to focal events. During the last decades, integrated approaches have uncovered the complexity of genetic mutations, epigenetic modifications as well as signaling pathway alterations that lead to malignant transformation of the cell of origin.

Classically established genetic alterations of GBMs have been initially described as follows:

- Primary GBMs: loss of heterozygosity (LOH) at 10q (70%), *EGFR* amplification (36%), *P16/INK4A* deletion (31%), and phosphatase and tensin homolog (*PTEN*) mutations (25%);
- Secondary GBMs: LOH 10q (63%) and *TP53* mutations (65%) [10].

LOH 10q is a frequent genetic event in both primary and secondary GBM. The tumor suppressor *PTEN* maps to 10q23 and is important in the regulation of cell proliferation, apoptosis and tumor invasion. There are cases in which *PTEN* is mutated even without chromosome 10 LOH, which suggest that *PTEN* is a critical gene in GBM pathogenesis [11].

Further efforts have widened the understanding of the genetic landscape of GBM. A multitude of genes have been described to be targets of amplification, like *EGFR*, *MET*, *PDGFRA*, *MDM4*, *MDM2*, *CCND2*, *PIK3CA*, *MYC*, *CDK4*, *CDK6* and *AKT3*. Other genes have been defined as targets of deletion, like *CDKN2A*/*B*, *CDKN2C*, *PTEN*, *PARK2* and *RB1* [12].

Further analyses revealed mutations in various genes, the most significantly mutated being the tumor suppressors *TP53* (42%), *PTEN* (33%), *NF1* (21%), *RB1* (11%) and *PIK3R1* (10%), and the oncogenes *EGFR* (18%) and *PIK3CA* (7%) [13, 14]. Specific patterns of mutations emerged, such as the mutual exclusion between NF-κB inhibitor α (*NFKBIA*) heterozygous deletion and *EGFR* amplification [15].

1.2.2 Glioblastoma core pathways

The understanding of the genetic landscape of GBM allowed an integrated approach that uncovered the consistent alteration of three critical signaling pathways in GBM, namely inactivation of retinoblastoma (RB) and p53 tumor suppressor pathways and activation of RTK/Ras/PI3K pathway [14]. A schematic view of the three pathways is shown in Figure 1.

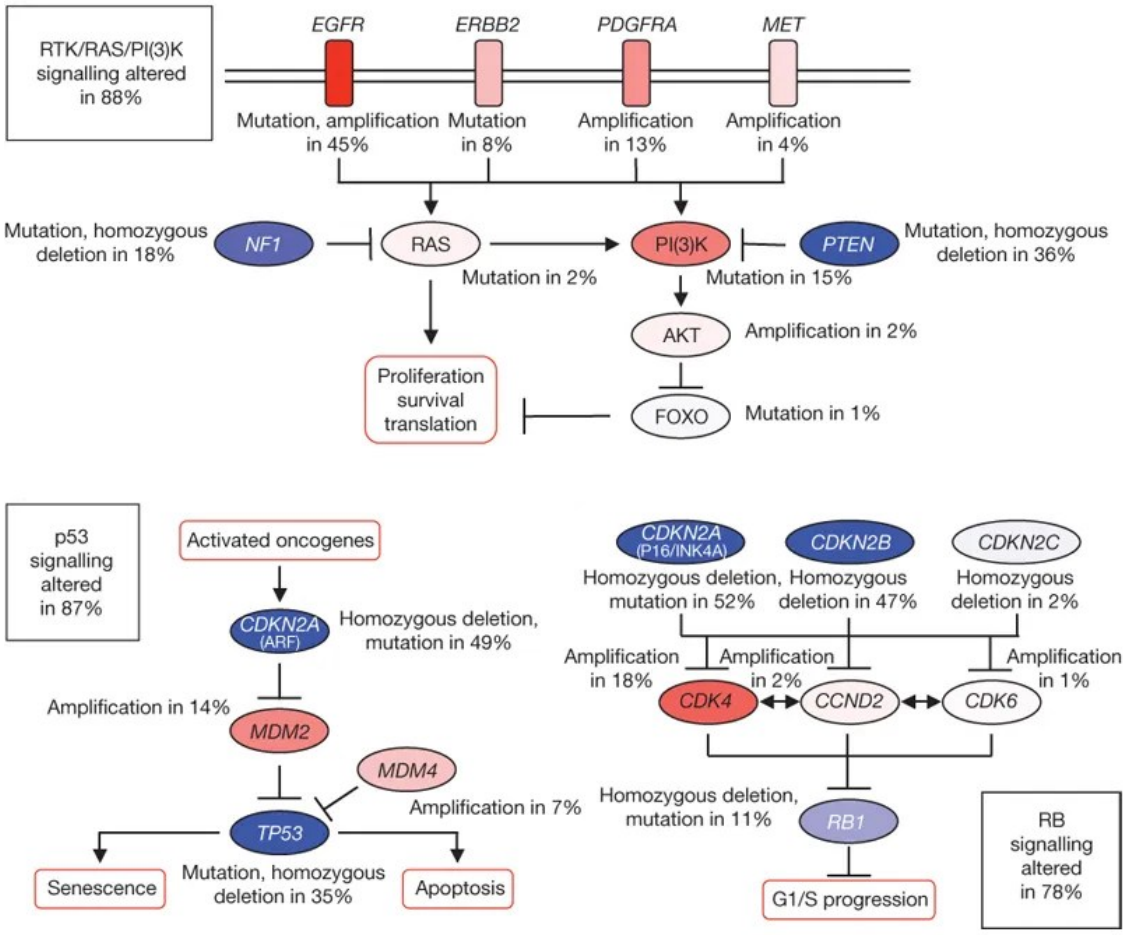

Figure 1: Frequent genetic alterations in three critical signaling pathways in GBM. Reprinted with permission from [14].

In the TCGA study, dysregulated p53 signaling occurred in 87% of the samples, mostly resulting from *CDKN2A* deletion (49%), *MDM2* (14%) and *MDM4* (7%) amplification, and mutation or deletion of *TP53* (35%). RB pathway impairment occurred in 78% of the samples and was due to the deletion of members of the CDKN2 family, or amplification of *CDK4* (18%), *CDK6* (1%), and *CCND2* (2%) and mutation or deletion of *RB1* (11%). The RTK/Ras/PI3K pathway was found to be activated in 88% of tumors as a result of alterations that include mutations or deletions in *NF1* and *PIK3R1* [12, 14].

Among the RTKs, EGFR, PDGFRα and MET play a substantial role in GBM. *EGFR* is amplified in 50% of the cases and associated with poor prognosis. About half of these amplified cases harbor also *EGFRvIII* mutation which has been linked to worse prognosis when compared to wild-type *EGFR* [12]. PDGF signaling is activated in glioblastoma mainly through *PDGFRA* amplification (15% of the cases) or upregulation of the ligands. The activation of c-Met occurs in about 5% of glioblastomas and especially in samples with shorter median survival. It also shows association with activation of *EGFR*/*EGFRvIII* [12]. *TERT* promoter mutations are also common in GBM [16].

1.2.3 IDH status in brain tumors

Data revealing IDH mutations in brain tumors shed light on the nature of primary and secondary GBMs [7].

There are three IDH isoforms, the cytosolic IDH1 and the mitochondrial IDH2 and IDH3. Wild-type IDH1 and IDH2 are enzymes which play a role in the Krebs cycle by converting isocitrate to α-ketoglutarate (α-KG), ultimately impacting on cell metabolism through production of NADPH [17]. The R132H substitution in IDH1 is the most frequently observed mutation. Such a modification in the residue R132 increases α-KG and NADPH bindning to the active site of IDH1. Mutations in IDH1/2 lead to depletion of α-KG and accumulation of the oncometabolite D-2-hydroxyglutarate (D2HG), a reduced form of α-KG [18-20]. High D2HG levels interfere with the action of a number of enzymes that use α-ketoglutarate as co-factor, including enzymes that have an important role in epigenetic regulation, leading to aberration in the differentiation program [18]. Prolyl-hydroxylation of collagen is also perturbed by D2HG, leading to defects in collagen maturation [21]. Induction of HIF-1α has also been shown to be triggered by mutant IDH [22]. Ultimately, the downstream effects of IDH1/2 mutation appear to lead to tumor growth by affecting processes such as metabolism, epigenetic modifications, basement membrane function and response to hypoxia [12]. However, the role of IDH1 mutation is controversial; different experimental approaches have suggested a tumor-suppressive activity [23].

IDH1/2 mutations are typical of secondary GBMs. Primary GBMs display mutant IDH1/2 only in less than 5% of the cases, while more than 80% of secondary GBMs carry mutant IDH1/2. Mutations of IDH1/2 have been described as an early gliomagenesis event leading to development of low-grade gliomas. This strengthens the hypothesis that secondary GBMs derive from the evolution of lower grade gliomas, sharing with them a common progenitor cell. Figure 2 summarizes the genetic pathways that lead to primary and secondary GBMs; they appear to be distinct biological entities requiring specific therapeutic approaches, also suggested by the better prognosis carried by secondary GBMs. IDH1 R132H mutation is nowadays used as a marker for the distinction of secondary from primary GBMs [7].

Interestingly, *IDH1* mutation is strongly associated with the glioma-CpG island methylator phenotype (G-CIMP) subgroup of GBM, identified following profiling of alterations in promoter DNA methylation [24].

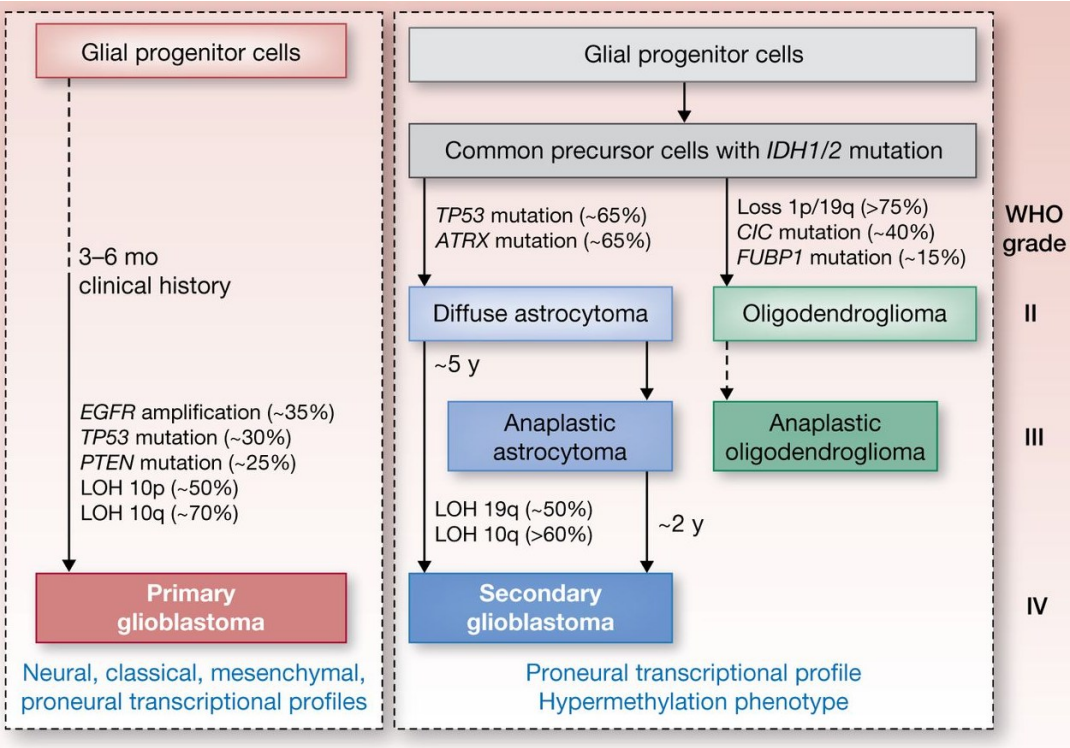

Figure 2: Genetic pathways to primary and secondary glioblastomas. Reprinted with permission from [7].

Ongoing efforts counting on more sophisticated technology and inclusion of a higher number of tumors are predicted to lead to a more accurate understanding of the molecular pathogenesis of GBM.

1.3 DIAGNOSIS AND CLASSIFICATION

A primary tool for GBM diagnosis is MRI, while the definitive diagnosis of GBM is based on histology. Tissue is collected by stereotactic biopsy or after tumor resection in order to perform standard histological staining, such as H&E staining, and additional molecular biomarker studies. [25, 26]. As shown in Figure 3, hallmarks of GBM histopathology are cellular polymorphism, nuclear atypia, mitotic activity, vascular alteration (thrombosis, prominent microvascular proliferation), necrosis and pseudopalisading necrosis. GBMs typically exhibit spatial heterogeneity and extreme cellular invasion into surrounding brain tissue [27-29].

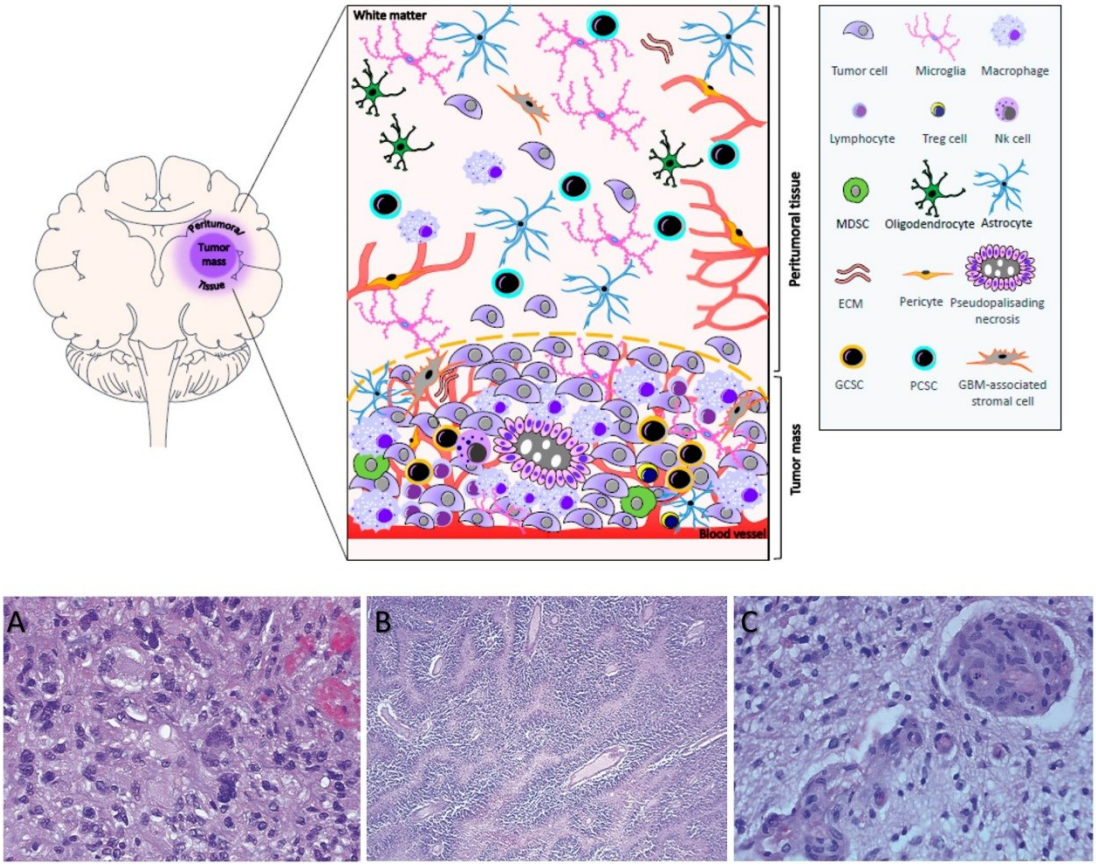

Figure 3: Pathological and cellular features of GBM. Elevated cellular density, pleomorphism and nuclear atypia (A). Cell necrosis and necrotic pseudopalisading (B). Microvascular proliferation and glomeruloid structures (C). Adapted with permission from [28, 30].

Taking into account the most recent findings, the latest WHO classification of CNS tumors has incorporated molecular features. In fact, molecular markers are acquiring increasing importance as a support to microscopy in brain tumors, including GBM, allowing an accurate diagnosis and giving indications about prognosis and response to treatment [25]. In particular, according to the 2016 WHO classification of CNS tumors, IDH mutation is used to classify GBMs as follows:

- Glioblastoma, IDH-wildtype (about 90% of cases, corresponding mostly to primary GBM);
- Glioblastoma, IDH-mutant (about 10% of cases, corresponding mostly to secondary GBM);
- Glioblastoma, NOS ("not otherwise specified", tumors for which a full IDH evaluation cannot be performed) [1].

In all gliomas, including GBM, IDH mutation is correlated with better prognosis [31], while it does not seem to predict response to therapy [12].

A variant of GBM has been added to the classification, within the IDH-wildtype group: the epithelioid glioblastoma. It is more common in children and younger adults and often carries

BRAF V600E mutation and lacks other IDH-wildtype frequent mutations such as *EGFR* amplification and losses on chromosome 10 [1].

The only known predictive marker of response to therapy in GBM is the epigenetic silencing of the *MGMT* gene. It encodes a DNA repair protein that removes the alkyl groups from the O6 position of guanine, thus interfering with the mechanism of action of chemotherapeutic alkylating agents like temozolomide (TMZ), which is standard of care for GBM [12]. *MGMT* status is hard to reveal in a reproducible way through immunohistochemistry but it can be assessed through methylation-specific polymerase chain reaction (MSP) [25]. Patients with methylated *MGMT* promoter display better response to therapy, given the fact that MGMT antagonizes the action of TMZ [32].

A number of additional biomarkers, including 1p19q deletion, *TERT* promoter mutation and *EGFR* mutation/amplification may nowadays be tested as clinical routine [33].

1.4 GLIOBLASTOMA HETEROGENEITY

GBM has classically been referred to as "*multiforme*" (from Latin, "of multiple shapes") based on its diverse histological features. Indeed, continuously emerging molecular data did not prove researchers wrong: GBMs appear to be "*multiforme*" even from a molecular point of view, displaying both inter- and intra-patient variability.

1.4.1 Molecular subtypes

Already before the in-depth efforts aimed at unraveling the GBM genetic landscape, a number of high-dimensional studies had highlighted the inter-case genetic differences of GBM and correlated that with prognosis [34-36]. Interestingly, these studies provided initial evidence that glioblastomas, all morphologically classified by histopathology in the same WHO grade IV class, can be better defined using molecular classification with regard to their nature, histopathological features and outcome.

A gene expression study of 200 GBMs was performed with the aim of expanding the knowledge about molecular subclasses of this disease [37]. Clustering analyses suggested 4 groups of GBM, named classical, neural, mesenchymal and proneural. These subgroups show defining gene expression patterns linked to different brain cell lineages. The proneural subtype has been linked to an oligodendrocytic signature, the classical group to an astrocytic signature, the mesenchymal to a cultured astroglial signature and, finally, the neural class with both oligodendrocytic and astrocytic signatures together with a strong enrichment of neuron specific genes [37].

The neural subtype signature consists of expression of neuronal markers, such as *NEFL*, *GABRA1*, *SYT1*, and *SLC12A5* [37]. This subtype has not been defined by exclusive gene abnormalities compared to other classes. This observation is nowadays explained by the fact that the assignment of tumors to the neural subtype is an artifact deriving from the collection and analysis of tumor margins contaminated by normal neural tissue, thus

invalidating the significance of this class [38, 39]. The remaining three classes are considered to be relevant; they carry distinctive molecular alterations and display clinical relevance. Accruing evidence suggests that they may derive from diverse cells of origin or be the result of cells in different stages of tumor evolution [9, 40].

The classical subtype shows chromosome 10 loss and gain of chromosome 7. Although chromosome 7 gain is also seen in other GBM subtypes, specific *EGFR* amplification, and consequent overexpression, or *EGFRvIII* mutations are highly frequent in the classical subtype. This subtype also shows a lack of *TP53* mutations. Focal 9p21.3 homozygous deletion, targeting *CDKN2A*, is frequently seen in the classical subtype and occurs in 94% of the cases with *EGFR* amplification. This genetic aberration is almost mutually exclusive with alterations in *RB1*, *CDK4*, and *CCDN2* (other members of the RB pathway). This suggests that in *EGFR* amplified classical GBM the RB pathway is deregulated solely through *CDKN2A* deletion. Members of the Sonic Hedgehog (SHH) and Notch pathways, as well as the neural precursor and stem cell marker nestin, are also highly expressed in the classical subtype [37].

The mesenchymal subtype shows predominant impairment of *NF1* through focal deletions of a chromosomal region at 17q11.2. Mesenchymal markers (such as *CHI3L1* and *MET*) and astrocytic markers (*CD44* and *MERTK*) are highly expressed in this subtype, suggesting aftermath of a process sharing similarities with epithelial-to-mesenchymal transition or dedifferentiation. The mesenchymal subclass has an overall higher component of necrosis and inflammatory infiltrates that can be connected to the high expression of members of the tumor necrosis factor super family pathway and NF-κB pathway [37]. *NF1* impairment has been shown to drive tumor-associated microglia/macrophage (TAM) recruitment in the mesenchymal subtypes; TAMs may be at the basis of an NF-κB mediated proneural-to-mesenchymal transition, observed in proneural patients who display a mesenchymal GBM recurrence along with radioresistance [39, 41].

The proneural class is defined by alterations in *PDGFRA* and *IDH1*. Amplification of *PDGFRA* at 4q12 occurs with or without point mutations in the gene at higher rates compared to other classes. Most of the *IDH1* mutations were found in this subtype, mainly in cases without *PDGFRA* alteration. Also, *TP53* mutations or LOH were described mainly in this subtype. Chromosome 7 amplification paired with chromosome 10 loss are events that are commonly seen in GBM but displayed less prevalence in this subtype accounting roughly for half of the samples analyzed. Oligodendrocytic development genes (*PDGFRA*, *NKX2-2*, and *OLIG2*) are highly expressed in this subtype [37]. *OLIG2* has been suggested to down-regulate *CDKN1* and thereby contribute to increased proliferation [42]. The proneural subtype also displays expression of proneural development genes, such as *SOX* genes [37].

Subtype mosaicism and subtype conversion have been observed in GBM patients. Thus, the relationship between subgroups has been explored. Mathematical modeling has suggested that chromosome 7 gain and chromosome 10 loss followed by loss of *CDKN2A* and/or mutation in *TP53* are early events leading to a tumor with proneural-like characteristics.

PDGFA and *PTEN* are key drivers of further alterations. Subtype-specific mutations arise at a later stage [40].

Patient outcome and response to intensive treatment has been evaluated in these groups. Mesenchymal and classical subgroups seem to benefit more from intensive treatment compared to the proneural group. At first, patients with proneural GBM showed a trend of longer survival [37]. Interestingly, it was later on demonstrated that the survival advantage of the proneural class derived from a subgroup of tumors, the G-CIMP GBMs [24, 37]. This is in line with younger age, methylated *MGMT* and *IDH1* mutation displayed by G-CIMP glioblastoma patients, and the fact that nearly all secondary GBMs analyzed in the study fell in the proneural class.

1.4.2 A deeper layer of heterogeneity

The advent of single-cell technologies has allowed in-depth studies designed to analyze the complexity of cell populations in a given sample. Techniques such as single-cell RNA sequencing can in fact provide data on the transcriptomic profile at the single-cell level [43]. Studies exploiting such methodology can help to unravel the complexity of human tissues and their pathological counterparts, allowing the identification of cell types and functions at higher resolution [44]. Such approaches have also been applied to the brain.

Recent efforts have described different transcriptomic statuses of malignant cells within the same GBM case [45-47]. Different malignant cells in the same GBM specimen showed mosaic gene expression of different RTKs, including different *EGFR* variants. This observation has important consequences from a therapeutic point of view, given the fact that RTKs are commonly explored therapeutic targets [45]. An unbalanced expression of the targeted RTK within the same tumor can invalidate the efficacy of a novel therapeutic approach. It is therefore important to further explore intra-tumor variability of GBMs.

In a recent study comparing the single-cell transcriptome data of malignant cells belonging to different GBM specimens, four cellular states were defined to drive GBM malignancy. These cellular states resemble neural progenitor cells (NPCs), oligodendrocyte-progenitor cells (OPCs), astrocytes and mesenchymal cells. At least two of the cellular states occurred in each tumor analyzed in the study, but the majority of the cases contained all four states [46]. Different distribution rates of the cellular states within the same tumor seem to recapitulate one of the three GBM subtypes (proneural, classical or mesenchymal) as defined by Verhaak et al., suggesting that bulk GBM transcriptome sequencing allows subtyping at a lower grade of resolution [45, 46]. Malignant cells showed plasticity with regard to transition between states, and genetic factors were indicated as responsible for the shaping of state distribution within the same tumor. *EGFR* alterations were associated with higher proportion of astrocyte-like cells. *CDK4* amplification was found to be associated with high frequency of NPC-like state and *PDGFRA* amplification with OPC-like state. Finally, abundance of mesenchymal-like state is favored by deletions at chromosome 5q and *NF1* alterations [46].

1.4.3 Cell of origin

The identification of the cell of origin of GBM is an area of continuous studies. On one hand, studies mentioned in the previous sections suggest that GBMs, given their extensive level of heterogeneity, hold their origin in different cell types. This concept is supported by a number of observations. In fact, GBMs resemble normal neural and glial cell types with regard to morphology and marker expression [9]. In addition, transcriptional profiles of the different GBM subtypes resemble that of normal glial cell types [37]. Moreover, different cell types such neural stem cells, glial precursor cells, oligodendrocyte precursor cells and astrocytes have shown potential of giving rise to gliomas in mouse models [48, 49]; different cells of origin have also been associated with a specific GBM subtype, malignancy and response to drugs [49-51].

On the other hand, other models have suggested that a single cell type of origin may give rise to the different linages of GBM cells, representing different stages of tumor evolution [40, 46].

Continued studies aimed at better clarifying the cell of origin of GBM are relevant to improve the understanding of the disease and the development of new therapies.

1.5 CURRENT AND EXPERIMENTAL TREATMENT

Given the highly infiltrative nature of GBM, which represents the basis for its recurrence, the diseased tissue cannot be completely resected. Surgery is therefore not curative, and virtually all patients experience recurrence that will eventually lead to death [52]. Surgery alone leads to a median patient survival of only 3-6 months [53].

After being diagnosed with GBM, patients are subjected to surgical resection and then generally follow a protocol of radiation with concomitant and maintenance TMZ [54]. In the US, patients can be offered tumor treating fields (TTFields, see §3.2.3, p. 32) together with maintenance TMZ, given the fact that this regime has been shown to improve progression-free survival (PFS) and overall survival (OS) compared to TMZ alone [55]. After recurrence, the protocol can vary largely depending on clinical characteristics of the patient; surgery and radiotherapy (including gamma-knife radiosurgery) can be considered, whereas patients mainly receive TMZ rechallenge and nitrosureas. Bevacizumab is also considered for recurrent GBM in the USA, while it is not approved for this use in the European Union [26]. Further details about therapeutic approaches are provided in §3 (p. 31).

Efforts are ongoing to understand the complexity of this disease and identify critical pathways that are altered in GBM and may become targets for therapy, hopefully improving the outcome [56]. As already mentioned, high inter-tumoral GBM heterogeneity makes tumor targeting particularly challenging [45]. GBMs are highly heterogeneous also spatially within the same tumor, reflecting their transcriptome heterogeneity. Different regions of the same tumor vary with regard to oxygenation, proliferation, infiltration and vascularization [52].

Other than MGMT methylation, there is a lack of predictive markers for therapy. Failure of new experimental therapeutic approaches has been assigned to the intra-tumor heterogeneity that allows multiple resistance mechanisms to manifest. It is thus unlikely that one molecule alone will be able to cure GBM, because of the cell-cell genetic background differences and resistance mechanisms [52]. As an example, treatment-naïve TMZ-resistant clones have been identified using single-cell clonal analysis. These clones further showed different sensitivity to a panel of chemotherapeutic molecules [57]. This indicates the need for simultaneous pathway inhibition in GBM therapy.

2 TUMOR MICROENVIRONMENT IN GLIOBLASTOMA

The brain tumor microenvironment, composed of different cell types and extracellular matrix, has emerged as an interesting study field for a broader understanding of brain tumors and improved therapy design. A complicated network of crosstalk between malignant cells and the tumor microenvironment remarkably alters several properties of the tumor. The brain tumor microenvironment is crucial in both primary tumors such as GBM and metastatic brain disease that frequently arises from lung, breast and skin cancer [58].

2.1 ROLE OF THE MICROENVIRONMENT IN CANCER

Cancer is a complex disease. Several efforts in the history of cancer research were aimed at understanding the mechanisms through which a normal cell transforms into a malignant cell, acquiring capacities that include sustained proliferative signaling, evasion of growth suppression mechanisms, resistance to apoptosis, replicative immortality, angiogenesis, and activated invasion and metastasis [59]. To address this matter it is necessary to analyze not only the malignant cells, but also components of the tumor microenvironment [59]. In fact, a number of cell types functionally interact with the malignant cells. Cells of the tumor microenvironment can become functionally and phenotypically altered, and regulate important aspects of tumor biology, including response to chemotherapy. Notably, these interactions between cells of the tumor microenvironment and malignant cells may also represent interesting novel therapeutic targets [60].

The correct function of the healthy brain is made possible by the interactions between the different cell populations. Astrocytes, microglia and oligodendrocytes are the main cell populations collectively named “glial cells” of the mature central nervous system, or alternatively “neuroglia”. The term “glia” comes from Greek and means “glue”, alluding at the connective role of these cells, as opposed to the primary functional role of the neurons [61]. This is an old definition and is not complete, given the fact that glial cells play meaningful roles in brain physiology and pathology.

Astrocytes are star-shaped cells with elaborate processes. They support neural signaling by regulating the chemical environment and participating in the formation of the blood brain barrier [62]. They are connected through gap junctions to each other, and receive signals from neurons and can signal back to them [63]. Astrocytes regulate synaptogenesis during brain development by secreting factors that include the neuroprotective activity-dependent neurotrophic factor (ADNF) [64]. They also play an important role in regulating neurotransmitter uptake and ion homeostasis. Astrocytes can basically interact with all CNS cells [65].

Oligodendrocytes hold as main task the myelination of neuronal axons, making the transmission of electric signals at extreme speed possible [62]. In addition, oligodendrocytes secrete growth factors that act on the neurons, such as brain-derived neurotrophic factor, NT-3, insulin-like growth factor-1, and TGF-β [64].

Microglial cells are the resident macrophages of the brain that origin from either hematopoietic cells or neural precursors. They have scavenging functions as macrophages, and also secrete a spectrum of cytokines that play a role during inflammation [62]. Microglia and astrocytes are implied in neuroplasticity processes by releasing trophic factors that comprise fibroblast growth factor (bFGF) and nerve growth factor (NGF). Additionally, astrocytes can up- or down-regulate microglial function by secreting factors such as TGF- β or IL-12 [64].

The physiological equilibrium between these cell types can drastically change in cancer; the following paragraphs will address this issue. In fact, GBMs are rich in components of the microenvironment. The malignant cells are immerged in a multitude of cell types such as astrocytes, macrophages, microglia, endothelial cells and stem-like cells [66]. A mouse study compared stromal cells from healthy brain to those from GBM xenografts, named tumor-associated glial cells (TAGs). Genes associated with self-renewal and immature cell types were upregulated in TAGs, which were also able to promote tumor growth in a co-injection *in vivo* model [67].

2.2 TUMOR-ASSOCIATED ASTROCYTES

The astrocytes represent between 20% and 40% of the glial cells in the brain, depending on the region [68]. They are very important in several physiological processes and have been described to play roles in primary and secondary brain tumors with regard to tumor proliferation, invasion and response to treatment [69].

Astrocytes react to damages in CNS with a process called astrogliosis, with the aim of repairing the affected tissue. Dysfunctions in astrogliosis can however exert harmful effects. Astrogliosis is determined by a spectrum of changes in the astrocytes with regard to proliferation, metabolism and cell signaling. Many astrocytes in the healthy brain, but not all, express GFAP; following CNS injuries, GFAP is upregulated and basically expressed by all reactive astrocytes [70, 71]. The changes that astrocytes undergo during astrogliosis seem to be important in glioblastoma and other brain tumors, generally leading to pro-tumorigenic effects [72]. GBM xenografted mice show a peak of astrogliosis 3 days after implantation, with GFAP upregulation happening in parallel with tumor growth. In these settings, the extent of astrogliosis has also been shown to strongly correlate with tumor size [73]. Astrocytes thus appear to be a complex cell type, which can turn to tumor-associated astrocytes (TAAs) under the influence of a malignancy like GBM [74].

By comparing gene expression of TAAs and normal astrocytes in PDGF-driven murine gliomas, the MHC Class II pathway was found to be active in the former. This signature was also shown to predict survival in human proneural GBMs specifically [75].

Recent studies, also taking advantage of single-cell technologies, have explored the previously poorly understood significance of astrocytes subpopulations [76-78]. This poses the basis for studies aimed at understanding the biomarker validity of specific TAA

subpopulations. For instance, a subpopulation of $pSTAT3^+$ astrocytes in brain metastases was associated with shorter patient survival [79].

2.2.1 Effects on tumor proliferation

The effects of the astrocytes on tumor growth have been only marginally explored in the past years. It was observed that secretion of TGF-β, IL-6, bFGF, EGF and growth/differentiation factor-15 (GDF-15) by the astrocytes may play a role in primary brain tumors and brain metastases [69, 74]. Reactive astrocytes of the tumor environment also release CHI3L1, driving MAPK signaling and favoring proliferation in GBM cells [80]. Furthermore, GBM-derived extracellular vesicles have been shown to stimulate the astrocytes to release growth factors, which in turn can stimulate GBM growth [81].

2.2.2 Effects on tumor invasion

Collectively, multiple studies have indicated the TAAs as important mediators of brain tumor invasion. GBM invasion mostly occurs along blood vessels, also exploiting astrocytic end feet [82].

Astrocytes were described to be capable of modulating levels of the pro-form of matrix metalloprotease-2 (MMP-2), and in presence of a GBM cell line they were able of converting it to active MMP-2, a proteolytic enzyme implicated in the invasiveness of glioma cells [83]. In an *in vitro* model the astrocytes stimulated GBM invasion through a co-culture dependent increase in IL-6 production that leads to MMP-14 and MMP-2 activation in GBM cells [84].

In another study, the role of glial-derived neurotrophic factor (GDNF) in glioma progression was investigated. Astrocytes secreted GDNF, which acted on GBM cells and stimulated their invasiveness *in vitro* involving ERK and Akt pathways [85]. A mouse study using a GDNF co-receptor (expressed by GBM cells) inhibitor on mice injected with tumor cells showed development of significantly smaller tumors in the treatment group [85].

High expression of receptor activator of NFκB ligand (RANKL) has been shown to correlate with enhanced invasion in tumor models and TAA accumulation. Low RANKL-expressing GBM cells were engineered to overexpress this factor and subsequently implanted in mice, where they showed enhanced invasion. The astrocytes were activated by RANKL through the NFκB pathway and secreted a number of factors, including TGF-β, that can stimulate glioma cell invasion [86]. Moreover, TAAs enhanced *in vitro* invasion of glioblastoma stem-like cells (GSCs) by chemokine and cytokine secretion [87].

Astrocytes also express connexin43 (Cx43), a major gap junction protein. A study compared glioma invasion in mice carrying wildtype Cx43 astrocytes or C-terminal truncated Cx43 mutant astrocytes, showing that abolishment of Cx43 function was sufficient to decrease glioma spreading [88].

2.2.3 Effects on tumor sensitivity to treatment

A number of GBM cell lines have been shown to acquire astrocyte-derived chemo-resistance to TMZ [89, 90]. Chemoprotection relies on physical contact between astrocytes and GBM, as transwell assays have shown. Cx43 is the major component of gap junctional communication in astrocytes. When inhibiting gap junctional communication between astrocytes and GBM cells in these experiments, the chemoprotective effect of astrocytes was lost. Gene-expression profiling revealed that astrocytes alter drug resistance, anti-apoptosis and survival genes in GBM through gap junctional communication [91].

GBM cells have also been shown to stimulate conversion of normal astrocytes into reactive astrocytes *in vitro*, enhancing their capability of delivering *MGMT* mRNA back to GBM cells via exosomes. Such a mechanism can convert GBM cells from TMZ-sensitive to TMZ-resistant and could underlie yet another TAA-driven chemoprotection phenomenon [92].

In addition, GSCs displayed a reduction in radiosensitivity when co-cultured with astrocytes, together with activation of STAT3. Inhibition of STAT3 has shown potential in enhancing GSCs radiosensitivity *in vitro* and orthotopic xenografts [93].

2.2.4 Other tumor-supportive effects

TAAs have also been shown to support GBM survival in distinct ways. For instance, resistance to hypoxia and vascularization are enhanced by TAAs. When they are exposed to hypoxic conditions, TAAs secrete CCL20 which binds the CCR6 on GBM cells, ultimately leading to activation of NF-κB and HIF-1 and resistance to hypoxia. This observation is supported by *in vivo* data: CCR6-deficient GBM xenografts display in fact slower growth and poorer vascularization compared to control [94].

Vasculogenic mimicry is a process of vascular-like channel formation that is independent of endothelial cells and correlates with prognosis in high-grade gliomas. Astrocytes enhanced vasculogenic mimicry in an *in vitro* model relying on TGF-β1. Galunisertib is a TGF-β1 inhibitor that was shown to inhibit astrocyte-dependent vasculogenic mimicry and tumor growth in mice [95].

Tumor necrosis factor receptor superfamily 9 (TNFRSF9) inhibition led to tumor eradication and prolonged survival in a glioma animal model [96]. Gliomas displayed a higher expression of TNFRSF9 when compared to normal brain tissue, and the main source of this factor was a subclass of reactive astrocytes found in perivascular and peritumoural areas. This phenomenon was especially prominent in IDH1 mutant gliomas [97].

Astrocytes, microglia and tumor cells were shown to interact with each other, ultimately mediating activation of the JAK/STAT pathway in the astrocytes and secretion of anti-inflammatory cytokines including IL-10. This crosstalk ultimately causes reactive astrocytes to favor an immunosuppressive environment in GBM [98].

Using an *in vitro* approach, reactive astrocytes have also been described as a key component that leads to a tumor-supportive postsurgery microenvironment, potentiating tumor aggressiveness. [99].

In summary, several studies have explored diverse and complex ways through which TAAs affect processes in GBM such as vascularization, immunoprotection and malignant cell survival, apoptosis and invasion [74].

2.3 TUMOR-ASSOCIATED MICROGLIA/MACROPHAGES

The resident macrophages in the brain are called microglia. Together, TAMs represent approximately 30% of glioma tissue [100]. The origin of TAMs has been debated and controversial until recent studies provided new evidence. Microglia have been shown to originate during embryogenesis from primitive myeloid precursors in the yolk sac, following different waves of hematopoietic events [101, 102]. Macrophages are bone-marrow-derived cells that migrate to the brain in pathological settings [103]. Efforts aimed at clarifying the ontogeny of TAMs have suggested that both circulating monocytes and resident microglia contribute to the brain tumor TAMs, which keep to some extent memory of their source [104]. Furthermore, it was observed in genetically engineered mouse models that microglia-derived TAMs locate mostly in the peritumoral area and perivascular TAMs are mostly monocyte-derived [105].

The clinical significance of TAM infiltration in gliomas has not been definitely clarified; studies exist associating presence of TAMs with better or worse patient survival [106]. This also suggests the need for a better characterization of TAM subpopulations that may have diverse functions and impact on the biology of different GBM subtypes [107]. Mesenchymal GBMs have been described to contain the highest quantity of TAMs compared to classical and proneural GBMs. Furthermore, high levels of *AIF1* expression (a TAM-specific marker) correlated with worse and better survival respectively in proneural and mesenchymal GBMs from a TCGA dataset [108].

Experiments performed *in vitro* have captured a first degree of polarization of TAMs, classifying them into M1 (pro-inflammatory) and M2 phenotypes (anti-inflammatory). For both states there are data indicating corresponding mediators that lead to polarization. For instance, ligands of toll-like receptor 4 (TLR4) and IFN-γ are usually associated with the acquisition of M1 polarization, whereas IL-4, IL-10 and IL-13 are associated with M2 polarization [109]. This compartmentalized view of TAMs polarization is hard to translate *in vivo*, where the biology of TAMs seems more complicated and plastic [110]. Nevertheless, the expression of the M1-polarization marker CD74 has interestingly been associated with better prognosis in high-grade glioma patients, compatibly with an anti-tumoral immunoenvironment [111].

Microglia recruited by GBMs display distinct features compared to functional, pro-inflammatory microglia seen in non-tumoral diseases [66]. A number of factors released by GBM cells have been reported to be chemo-attractants for TAMs, such as monocyte

chemotactic protein-3 (MCP-3) [112], colony-stimulating factor 1 (CSF-1) [113], granulocyte-macrophage colony stimulatory factor (GM-CSF) [114] and EGF [115]. The most important factors appear to be CCL2 and CX3CL1 [106]. In general, all these factors induce an M2-polarization of the TAMs [116]. In GBM experimental models, malignant cells produce Ccl2 which attracts macrophages, and the inhibition of Ccl2 and its receptor Ccr2 prolongs mouse survival [117].

A study from 2002 showed for the first time that microglia could stimulate glioma invasion using a Boyden chamber. When further stimulated with GM-CSF or LPS (known to activate microglia), they enhanced glioma cell motility to a higher extent [118]. This finding was recapitulated in an experiment using mouse microglia-depleted brain slices, and the stimulation of glioma invasion was attributed to an increase in the activity of MMP-2 [119]. Glioma cells, which themselves release pro-MMP-2, also produce factors that stimulate microglia to release membrane type 1 metalloprotease (MT1-MMP) which is responsible for the conversion of the pro-enzyme to active MMP-2 [120]. Also the CX3CL1/CX3CR1 pathway activation has been connected to metalloproteinase activity enhancement; furthermore, polymorphisms of the allele CX3CR1 (upregulated in microglia in the context of glioma) have been associated with prognosis in glioma patients. The common CX3CR1 was described to be a favorable prognostic factor in glioblastoma, associated with less microglia infiltration [121, 122].

It was also shown in a mouse model that microglia depletion led to a 80% reduction in glioma mass, while at the same time another study on the same model demonstrated that macrophage depletion had the opposite effect, a 33% increase in glioma growth, suggesting a complex relationship between glioma and TAMs [120, 123].

Colony stimulating factor-1 (CSF-1) is important for macrophage differentiation and survival. In another mouse model, a CSF-1R inhibitor was used with the intent of achieving a reduction in TAMs and thus a better survival. This study showed that glioma growth and progression were blocked using a CSF-1R inhibitor, but the TAMs were still present in the tumor. Further analyses showed that these TAMs had been re-educated, leading to a reduction in the proportion of M2-polarized macrophages [113], that have been described in many tumors as pro-tumorigenic [124]. It was subsequently shown that tumors recur in 50% of the mice after continuous CSF-1R inhibition. The mechanism behind the resistance is microenvironment-driven. In fact, transplantation of resistant tumors in naïve hosts reestablished sensitivity to CSF-1R inhibition. Macrophages were shown to secrete insulin-like growth factor-1 (IGF-1) thus activating IGF-1 receptor (IGF-1R) on tumor cells that responded with activation of phosphatidylinositol 3-kinase (PI3K) pathway [125].

In TAMs isolated from mice injected with GBM cells, genes coding for osteoactivin and the secreted form of osteopontin were found to be highly expressed compared to control cells. The two genes have shown correlation with poor survival in human GBM following patient dataset analysis [126]. TAM-derived osteopontin has also shown to lead to suppression of glioma growth [127] and reduction in apoptosis [128], depending on experimental settings.

The mechanisms that lead to TAM reprogramming and their precise role in gliomas are still to be clearly defined. Many studies have shown pro-proliferative effects of TAMs, together with capability of disrupting the extracellular matrix (ECM) and promoting glioma invasion, while others proposed alternative effects on malignant cells; the actual proportion of microglia and macrophage in the glioma/GBM is also still controversial [106]. This likely reflects the plasticity and heterogeneity of these cells and a complicated relationship with malignant cells, together with lack of a comprehensive view on different TAMs subpopulations and functions. Single-cell RNA sequencing strategies are a possible tool to further clarify the role of TAMs in brain tumors [129].

2.4 BRAIN TUMOR VASCULATURE

GBM growth, progression, invasiveness and resistance to treatment are also strongly dependent on tumor-mediated alterations of the brain vasculature. Neurons, astrocytes, endothelial cells, pericytes and ECM components regulate blood supply in the healthy brain [130]. A distinct feature of the brain vasculature is the blood-brain barrier (BBB), an interface structure where endothelial cells and astrocytes play a major role in regulating the exchanges between peripheral circulation and CNS. In pathological conditions such as GBM, the disruption of the BBB leads to serious consequences for the CNS [131]. For instance, brain-tumor-related edema, characterized by water accumulation in the brain with subsequent increase in intracranial pressure, is one of the primary causes of death in glioma patients [130].

GBMs are among the most angiogenic and vascularized tumors. The structure of the vasculature in glioblastoma is altered if compared to the healthy brain [132]. Areas of angiogenesis are characterized by microvascular proliferation (MVP), where endothelial cells, pericytes and smooth muscle cells undergo hyperplasia. MVP has also been associated with increased malignancy [66]. The vascular architecture has been shown to be the niche for neural stem cells (NSCs) in the healthy brain [133] but also to play a role in brain tumors in hosting brain tumor stem cell (BTSCs) [66].

Endothelial cells are involved in brain tumor progression through the regulation of oxygen and nutrient delivery and the secretion of factors that act on the BTSCs attracting them, maintaining them in a stem cell-like state, and stimulating their tumorigenicity [66, 134]. Nitric oxide and Notch signaling are thought to be implicated in this process [66]. The perivascular niche (PVN) therefore assumes an important role in GBM biology. Pericytes have been described to play an essential role in increasing tumor vascular permeability and to be implicated in tumor progression, especially NG2-expressing pericytes [66]. HIF-1α has also been shown to recruit bone marrow-derived endothelial and pericyte progenitors and promote neovascularization in glioblastoma [135].

Around the vasculature there are also reactive astrocytes that play an important role in tumor progression [66]. Astrocytes residing in the PVN produce SHH which affects BTSC self-renewal and growth and is correlated with glioma grade [136]. The Gli signaling, effector of

the SHH pathway, is also thought to be an important driver of neural stem/progenitor cell hyperproliferation and subsequent brain tumor initiation [137, 138].

In the initial phase of vascular remodeling, tumor cells organize themselves in close contact with existing vessels and start destroying them in a process defined “vessel co-option” [130]. Subsequently, endothelial cells actively direct angiogenesis, which is the most common mechanism of vessel formation in GBM. Degradation of the extracellular matrix activates the endothelial cells that are attracted by the malignant cells expressing pro-angiogenic factors, ultimately mediating endothelial cells survival and adhesion. The endothelial cells then recruit the pericytes through the expression of PDGF, achieving in this way the formation of the basement membrane [66].

New vessel formation in glioblastoma is mediated by pro-angiogenic growth factors. VEGF and SDF-1α are among the most important regulators of angiogenesis, secreted by cells like GSCs [139-141]. Hypoxia induces activation of HIF-1α in glioblastoma cells. *VEGF* is a transcriptional target of HIF-1α, and the tumor vasculature express the receptor VEGF receptor 2 (VEGFR2) [142, 143]. VEGF also mediates endothelial progenitor cell recruitment and proliferation, leading to their differentiation to endothelial cells and *de novo* vessel formation, a process known as vasculogenesis [130].

Through analyses of an *in vivo* model, it was shown that VEGF is also released through a mechanism that involves pleiotrophin (PTN), a small angiogenic cytokine. PTN is expressed in the brain during development and is found to be upregulated in tumors. By activating anaplastic lymphoma receptor tyrosine kinase (ALK) on perivascular cells, it mediates VEGF release in close contact to the vasculature, thus supporting vascular abnormalization in GBM [144].

Another factor that contributes to tumor vessel abnormalization is the C-Type Lectin CD93, which activates β_1 integrin signaling [145]. In an orthotopic mouse model of GBM, wild-type mice showed shorter survival when compared to $CD93^{-/-}$ mice. In the former, CD93 expression was mainly restricted to the vasculature that was found to be aberrant, with malformed vessels that recapitulated the situation of human GBMs [145, 146]. Laminin-411 ($\alpha_4\beta_1\gamma_1$) has been shown to increase the expression of Notch pathway members in endothelial cells and astrocytes, specifically due to the laminin β_1 chain. High vascular laminin-411 expression in human GBM samples has been associated with higher malignancy, recurrence, expression of stem cell markers and shorter patient survival; this may be due to a tumor-microenvironment crosstalk. Furthermore, longer survival and slower tumor growth were observed in a CRISPR/Cas9 laminin-411-depleted GBM mouse model [147].

The abnormal status of tumor vasculature is a hallmark of GBM and represents a target for novel therapies. Furthermore, there are indications for the association between failure of antiangiogenic, VEGF-targeted therapy and tumor vessel status, indicating a potential of vascular features to be developed to biomarkers for response to anti-angiogenic treatment

[132]. MicroRNAs have also been implicated in the alteration of endothelial cells in GBM and as potential therapeutic targets [148, 149].

Vasculogenic mimicry, mentioned in §2.2.4 (p. 24), is another important mean of tumor vascularization, independent of angiogenesis or endothelial cells. This process has been associated to worse prognosis and resistance to radiotherapy in gliomas [130, 150].

An additional mechanism of tumor vessel shaping is the transdifferentiation of GSCs into endothelial cells [151] and pericytes [152]. High pericyte coverage has been associated to shorter survival in chemotherapy-treated GBM patients. Interestingly, it has been shown in a mouse model that targeting GSC-derived pericytes disrupts the blood-tumor barrier while leaving the BBB intact, enhancing therapeutic delivery to the tumor [153].

3 THERAPY

3.1 STUPP REGIMEN

As mentioned in §1.5 (p. 18), the basis for the current GBM therapy is referred to as "Stupp regimen", based on a study from 2005 analyzing two cohorts that received either chemotherapy alone or concomitant chemotherapy and TMZ. From the study it emerged that the OS was significantly longer in the group that received the combined therapy compared to radiotherapy alone (14.6 and 12.1 months, respectively) [54].

MGMT methylation status has been demonstrated to correlate with response to therapy. Patients with *MGMT* methylation benefit from combined therapy more than patients with unmethylated *MGMT* (respective median OS: 21.7 and 12.7 months). However, due to a lack of better alternatives, combined therapy is still given to both groups of patients [32].

3.2 NOVEL THERAPEUTIC APPROACHES

Given the dismal prognosis of GBM, a constant investigation of novel therapeutic approaches has been performed during the last decades. On one hand, a few trials have addressed the improvement of surgical intervention (e.g. use of fluorescent dyes like 5-ALA to visualize tumor margins during operation), radiotherapy (e.g. gamma-knife radiosurgery) protocols and imaging techniques [154, 155]. The major trend has been the evaluation of systemic therapies; the following sections will focus on the development of such novel pharmacological tools for the treatment of GBM. Among the hundreds of GBM clinical trials present on ClinicalTrials.gov, the majority involve the use of either small molecules or biological products, and the most promising approaches so far seem to be immunotherapy and TTFields (described in §3.2.3, p. 32) [156].

3.2.1 Angiogenesis targeting studies

As explained in §2.4 (p. 27), brain tumors are highly vascularized and display abnormal vessels with altered permeability. They express high levels of vascular endothelial growth factor A (VEGFA) that is responsible for increased angiogenesis and vessel leakiness. The levels of VEGFA in brain tumors are increased by hypoxia and acidosis, and cells of the tumor microenvironment are also a source of VEGFA [157]. Antiangiogenic therapy has thus been explored in GBM.

Bevacizumab was developed in 1993. It is a humanized variant of an anti-VEGF antibody that was able to suppress tumor growth in vivo [158]. Two big double-blinded randomized phase III studies have looked at the outcome of newly diagnosed GBM patients treated with bevacizumab, thus targeting angiogenesis. Both studies showed similar improvements of PFS, but failed to demonstrate increased OS [159, 160]. A retrospective study of one of the bevacizumab trials aimed at evaluating the association between GBM molecular subgroups and benefit from bevacizumab treatment. The study has reported an improved OS for patients of the IDH1 wild-type proneural patients [161]. Even if first-line treatment of GBM with

bevacizumab did not lead to positive results, this drug has been approved by FDA for the treatment of recurrent GBM in the US. Two clinical trials have in fact demonstrated that patients with recurrent GBM benefit from this second-line treatment based on response rate assessment [162].

Other studies have explored different angiogenesis-targeting approaches in GBM. A randomized, open-label phase III study explored the efficacy of cilengitide in newly diagnosed glioblastoma [163]. Cilengitide is an anti-angiogenic drug developed in the 90s that targets the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [164] which are overexpressed on tumor cells and vasculature and were indicated as mediators of tumor-microenvironment crosstalk [163]. This study also failed to prove increased OS in the cilengitide group [163].

3.2.2 Imipridones

ONC201 is the first-in-class of a new family of drugs named imipridones. It antagonizes members of the dopamine receptors family D2, particularly the dopamine receptor D_2 (DRD2), ultimately inactivating Akt and ERK with anti-proliferative and pro-apoptotic effects. The drug has been described to affect tumor cells but not normal cells and to be relevant for different types of cancer [165].

ONC201 has shown promising results during a phase I clinical trial for GBM which highlighted a durable response in a secondary GBM patient carrying the H3 K27M mutation [166]. H3 K27M mutant gliomas exhibit an increased DRD2 expression [167]. Mutation in H3 K27M is a hallmark of diffuse midline gliomas in children and young adults. Clinical trials are evaluating the efficacy of ONC201 in high-grade gliomas, including glioblastoma and diffuse midline glioma, H3 K27M mutant or wildtype [166, 168].

3.2.3 Tumor treating fields

Preclinical data has shown that low-intensity, intermediate-frequency (200 kHz) alternating electric fields interfere with the mitotic process [169]. These electric fields are called TTFields and have been studied as therapeutic approach in a phase III clinical trial comparing chemotherapy-free and TTFields treated patients with chemotherapy active patients, showing increased quality of life but failing to demonstrate increased OS in the intent-to-treat group [170]. After demonstrating the existence of synergy between TTFields and chemotherapy [171], a new phase III study was designed to compare chemoradiotherapy followed by maintenance treatment consisting of TTFields either with or without TMZ. This study has demonstrated a median survival of 20.5 months in the combined TTFields and TMZ group, significantly higher than the group that received TMZ alone, of which the median survival was 15.6 months [172]. As mentioned in §1.5 (p. 18), only GBM patients in the US can currently receive TTField treatment.

3.2.4 Immunotherapy

Another field of cancer treatment is immunotherapy, which consists of a number of techniques aimed at eliminating malignant cells by exploiting the host's immune system. It

can be divided into active and passive cancer immunotherapy. Active immunotherapy aims at directly stimulating the host's immune system through the infusion of tumor associated antigens, for example. Passive immunotherapy mainly uses antibodies directed against tumor associated antigens or *ex vivo* cultured immune cells that are delivered to the patient [173].

During the last years, several approaches have been tried with the intent of targeting putative tumor associated antigens (as EGFRvIII, IDH1, and even multipeptide vaccines). Other approaches used dendritic cells loaded with tumor lysate, tumor peptide, mRNA or glioma stem cells. The studies suggest a need for optimization of the protocol with regard to parameters such as timing and combination with alternative therapies, together with choosing the appropriate biological target. Nevertheless, there is a number of studies that are currently active and recruiting patients, mainly in phase I and II [173].

3.2.4.1 Personalized immunotherapeutic vaccine

A completed phase I trial has shown promising results in patients that received a personalized immunotherapeutic vaccine where standard of care had failed. These patients were treated with Gliovac™, a vaccine preparation composed of autologous (derived from the same patient) and allogenic (derived from other patients) antigens, administered together with GM-CSF. The 6-month survival in the patients that received Gliovac™ was 100%, compared to 33% in the control group. A phase II study (NCT01903330) is ongoing given the initial promising findings on the efficacy of this approach [174].

3.2.4.2 EGFR-directed immunotherapy

Rindopepimut is a vaccine composed of a peptide directed against EGFRvIII linked to keyhole limpet haemocyanin (an immunogenic metalloprotein). A phase III trial has failed showing increased patient OS and the study was terminated [175]. Other approaches targeting EGFR with immunotherapy are under study, such as the use of ABT-414, an antibody-drug conjugate targeting EGFR and delivering the antimitotic mafodotin (monomethyl auristatin F, a potent antimicrotubule agent) in recurrent GBM patients. The phase I study has highlighted a 6-month OS rate of 72.5%, together with some expected ocular toxicities, although the therapeutic agent had shown to target mainly the tumor and not healthy tissue [176]. This has led to the completion of phase II trials where a better long term OS was observed [177]. Unfortunately, the phase III study of ABT-414 did not meet OS primary endpoint and was terminated [178]. Failure of such approaches may be explained by the heterogeneity of *EGFR* mutations, which may lead to outgrowth of *EGFR* mutation-independent clones, and mechanisms of resistance that include opportunistic suppression of mutant EGFR upon drug treatment [179, 180].

3.2.4.3 CAR T cells

Patient-derived T cells can be engineered to recognize a tumor specific antigen through the expression of a chimeric antigen receptor (CAR), obtaining what is defined as CAR T cells. CAR T cells have shown relevance in the treatment of liquid tumors and their activity has

therefore been explored in solid tumors, including GBM [181, 182]. Studies exist showing the potential of CAR T cells in GBM, targeting among others IL-13 receptor alpha 2 (IL-13Rα2) [183], carbonic anhydrase IX (CAIX) [184] and EGFRvIII [185]. In a phase I clinical trial (NCT02208362), IL-13Rα2 targeting led to GBM regression and sustained immune response for 7.5 months after treatment with CAR T in one patient with recurrent GBM [183]. Further results from the trial have not been posted yet. Anti-CAIX CAR T have shown initial promising results *in vitro* and in a GBM mouse model, displaying a 20% cure rate [184]. The phase I trial NCT02209376 analyzed feasibility and safety of anti-EGFRvIII CAR T infusion in 10 patients with recurrent GBM. Some of the patients underwent second surgery, allowing sampling of the tumor and evaluation of the response to CAR T treatment; it was possible to observe an increased T cell trafficking and antigen loss. The median OS was around 8 months for the patients object of the study, with one of them staying alive with stable disease for 18 months [185]. CAR T cells seem indeed to induce a certain response in the patients, but they have to face challenges such as tumor heterogeneity and tumor microenvironment-mediated adaptation and escape from the immune response [156].

3.2.4.4 Dendritic cell vaccine

Promising results have been achieved in a phase III study on the dendritic cell vaccine DCVax®-L in patients with newly diagnosed glioblastoma. The vaccine consists of patient-derived dendritic cells pulsed with autologous tumor lysate. The intent-to-treat population received DCVax®-L plus TMZ, and out of 331 patients (median OS of 23.1 months), 100 extended survivors surprisingly displayed a median OS of 40.5 months. The study has shown safety, feasibility and initial efficacy as measured by improved OS and will be continued [186].

3.2.5 Gene-mediated cytotoxic immunotherapy

Therapeutic adenoviral delivery of the herpes simplex virus thymidine kinase (TK) has been investigated in a Phase II clinical trial for GBM. The patients received local delivery of the biological agent to the tumor bed. Subsequently, patients took an oral formulation of the pro-drug valacyclovir, which is activated by the TK in GBM cells leading to apoptosis. In patients with gross total resection, median OS was 25 months in the group receiving aglatimagene besadenovec (the adenovirus delivering TK) together with standard of care and 17 months for the patients receiving standard of care only [187]. New studies are further evaluating this approach in combination with other therapeutics, also led by the fact that the modulation of the immune system seems to be a relevant component of this therapeutic approach [156].

3.2.6 Macitentan

A phase I trial focused on targeting astrocytes and endothelial cells in GBM. Studies on *in vitro* models have shown that astrocytes and endothelial cells mediate TMZ chemoprotection in GBM, which can be reversed by inhibiting the endothelin signaling. GBM cells express the endothelin receptors ET_AR and ET_BR, while astrocytes and endothelial cells – especially

tumor associated endothelial cells – provide the ligand endothelin-1. These observations have been translated *in vivo* in studies providing preclinical validation of the concept [188].

Macitentan is an antagonist of ET_AR and ET_BR. In mice that were given a combination of TMZ and Macitentan after implantation of TMZ sensitive or resistant cells, the OS was significantly longer than in the single therapy or placebo groups [188]. The receptors are expressed in human GBM [189, 190]. The clinical trial (NCT02254954) has unfortunately been terminated due to low recruitment; nevertheless, the biology behind this study is still interesting.

3.2.7 Chloroquine

Chloroquine re-purposing for cancer has been explored following evidence of its anti-neoplastic potential. Originally designed as an antimalarial medication, chloroquine has shown radio/chemo-sensitizing properties [191]. The activity of chloroquine (an autophagy inhibitor) has been studied during the last decade as an adjuvant for TMZ therapy, giving positive results in mouse models [192] and in patients (14 months of difference in median OS) [193]; continued efforts are ongoing to clarify its potential [191].

3.3 OUTLOOK ON THERAPY STATE-OF-THE-ART

In general, the studies mentioned in this section have demonstrated promising potential for further development, given the fact that they demonstrated improvement of OS in certain cases. Many other studies unfortunately fail translation to the clinics due to unfavorable efficacy or safety [194]. When new therapeutic approaches show positive results, they also nearly always uncover the need for a deeper understanding of the biology of GBM and its microenvironment. In fact, new therapeutic agents have several challenges to face, including GBM heterogeneity, BBB penetration, drug resistance, identification of both suitable target and population characteristics, and tumor microenvironment mediated therapeutic escape [156].

4 STRATEGIES FOR THE IDENTIFICATION OF NEW GLIOBLASTOMA TARGETING DRUGS

Advances in the understanding of cancer biology have uncovered new possibilities for drug discovery and development. As discussed before, new therapeutic targets have been identified thanks to the insights regarding the alterations that lead to cancer, including GBM. Nevertheless, despite a number of compounds that have to some extent displayed efficacy, the majority of the compounds fail when brought to clinical trials.

There are several reasons that can lead to this failure. Some of them include drug resistance, alternative mechanisms of escape from the therapeutic effect and the fact that the tumor microenvironment complicates the picture of cancer biology, mostly by giving support to malignant cells. Eventually, these reasons reflect the fact that cancer is an extremely complex disease that is studied using rather simple *ex vivo* models. When a therapeutic agent is found to work on a specific model, subsequent successful validation steps become exponentially challenging in mouse models and especially later in human.

New technologies have made possible the screening of very large numbers of compounds in a given model. High-throughput screening (HTS) of compound or biological libraries is a cost effective and streamlined way of finding new therapeutics. HTSs can be used to interfere with a specific target or phenotype.

The trend of the last decades in drug discovery has followed a pipeline involving molecular-target based HTS and hit finding, with subsequent computer-assisted lead identification and optimization [195]. This approach benefits from a wide understanding of a certain condition object of the study, and it allows screening of small molecules or biologicals such as monoclonal antibodies against a given target. Target-based assays investigate a specific biological hypothesis relying on one sole target. Novel therapeutic agents that are identified in this way can be very strong modulators of the proposed target; at the same time, such a fine modulation may not be relevant at all for the disease under investigation [196].

Phenotypic assays are a promising substrate for HTS, especially given the fact that they do not require prior knowledge of the specific target or mechanism of action. They can help to identify novel compounds that directly interfere with a cellular process (e.g. proliferation, apoptosis) [196]. Once an interesting compound is identified, the following process of target deconvolution is challenging, especially in case of libraries of unknown compounds. There are nevertheless assays including cellular thermal shift assay coupled with mass spectrometry (MS-CETSA) or *in silico* analyses that can help to identify the target of a given compound [197, 198]. Phenotypic assays have a higher potential of contributing to the development of first-in-class drugs than target-based assays [196].

There are a number of assays that are suitable for HTS of compounds in biomedical research. The activity of several compounds can be evaluated on each hallmark feature of cancer by choosing the appropriate assay and the right model [59, 199]. HTS has contributed to the

discovery of relevant therapeutics during the past 20 years, leading to a development of the methodologies granting enhanced quality of produced data and feasibility [200, 201]. Phenotypic HTS assays have also started to contribute to the approval of new therapeutics, appearing as a very powerful resource [202].

Nevertheless, screening a high number of compounds on a disease-irrelevant model can also underlie the risk of long-term failure of drug development. Ordinary cell-line screens with validation in xenografts have widely been used to develop drug candidates that unfortunately most often have failed to show clinical efficacy. New disease-relevant models are needed in order to allow development of new therapeutics by testing them on a system that recapitulates the pathophysiological properties of the disease object of investigation [203].

Most of the research for new therapeutics in oncology has been pursued on cell lines that have been grown for an extended time in culture conditions on plastic substrates. These cell lines may become different from the original cancer that they are supposed to represent [203]. Such cell lines and derived xenograft models used in drug discovery do change in culture and lose the contribution of the original tumor heterogeneity and the tumor microenvironment; furthermore, they may display limitation due to misdiagnosis and contamination [204].

Alternative approaches to overcome this problem are represented by the use of fresh patient-derived cultures/xenografts, co-culture systems and organotypic models.

Patient-derived cultures can better retain the complexity of the original tumor; they can also be directly transplanted to immunodeficient mice as patient-derived tumor xenografts and transferred from mouse to mouse without being affected from *in vitro* culture, this way representing an interesting model for drug validation following HTS [200, 205]. Following HTS of compounds on seven GBM patient derived-cultures, synergy between two test compounds was shown to be lethal in a subset of the cultures, suggesting potential for precision medicine in GBM [206]. A collection of patient derived human glioblastoma cell cultures has been generated containing 48 well characterized cell lines of all molecular subtypes [207]. Such a collection has been used to discover novel potential therapeutic targets for GBM [208, 209]. *In silico* approaches may also be integrated with patient-derived xenograft studies to identify novel treatment modalities [210].

Isolating and focusing on cancer stem cells is also a relevant way of analyzing the activity of new drugs, given the fact that they may represent the population of cells that resists therapy [204, 211]. In a HTS format, a pyrimidine synthesis inhibitor was found to inhibit proliferation, survival, and stemness of glioblastoma-initiating cells *in vitro* and reduce tumor mass *in vivo* [212].

An appropriate way of increasing the likelihood of finding therapeutics that can enhance patient survival is to take into strong consideration the role of the tumor microenvironment. Many changes in cultured cancer cell lines are due to the missing crosstalk with cells of the tumor microenvironment. Co-culture models can be used for HTS of compounds to increase

the likelihood of more accurately reproducing key features of a disease *in vitro* [204]. Co-culturing GBM cells with cells such as astrocytes or TAMs can uncover features of a given compound that would be ignored if it was tested on a monolayer of GBM cells; for instance, a pro-tumorigenic crosstalk with cells of the microenvironment can be targeted in such settings, and microenvironment-mediated resistance to a given compound can be underscored.

Cell growth in 3D models has also been shown to better represent the original tumor conditions. Furthermore, it has been shown that cells grown in 3D settings may respond differently to drugs compared to 2D culture [213]. HTS of compounds in 3D conditions may increase the chance of identifying clinically relevant therapeutic agents.

Spheroids and organotypic models display less compliance to HTS formats, but can represent interesting substrates for drug validation; they better reproduce part of the original tumor properties [200]. A human GBM organotypic slice culture model can be used for the study of GBM in settings that are as close as possible to human, providing a relevant framework for drug validation in addition to mouse models [214].

A zebrafish GBM model has been proposed as suitable for HTS of drugs. GBM cells can be transplanted in an automated and fast way in zebrafish embryos (thousands of embryos per hour), avoiding a time-consuming and challenging intracranial transplantation. The resulting xenografts have been described to recapitulate human GBM and features of the BBB [215].

The stepwise organization of clinical trials in oncology has with time become more elastic, given the nature of oncological diseases. Traditionally, phase I studies were mainly meant to assess safety and pharmacokinetics of a new therapeutic agent in a limited number of healthy volunteers [216]. In oncological clinical trials, phase I studies provide with initial information about the efficacy of a new therapeutic target, which is further validated with phase II and III studies where the superiority/synergy with standard-of-care treatment is also assessed [217]. Choosing the right population that may benefit of a novel therapeutic target is particularly important in cancer; this is possible for example by incorporating presence of the specific alteration towards which the drug is directed in the patient inclusion criteria. The discovery of new clinically relevant biomarkers based on studies of both the malignant cells and components of the tumor microenvironment can help to uncover therapeutic effects that would be hidden within a non-stratified population of enrolled patients [217]. Properties of the complex microenvironment of GBM have shown interesting associations with patient survival and response to treatment, as described in §2 (p. 21). The GBM tumor microenvironment needs thus to be further investigated for novel biomarkers and taken into account in the study of the efficacy of new therapeutics.

In the GBM drug discovery field, the challenges described so far (tumor heterogeneity, therapy resistant glioblastoma-initiating cell, influence of the microenvironment) are complicated by the presence of the BBB. A suitable drug for GBM treatment, in fact, should be able to cross the BBB and reach the tumor. *In vivo* experiments following HTS and compound optimization can help to understand the potential of a new therapeutics of crossing

the BBB. Pathway redundancy and low frequency of common mutations in GBM are also a limiting factor for the identification of drug candidates [218]. Given the complexity of GBM, it appears necessary to target it from multiple angles, rendering combination therapy approaches largely indicated. Because of all the reasons mentioned in this section, it is crucial for the discovery and development of drugs in oncology and especially GBM to choose the most relevant models for HTS and compound validation.

5 PRESENT INVESTIGATION

5.1 AIMS

The general aim of this thesis was to investigate the role of astrocytes in GBM. Astrocytes were studied with regard to their involvement as regulators of tumor growth and patient survival, and subsequently investigated as a therapeutic target.

Specific aims were:

- to study the potential of astrocytes as enhancers of GBM growth *in vitro* and *in vivo*;
- to explore the impact of an "astrocyte signature" on patient survival;
- to examine the prognostic significance of a PDGFRα^{+}/GFAP^{+} subset of astrocytes in a GBM cohort;
- to identify compounds that are able of interfering with the crosstalk between GBM cells and astrocytes by inhibiting the astrocyte-driven tumor growth.

5.2 RESULTS

5.2.1 Paper I

Astrocytes enhance glioblastoma growth

Cells of the tumor microenvironment play a crucial role in the biology of GBM. The role of astrocytes has received only marginal focus until recently. In particular, we have investigated the role of astrocytes in GBM growth with *in vitro* co-culture models and an *in vivo* co-injection model. Furthermore, we analyzed the crosstalk between GBM cells and astrocytes by evaluating perturbations in gene expression of naïve cells with co-cultured cells. We then used such gene-expression alterations to speculate on how GBM-conditioned astrocytes affect patient survival.

We co-cultured GBM cells with astrocytes and subsequently sorted each population out via FACS. We compared gene expression of GBM-conditioned astrocytes to that of naïve astrocytes, identifying a number of genes (e.g. *PLA2R1*, *ADA*, *TLR4*, *ANKRD1*) whose expression levels are changed upon co-culture with GBM cells. We used this collection of differentially expressed genes to assemble an "astrocyte signature" that we then screened against GBM gene expression data from a TCGA cohort, detecting significantly shorter survival in those patients with a high astrocyte signature score. This observation was validated in an additional cohort. The astrocyte signature score was found to be higher in the mesenchymal subgroup of GBM and to correlate with unmethylated *MGMT* promoter.

Using a format similar to the aforementioned one, we identified deregulated genes in GBM after exposure to astrocytes. Periostin (*POSTN*) and serglycin (*SRGN*) were found to be upregulated in astrocyte-conditioned GBM cells, and associated with the high astrocyte signature group of the TCGA cohort.

We evaluated GBM growth in the presence or absence of astrocytes. Using commercially available GBM cell lines and a patient derived GBM culture, we demonstrated that astrocytes enhance GBM growth *in vitro*. In addition, results were reproduced using a primary astrocyte culture. Further analyses suggested specific astrocyte-dependent growth enhancement and increased S-phase entry of GBM cells.

We tested our hypothesis *in vivo* using a NOD scid mouse model, comparing survival of mice that received mono-injection of GBM cells to that of mice that received co-injection of GBM cells together with astrocytes. Co-injected mice displayed a shorter survival. Interestingly, *POSTN* and *SRGN* were found to be upregulated in tumors from such group of mice. This supports the hypothesis that astrocytes contribute to increased GBM aggressiveness.

In summary, this study provides evidence of a previously unrecognized effect of the astrocytes on GBM growth using multiple approaches. The study also proposes a gene signature reflecting activated status of GBM-associated astrocytes, which are implied as enhancers of GBM aggressiveness through correlative analyses of clinical samples. The study also presents putative mediators of the GBM-astrocyte crosstalk and a mouse model suitable for further investigations.

5.2.2 Paper II

Platelet-derived growth factor receptor α/glial fibrillary acidic protein expressing peritumoral astrocytes associate with shorter median overall survival in glioblastoma patients

In the process of GBM growth and invasion, malignant cells crosstalk with cells of the microenvironment, including astrocytes. The relationship between the astrocytes and malignant cells affects GBM biology in a pro-tumorigenic manner. Astrocytes have been described to display heterogeneity in physiological and pathological conditions. PDGF signaling is involved in GBM and astrocyte biology. The significance of astrocyte subsets in GBM has not been widely explored; in this paper we studied the potential of a $PDGFR\alpha^+$ astrocyte subset in the GBM peritumoral area (PTA) by performing analyses of immunohistochemically stained tissue.

By screening a cohort of 45 patients for which PTA was available in the tissue, presence of $PDGFR\alpha^+/GFAP^+$ astrocytes were encountered in 13 cases. Given the infiltrative nature of GBM and potential marker overlapping between malignant cells and astrocytes, experiments were performed to support the non-malignant nature of identified $PDGFR\alpha^+/GFAP^+$ cells. Evaluation of Ki67 status and *EGFR*, *PDGFRA* and chromosome 7 copy number variation supported the idea that $PDGFR\alpha^+/GFAP^+$ astrocyte-like cells in the peritumoral area were host cells.

Total $GFAP^+$ astrocyte density was found to vary significantly throughout the cohort. Presence of the double positive astrocytes was associated with older age, and not associated to other clinico-pathological characteristics (sex, *MGMT* methylation). Presence of double

positive astrocytes was found to be associated with astrocyte density. Interestingly, presence of double positive astrocytes, but not total astrocyte density, significantly correlated with shorter patient survival. A multi-variable analysis subsequently confirmed the survival association, interestingly showing independence from high age.

This study has thus identified the presence of PDGFRα^{+}/GFAP^{+} astrocytes in the PTA as a candidate biomarker for poorer prognosis in GBM. Further studies on this subset may reveal previously unrecognized functions of this astrocyte subset.

5.2.3 Paper III

A high-throughput screen to explore the astrocyte-driven enhancement of glioblastoma growth as a candidate therapeutic target

Novel therapeutic approaches are particularly needed in GBM, given the dismal prognosis despite intensive therapy and continued attempts to develop new drugs. Lack of focus on the contribution of the tumor microenvironment to cancer is among the reasons that may lead to failure of the development of a new therapeutic agent.

Given the role of astrocytes as enhancers of GBM growth, we set up this study to identify compounds that interfere with such pro-proliferative activity of the astrocytes. This study is also motivated by the need of screening compounds on a relevant model system that recapitulates human *in vivo* settings, instead of solely focusing on the malignant cells. Such an approach may increase the clinical translation potential of identified compounds.

We optimized our phenotypic assay described in Paper I in order to make it suitable for HTS of compounds. Using a collection of 1200 known and approved drugs (the Prestwick library), we searched for active compounds that were able of interfering with the astrocyte-driven enhancement of GBM cell growth.

Out of 205 compounds found to be active, 17 displayed specific activity in co-culture settings. Further validation assays confirmed three of these compounds as specifically interfering with the crosstalk between GBM cells and astrocytes. One of these candidate compounds was further validated on an additional cell line, where it displayed specific activity on the astrocyte-driven enhancement of GBM cell growth, while not affecting the autocrine GBM cell growth enhancement.

Preliminary analyses showed basis for further validation of the identified compounds, suggesting the astrocyte-GBM crosstalk as a potential therapeutic target in GBM.

5.3 CONCLUSIONS AND FUTURE PERSPECTIVES

GBM is an aggressive disease with an extremely poor prognosis and urgent need for new therapeutic approaches. No prominent advances have been made with regard to therapy in the last few decades, despite continued studies. Drug discovery and development in GBM is a complex field that relies on one hand on the availability of innovative technologies and

methodologies and on the other hand on a deep understanding of cancer biology; both subjects have developed remarkably during the last couple of decades.

GBM biology has been profoundly investigated, revealing the complexity of this disease. Targeting GBM is challenging given its complicated and heterogeneous genetic landscape and the intricate crosstalk between malignant cells and the tumor microenvironment. In paper I, we describe tumor-growth enhancing properties of the astrocytes in GBM, together with a crosstalk that mediates two-way gene expression changes in both the malignant cells and the astrocytes. These observations are recapitulated *in vivo*, translating in poorer survival of astrocyte/GBM cell co-injected mice, and *in silico*, where an astrocyte signature correlates with poorer patient survival in two GBM cohorts. Results from Paper I are supported by those in Paper II, where we show that the presence of a subset of $PDGFR\alpha^{+}/GFAP^{+}$ astrocytes in GBM patients' PTA correlates with poorer patient survival, suggesting biomarker properties of this astrocyte subset, and postulating an unidentified function of such an astrocyte subset. Our results from Paper I and II are in line with other studies mentioned in this thesis that describe a critical role of the tumor microenvironment at multiple levels of GBM biology.

Paper I and Paper II suggest further studies to improve elucidation of the importance of the astrocytes in GBM. The absence of a widely recognized TAA marker represents a challenge for the characterization of TAA populations. The astrocyte signature could be analyzed in additional cohorts, also with regard to its correlation with response to treatment. In fact, the association between high astrocyte score and unmethylated *MGMT* promoter – a negative predictive factor for response to therapy – suggests that astrocyte and malignant cell co-option may lead to chemotherapeutic resistance. The nature and molecular components of the crosstalk between GBM cells and astrocytes should also be clarified with mechanistic studies. Such studies may take advantage of the collection of deregulated genes in GBM cells and astrocytes following co-culture and the mouse model described in Paper I. The $PDGFR\alpha^{+}/GFAP^{+}$ astrocyte subset should be further analyzed in additional cohorts; the definition of its role may benefit of mechanistic studies using mouse models. Additional cohort studies may exploit IDH mutated GBMs to discriminate between malignant cells and astrocytes, allowing for instance the investigation of the role of this astrocyte subset in GBM invasion. In addition, the $PDGFR\alpha^{+}/GFAP^{+}$ astrocyte subset should be analyzed with regard to vascular and immunological properties of GBM.

The tumor microenvironment is important also as a therapeutic target in cancer, including GBM, as studies have suggested. High-throughput technologies have enhanced the implementation of drug screenings, reducing their cost and time of realization and providing accurate high-content techniques for the investigation of drug activity; despite this, it is complicated to setup an appropriate model to perform HTS of compounds that entirely recapitulates the human disease. The tumor microenvironment modulates hallmarks of GBM and should be taken into account to systematically investigate the activity of novel candidate therapeutic agents. In Paper III we performed HTS of compounds on a co-culture model of astrocytes and GBM cells with the aim of finding compounds that are able of interfering with

the astrocyte-driven enhancement of GBM growth. Three such compounds were identified, suggesting further studies to investigate their target and mechanism of action. The chemical properties of the identified compounds did not render them immediately suitable for *in vivo* validation. We have in fact analyzed the Prestwick library, composed of known and approved drugs. Repurposing existing drugs is particularly challenging in GBM, primarily because they should be able to cross the BBB to reach the target. Nevertheless, identified compounds can be a tool to unravel the crosstalk between astrocytes and glioblastoma by describing their mechanism of action, aided by *in vitro* and *in silico* approaches, and possibilities for their optimization to work in GBM settings may be further evaluated. In general, results from Paper III suggest continued HTS of wider and diverse libraries of compounds on our model to discover and develop candidate therapeutic agents for GBM, and the evaluation of their synergistic effect with standard therapeutics in GBM.

Collectively, Papers I-III recognize important roles of the astrocytes in GBM biology: they support tumor growth, predict patient survival and can be used as a therapeutic target. Our results suggest studies aimed at better understanding the crosstalk between malignant cells and astrocytes in GBM and exploiting it to design new therapeutics for this terrible disease.

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