

Review

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Review

Challenges and Promise for Glioblastoma Treatment through Extracellular Vesicle Inquiry

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The article is dedicated to Sveva and her family, remembering her passionate and creative love for life and people.

Abstract: Glioblastoma (GB), even though not frequent, is an extremely aggressive brain tumor, with a strong impact on patient outcomes, in terms of both length and quality of life. The protocol established by Stupp and coworkers in 2005, based on radiotherapy and temozolomide chemotherapy, after maximum safe surgical resection, remains the gold standard for GB treatment. However, it is evident nowadays that the extreme GB intratumoral and intertumoral heterogeneity, invasiveness and recurrence are not compatible with a routinary and unfortunately ineffective treatment. In this review article, we summarize the principal challenging issues in finding new valuable therapies for GB and focus on the impact that extracellular vesicle (EV) research and exploitation can have in the field. EVs are natural particles delimited by a lipidic bilayer and full of functional cellular content, released and uptaken by cells as key means of cell communications. Moreover, being stable in body fluids, well-immunotolerated, able to cross physiological, inter-species, and inter-kingdoms barriers, and to target specific cells, releasing inherent or externally loaded functionally active molecules, EVs can be potentially ideal allies in fighting GB and assuring to GB patients a better prognosis. Here we report the main results obtained until now, focusing on both EV sources and molecular cargo used in the different functional studies, mainly *in vivo*. Finally, we perform a SWOT analysis reporting both the main advantages and the pitfalls of developing EV-based GB therapeutic strategies with the aim of highlighting the main directions to explore for realizing the promise of EV exploitation for GB treatment.

Keywords: glioblastoma; extracellular vesicles; therapy; drug-delivery

1. Introduction

Gliomas are the most frequent intracranial tumors in adults. The first reports of gliomas date back to the early 19th century thanks to the British Berns and Abernety, whereas the first comprehensive histomorphological description was made by the German pathologist Rudolf Virchow in 1865. Studying gliomas, Virchow first introduced the concept of “neuroglia”, from which gliomas derive as a connective tissue of the brain and the spinal cord, formed by star-shaped units, interconnected by fine fibers, in which the nervous elements are immersed”[1,2]. The term glioblastoma (GB) was first used in 1927 by the neuropathologist Percival Bailey and the neurosurgeon Harvey Cushing, who developed the first systematic classification and histological description of gliomas, giving the base for the glioma modern one [3]. Since then, glioma classification has been updated several times by the World Health Organisation (WHO), introducing different nomenclature and diagnostic criteria. In the fourth edition of the WHO Classification of CNS Tumors (WHO CNS4), the term “multiforme” was abolished [4]. Moreover, in WHO CNS4 and even more in the fifth and last edition of 2021 (WHO CNS5) besides the traditionally used histological and immunohistochemical features, molecular (genetic and expression data) parameters were also included, establishing a different approach to both CNS tumor nomenclature and grading, and emphasizing the importance of integrated diagnoses [5–7].

According to the WHO CNS tumor classification, GB is the highest and most severe prognostic grade, namely “grade IV” glioma, and the most aggressive and lethal among all primary brain

tumors, with a median overall survival with no treatment at most 4 months, otherwise 14-17 months after diagnosis and a five-years survival rate of 5-6% [4,5,8,9]. Although considered a rare tumor, with an incidence rate of about 3 per 100,000 people, GB accounted for 14.5% of all CNS tumors and for 48.6% of the malignant ones, based on the data collected in the central brain tumor registry of the United States (CBTRUS) since 2014 to 2018. [8]. The tumor has a slightly higher frequency in men than in women and a higher incidence in Caucasian than in African or Asian populations. The annual age adjusted incidence of GB increases with age from 0.15 per 100 000 in children to 15.03 per 100,000 in patients over 75, with a median age at diagnosis of 65 years. In pediatric age, however, although rare, GB constitutes one of the groups of neoplasms with the worst prognosis [8,10]. Unfortunately, more recent studies indicate a rise in incidence of both GB and brain tumors in general, as highlighted by the Global Cancer Statistics of 2020 [11]. The etiology of GB is unknown with the only identifiable risk factor being exposure to ionizing radiation. Other possible contributing factors include aging, non ionizing radiation, and air pollution [12,13].

GB mostly develops in the cerebral hemispheres and, depending on the brain area affected and due to increased intracranial pressure, patients can show different clinical features, with a wide range of fast-developing and life-changing symptoms. These include neurological symptoms such as severe headache, loss of vision or alteration of the language, persistent weakness, as well as psychological and psychiatric symptoms, comprising unpredictable personality changes, that can severely compromise the quality of life and even the autonomy of patients, with strong impact on patient's family and entourage [14–17]. In most cases, diagnosis occurs only at the onset of symptoms, and is mostly based on magnetic resonance imaging (MRI), with the relative technical limitation in specificity (difficulties to exclude other diagnosis) and sensibility (spatial resolution of 2-3 mm) [18]. Since 2005 the most widely adopted therapy for GB consists of surgical removal of the tumor mass, followed by radiotherapy and chemotherapy, according to the protocol identified by Stupp and coworkers (Stupp et al., 2005). The surgical removal of GB is quite complex due to the high number of cells composing the tumor and their very high infiltration power in the surrounding healthy tissues, both causes of the high recurrence rate, and is strongly dependent on the location and accessibility of the tumor mass. Where possible, extensive surgical therapy is crucial to improve patients' prognosis and lower the risk of recurrence, even though this might increase the risk of postoperative neurologic deficits [19]. After surgical resection and if this is not possible, patients receive radiotherapy in combination with chemotherapy, of which Temozolomide (TMZ), a DNA alkylating agent, is the chemotherapy of choice, [20].

More recently, alternative therapies, such as extended adjuvant TMZ treatment, use of the monoclonal anti-VEGFA antibody bevacizumab or the receptor tyrosine kinases inhibitor regorafenib, magnetic tumor-treating fields, and immunotherapies have been tested in clinical trials, alone or in combination with already established treatments [21–24]. Moreover, to clearly identify the most promising interventions, several meta-analysis studies have been comparing the efficacy of the different treatments across different randomized clinical trials, highlighting some encouraging results such as the ability of Bevacizumab in combination with TMZ to slightly increase progression-free survival (but not patient overall survival) serving as a salvage regimen for recurrent GB [25–28]. Until now, however, no breakthrough therapies leading to extensive and durable survival of patients have been found and survival rates for GB patients have shown no notable improvement in population statistic-based studies [10]. Therefore, there is a striking need for effective and less invasive diagnostic as well as therapeutic tools to early discover GB onset, and, even more, to block GB progression and avoid its recurrence after surgery.

Improving the knowledge of GB onset and progression at molecular, cellular, and systemic level is critical to unravel new key therapeutic targets and implement effective therapeutic strategies. In this scenario the discovery of natural and functional nanoparticles layered by a lipid membrane, called extracellular vesicles or EVs, both released and up-taken by cells in physiological and even more in pathological conditions, such as tumorigenesis, brings with it a strong potential for inspiring new, more effective and less invasive diagnostic and therapeutic approaches for GB patient

treatment. In this review, we will focus on the significance of EVs for the development of new promising therapeutic strategies against GB.

2. Main issues in GB therapy

The poor prognosis of GB and the objective difficulties in developing successful therapies is due to the complex and peculiar set of features characterizing this type of tumor. In this paragraph we summarize the main GB attributes (Figure 1) to also highlight the direction towards basic investigation, research and development and technology transfer should look at.

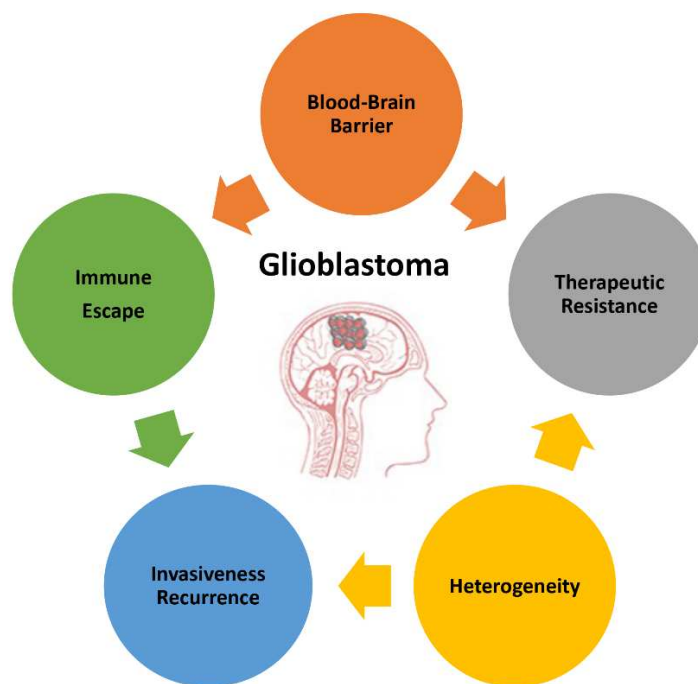


Figure 1. Main features of Glioblastoma responsible of his high malignancy and poor prognosis.

2.1. Blood-brain barrier

The blood-brain barrier (BBB) is a very selective and specialized barrier, preserving the homeostasis of the CNS, protecting and isolating the brain from potentially dangerous molecules transported in the blood such as virus and other pathogens. It has a complex structure formed by multiple layers, including brain microvascular non-fenestrated endothelial cells sealed by junctional complex, surrounded on the outer face by the end feet of glial cells. The physiological barrier is the result of a series of physical, transport, and metabolic processes inside endothelial cells, highly coordinated by interactions with different vascular, immune, and neural cell types [29–31]. Therefore, the BBB is not only a physical barrier, but it actively acts through endothelial efflux transporters to pump back in the bloodstream small lipophilic molecules that can diffuse through the plasma membrane. Even though the BBB has an evolutionary precious role in preserving brain health and functions, unfortunately, in case of brain pathologies, such as GB, its existence has the disadvantage to severely hamper the access to the brain of therapeutic molecules, strongly limiting their clinical success [30–32]. Moreover, as the barrier works in both directions, the flow of molecules from the brain to the bloodstream is also restricted, reducing the diagnostic potential of liquid biopsies based on blood samples in brain diseases [18].

Interestingly, the integrity of the barrier is compromised in many brain pathologies, including GB, but this is not enough to enhance drug accumulation and efficacy. This is in part due to the

extremely heterogeneous barrier disruption and the persistence of intact barrier besides tumor areas [31,33–35]. Moreover, in GB, the impaired BBB, also referred as blood-tumor barrier (BTB), is able to function synergistically with intact BBB to create a tumor-supportive adaptive environment, paradoxically permitting the transport of pro-tumoral molecules while limiting the entry of antitumoral ones [36,37].

2.2. Heterogeneity

GB is a highly dynamic and fast evolving tumor, characterized by a strong molecular and cellular heterogeneity inside the same tumor at a fixed time point, as well as at different times during tumor development and even more in different patients [38,39]. The high inter- and intra-tumoral heterogeneity can affect tumor diagnosis and characterization, being challenging to achieve a comprehensive description of the whole molecular signature in such a heterogeneous tumor through surgical biopsies. To have better chances of exact diagnosis, biopsies should be several and repeated during GB progression, a practice that is not feasible nor sustainable for the patient [18]. Moreover heterogeneity makes it exceptionally challenging to target all tumor cells inside the same patient as well as to cure different patients with the same drug [40]. Regarding GB, we can distinguish a molecular and a cellular heterogeneity.

- Molecular:

In 2016, the WHO CNS4 classified gliomas into low grade gliomas or LGGs and GB, further subdividing GB into primary and secondary tumors [4]. Primary and secondary GBs are histologically indistinguishable, but they develop from different genetic precursors and therefore show different genetic alterations. Primary or de novo GB is characterized by no mutation in isocitrate dehydrogenase 1 (IDH1), accounts for 90% of all GBs and affects older patients, showing a worse prognosis. Among the most frequent genetic alterations, loss of heterozygosity (LOH) at 10q chromosome, mutations or deletions in phosphatase and tensin homolog (*PTEN*) gene, mutation or amplification of the epidermal growth factor receptor (*EGFR*) gene, and deletion of Cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*) gene have been identified. Secondary GB derive from LGGs or Astrocytomas, are rarer, usually diagnosed at a younger age and with a more favorable outcome. The most frequent genetic alterations include IDH1 gene mutations, LOH at 22q and 19q, and methylation of the O6 methylguanine-DNA methyltransferase (MGMT) promoter [41].

The GB molecular signature has been identified and listed in The Cancer Genome Atlas (TCGA) project, reporting a large-scale-multi-dimensional analysis at genomic, epigenomic and transcriptomic level [42,43]. These studies highlighted frequent alterations in core oncogenic pathways, involving the tumor protein p53, the receptor tyrosine kinase/Ras/phosphoinositide 3-kinase, and retinoblastoma. Moreover, bulk gene expression profile analysis identified three main GB molecular subtypes, that are the proneural (TCGA-PN), classical (TCGA-CL) and mesenchymal (TCGA-MES), harboring mutations in PDGFRA and IDH1 (PN), EGFR (MES) or NF1 (CL), respectively, with a better prognosis for PN than MES subtype [39,44]. However, during time switching of a GB tumor from a subtype to another has been described, i.e. from PN to MES, being this one of the main cause of resistance to treatments [45,46]. Recently, single cell RNA sequencing pointed out to the high level of intratumoral complexity, by showing the presence of transcriptionally different subclones each one with peculiar features in the same tumor sample [47].

- Cellular:

A second layer of heterogeneity is due to the developmental state of GB cells in the tumor. GB contains subsets of GB stem cells (GSCs), that resemble and could derive by neural stem cells (NSCs) [48–51]. GSCs are thought to represent the GB driving force, being able to maintain stemness and proliferation, but also to remain quiescent, and to escape both chemotherapy and radiotherapy, transferring these abilities to the other tumor cells, and condition the tumor microenvironment to support GB growth, progression and recurrency [52]. GSCs could be isolated thanks to the presence on the cell surface of CD133, CD44 and L1 cell adhesion molecule (L1CAM), but it is unknown whether different GSC markers isolate distinct cellular states or subtypes and whether tumors

generated with different subpopulations of GSCs give rise to GB of comparable or diverse cellular composition. Several studies showed that the GSCs in recurrent GB differed from the ones that initiated and maintained the primary tumor, displaying different surface markers (CD15, BMI1, and SOX2, instead of CD133), and being more aggressive. Finally, GB single cell sequencing analysis suggest that tumor cell hierarchy arising from GSCs might account for the intratumoral cellular and molecular heterogeneity [37,53,54].

2.3. Invasiveness and Recurrency

GB cells have been shown to invade normal brain tissues, penetrating the brain parenchyma and the perivascular space by degrading the extracellular matrix through the release of proteolytic factors and by forming membrane-derived extensions known as invadopodia, involved in cell migration [55,56]. Patterns, directionality and mechanisms responsible for the invasive behavior comprise cell-to-cell and cell-to-ECM adhesion mechanisms, ECM and cytoskeletal remodelling and overall features of epithelial-mesenchymal transition (EMT), showing a preference towards specific brain regions (subventricular zone) despite others, such as hippocampus and cerebellum. Due to this aggressive migrative behavior, GB cells escape complete surgical resection, with following reoccurrence within a few centimeters from its original location. Almost all GB tumors return after surgery, radiotherapy, and chemotherapy, showing lower response rate to conventional treatments and extremely few options of treatment. To date, management protocols for recurrent GB patients are still missing and most of them are not eligible for surgical re-resection [57]. Multiple studies have identified in GSCs the tumor initiating and clonogenic potential as well as the origin of therapeutic resistance, being therefore the main actor in GB recurrency. The unremoved GSCs, after debulking, migrate within the resection cavity and initiate and recapitulate the whole tumor[58–60].

2.4. Therapeutic resistance

Therapeutic resistance is one of the main causes of poor prognosis for GB patients. This feature increases over time, weakening all the current therapeutic protocols for recurrent GB; in most cases surgery is no longer possible and the chemo and radiotherapy fail in counteracting tumor progression [37]. Many of the GB features here described have been also connected to radio-and/or chemo-resistance. First of all, the BBB presence limits chemotherapeutic access to the brain [31]. Moreover, therapeutic resistance increases over time due to the molecular changes that characterize GB progression, including the switching from the PN subtype to the MES one, and the accumulation of the O6-methylguanine-DNA methyltransferase (MGMT), which is in charge of DNA repair, whose increased levels are associated to increased TMZ chemoresistance [61]. GSCs also play a key role, showing low sensitivity to both chemo and radiotherapies, further decreasing following repeated chemoradiation treatments [62,63]. Therapeutic resistance is mediated by increase of MGMT, as well as of anti-apoptotic proteins, and drug-efflux transporters, which reduce drug sensitivity, and activation of both DNA damage checkpoints and DNA repair mechanism induced by radiation [64,65].

2.5. Immune escape

Immunosurveillance of the CNS is inherently adapted to maintain neuronal function and minimize non-specific immune responses. Additionally, GB cells contribute to generate an immunosuppressive microenvironment, by secreting soluble factors, interleukins, prostaglandins, and also EVs which target microglia, monocytes, and macrophages, inducing a tumor supportive M2 phenotype, as well as T lymphocytes, impairing their antitumoral activity. The complex GB microenvironment, conditioned by GB itself, collaborates in allowing the tumor to evade the immune response, progress and also recur [37,66–68].

3. Inquiring EV for GB treatment: strategies, advantages and future perspectives

GB cells communicate with each other and with the tumor microenvironment. In this respect EVs play key roles, transferring functional molecules (DNA, mRNAs, miRNAs, lncRNAs, proteins, lipids and metabolites) and their related properties between cells [69]. EVs are released by cells, mainly through two mechanisms that are budding from the cell membrane (microvesicles or ectosomes), or exocytosis of intraluminal vesicles formed inside the multivesicular bodies present in the cytoplasm (exosomes)[70–72]. The first mechanism produces both small and large EVs, whereas the second mechanism gives rise more specifically to the smaller vesicles, due to physical cellular constraints [73]. EVs can travel through the bloodstream and other biological fluid and transport the messengers contained within at considerable distance from the site of origin. They are able to recognize specific cells through cell membrane interactions, and can be internalized in the target cells where EVs release their functional content [73–75]. EVs released by GB cells can condition non tumoral cells, such as macrophages, immune cells, astrocytes, endothelial cells to create a supportive microenvironment for GB growth and progression. On their side, tumor microenvironment cells release EVs that are uptaken by GB cells increasing their proliferation, invasiveness and malignancy. Several review articles have explored the role played by EVs in the cellular cross talk inside GB microenvironment [56,69,76,77]. Here we aim to address how EV biology can be exploited for bypassing current limitations in GB therapy and developing innovative and breakthrough therapeutic approaches (Figure 2).

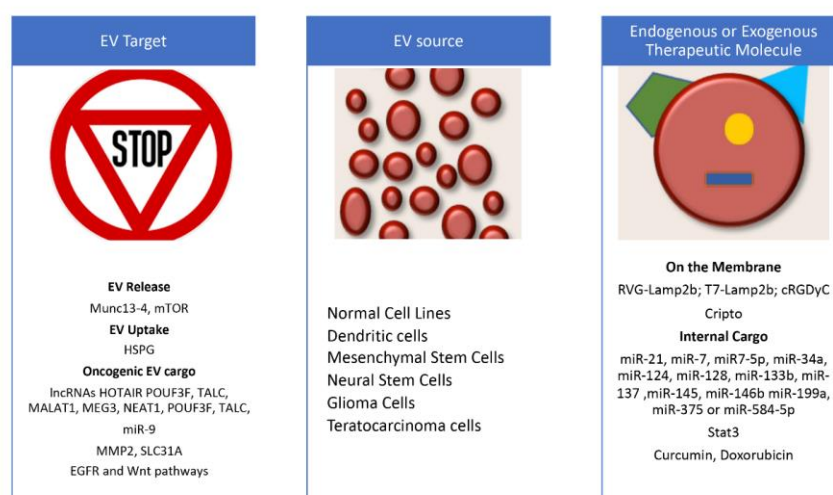


Figure 2. EV exploitation strategy for Glioblastoma treatment. The Figure schematizes on the left the identified targets to block EV release and uptake or impair specific oncogenic glioblastoma features, in the middle the EV sources and on the right the therapeutic molecules used in functional assays in Glioblastoma models (both in vitro and in vivo).

4. Targeting EV biology and cargo

First, being EVs so much implicated in the establishment of a supportive microenvironment for GB, and more in general tumor, progression, and the induction of key malignant features, such as proliferation, invasiveness and chemoresistance, therapeutic strategies focused on blocking EV release, uptake, and circulation might improve patient outcome, irrespectively of the highly tumor heterogeneity.

Numerous strategies have been identified, based on targeting of key mechanisms for vesiculation, targeting of EVs on route through hemodialysis or blocking EV uptake, including the use of natural substances (i.e. the antifungal agent ketoconazole), as well as of repurposed drugs such

as heparin and reserpine already used as anticoagulant and anti-hypertensive drugs, respectively [55,78]. In glioma cells heparan sulfate proteoglycans (HSPGs) have been identified as key modulators of EV uptake [79]. Decrease of HSPGs on the GB cell-membrane or the use of free heparin which competes with cell-membrane HSPGs for the binding to EVs, strongly reduces EV uptake [80]. However, heparin-mediated blocking of EV binding to target cells and following internalization is not specific of cancer cells [81,82]. The identification of selective strategies able to address tumor-derived EV biology, without affecting normal EVs, might be fundamental for the development of clinically suitable, effective and safe interventions, avoiding undesired off-target effects. As an example, recent studies have identified a specific Calcium dependent pathway inducing exosome release by cancer cells, involving Munc13-4, a Calcium-dependent soluble Nethylmaleimide-sensitive factor attachment protein (SNAP) receptor, upregulated in cancer cells [83]. In GB, increased levels of the mammalian Target of Rapamycin (mTOR) and consequent pathway activation is required for promoting EV release, through downregulation of the autophagy pathway, as well as for maintaining GSC tumor features, such as self-renewal, proliferation, migration and invasiveness [58]. Both mechanisms are potentially relevant for the development of novel approaches targeting GB-derived EV (GDEV) production.

In parallel, defining specific functionally relevant GB-associated EV cargo regulating the complex crosstalk inside GB environment, may also allow for specific and targeted therapeutic approaches. In particular, the last decade, gene/RNA therapy has significantly attracted the attention of the GB therapeutic field. Several long non coding RNAs (lncRNAs) with oncogenic activity have been found inside GDEVs [84]. Among these, the EV-associated lncRNAs POU3F3 and TALC can remodel GB microenvironment, acting on endothelial cells and microglia respectively, inducing angiogenesis and GB progression (POU3F3) or M2 microglia polarization with consequent secretion of the complement components C5/C5a and induction of TMZ chemoresistance in GB cells (TALC) [85,86]. Other GDEV-associated lncRNAs, such as MALAT1, MEG3, NEAT1 and HOTAIR play key role in promoting EMT and possibly chemoresistance when uptaken by GB cells both *in vitro* and *in vivo* [84]. Accumulating evidence shows that miRNAs also play essential roles in GB pathogenesis with a high potentiality to be exploited in targeted therapeutic approaches [87]. Among the others, miR-9, upregulated in GSCs, has been found in EV isolated from both GB cell cultures and patients, strongly implied in the GB malignant phenotypic traits including cell proliferation and migration [88]. miR-9 is also involved in the expression of the drug efflux protein P-glycoprotein, which contributes to increase TMZ drug resistance into GB cells. In this respect, anti-miR-9 molecules encapsulated in MSC-EV- were shown to be successfully delivered to GB cells *in vitro*, causing the reduction of the levels of P-glycoprotein, and enhancing TMZ sensitivity [89]. Therefore, a promising direction is represented by therapeutic approaches based on EVs loaded with siRNAs to target GB cells and downregulate the expression of the inherent oncogenic lncRNAs or miRNAs, inhibiting their EV mediated transferring to other tumor cells as well as non-tumoral cells of the GB microenvironment

5. EVs as drug-delivery systems in GB therapy

Recently, nanomedicine has dominated the field of tumor therapy with respect to different approaches, thanks to the abilities of nanoparticles to be loaded with specific therapeutic agents as well as surface decorated with specific targeting molecules. First, synthetic nanocarriers such as liposomes, have been used, with the crucial advantage of high loading and surface functionalization efficiency, but important limitations due to low tolerability, relatively short circulation times in biological environments, fast body clearance and last but not least low capability for BBB. Even though different strategies have been developed to increase BBB permeability and enhance nanoparticle access to the brain tissue, low biocompatibility and high risk of off-target effects are still key issues to overcome [30,90]

EVs show key advantages in this respect. Several studies have shown their low immunogenicity and cytotoxicity, low clearance from the phagocytic system, prolonged circulation time, high biocompatibility and good cell uptake [91,92]. Interestingly, EVs are able to cross interspecies and even interkingdom boundaries: EVs isolated from different sources, including microalgae, bacteria

and plants are easily uptaken by mammalian cells [93–97]. EVs can be loaded, through several physical/chemical/genetic strategies, with different functional cargoes, such as nucleic acids, proteins, natural substances or chemotherapeutics, that can be thus delivered to the cells. Moreover, the EV surface can be also functionalized with cell type-specific targeting ligands to enhance the interaction with specific cellular types [30,98]. EV encapsulation can enhance the solubility, stability, bioavailability, and in last instance the therapeutic effect of the molecule alone. Besides these features, EVs are also able to cross the BBB, being then ideal candidates for delivering therapeutic molecules to the brain to be used for treatment of brain pathologies [90].

The first study on the ability of engineered EVs to cross the BBB and efficiently delivery functional molecules to the brain was performed by Alvarez-Erviti and coauthors using EVs derived from mouse bone-marrow dendritic cells [99]. To confer brain targeting capabilities, dendritic cells were previously engineered to express recombinant fusion proteins, containing the central nervous system-specific rabies viral glycoprotein (RVG) peptide, that specifically binds to the acetylcholine receptor, fused to the N terminus of murine dendritic cell (DC)-derived lysosome-associated membrane protein (Lamp2b), a highly EV-associated protein. Isolated EVs, exposing the RVG-Lamp2b fusion protein on the membrane, were electroporated with specific siRNA and then intravenously injected into mice, showing specific targeting and siRNA delivery to brain neurons, microglia, and oligodendrocytes [99]. Besides this study, a similar approach was used in mouse models for Parkinson's disease [100], highlighted the great EV potential in penetrating the BBB and be exploited for the treatment of brain pathologies. More recently, Kim and coworkers [101] used the same Lamp2b-based approach to decorate EVs released from 293T cells with the Transferrin Receptor-binding peptide T7, able to target transferrin receptors naturally enriched on GB cell surface. The engineered T7-EVs were further modified to encapsulate antisense miRNA oligonucleotides against miR-21 (AMO-21). When injected intravenously into a intracranial GB xenotransplanted rat model, T7-EVs resulted much more efficient of both unmodified and RVG-decorated EVs to reach the brain, target GB cells and deliver AMO21, causing a reduction of GB size [101].

Besides ad-hoc modified EVs, EVs from other cell types have been successfully tested for GB treatment. EVs derived from a mouse lymphoma cells line (EL-4), loaded with the anti-inflammatory drug curcumin or with an inhibitor of the signal transducer and activator of transcription 3 (Stat3), were efficiently and noninvasively delivered (via intranasal route) to mouse brain microglia cells, causing a significantly delayed brain tumor growth in a glioma mouse model [102]. Moreover, EVs from a brain endothelial cell line (b.END3), naturally enriched with the CD3 tetraspanin, loaded with Doxorubicin, were able to cross the BBB into a xenotransplanted zebrafish brain tumor model (obtained by injecting GB cells into the zebrafish brain ventricle), significantly decreasing fluorescent intensity of xenotransplanted cancer cells and tumor growth markers [103].

MSCs are the better characterized source of EVs, used in several clinical trials, native or engineered, to target a plethora of different pathologies, including tumors [55,104]. MSCs can communicate with different cell types, and also GB cells, through direct contact by gap junctions and/or by contact-independent pathways (Biancone et al. 2012; Munoz et al. 2013). In fact stromal cells resembling MSCs are a key component of the GB microenvironment, where they can exert both tumor supportive and suppressive roles [105,106]. MSC-derived EVs carrying specific miRNA (miR-7, miR-34a, miR-124, miR-133b, miR-145, miR-146b miR-199a, miR-375 or miR-584-5p) were able to counteract GB tumor features, including cell proliferation, migration, and invasion *in vitro* by targeting specific molecules or pathways such as the Forkhead box (FOX)A2 (miR124a), Wnt pathway (miR133b), EGFR (miR-146b), SLC31A (miR-375) and MMP2 (miR-584-5p). Many of these MSC-EVs engineered with miRNAs were also tested *in vivo*, where they showed the ability to cross the BBB, promoting tumor regression in mouse xenotransplanted models [87]. A very promising study is the one involving MSC-EVs loaded with miR124a and systemically delivered in mouse models xenotransplanted intracranially with GB cells, being able to suppress tumor growth and prolong overall animal survival [107].

Tumor derived-EVs (TDEVs) can be also used as natural carriers for the delivery of antioncogenic molecules [55], thanks to their high tumor targeting and permeability, as a technological Trojan horse, as evoked by Simionescu and coauthors [87]. EVs isolated from engineered patient-derived GSCs carrying the miR-302-367 cluster, are internalized rapidly by neighboring GB cells, and are able to inhibit GB cell proliferation, stemness and invasion, through repression of Cyclin A, Cyclin D1, E2F1 and the CXCR4 pathways [108]. Moreover, GDEVs engineered with miR-124, miR-128, or miR-137 improved survival after injection in GB model mice when combined with chemotherapy [109], whereas GDEVs carrying miR-151a reduced chemoresistance in GB xenograft mouse models [110]. Cell treatment with miRNAs specific inducers can also be used, as valid alternative to external loading, for obtaining EVs carrying specific miRNA molecules. This is the approach used by Wang and coauthors (H. Wang et al., 2021), using traditional medicine substances to induce GDEV miR7-5p enrichment; GDEV carrying miR7-5p reduced GB formation and metastasis in GB nude mice models [111]. Obviously, the exploitation of GDEVs as drug carriers for GB therapy requires an extremely careful evaluation of the antioncogenic properties of the loaded therapeutic molecule vs the oncogenic potential of the GDEV intrinsic cargo and then of the relative cost-benefit assessment. Recently, Guo and coworkers [112] identified a saponin-mediated cargo elimination strategy to improve biosafety of GDEVs in GB therapeutic applications. Systematic analysis of the original proteins and RNAs together with functional *in vitro* and *in vivo* assays confirmed the high efficiency of the method in eliminating GB-EV cargo and its inherited abilities in promoting GB progression without affecting uptake by GB cells. Furthermore, saponin-treated GDEVs loaded with Doxorubicin displayed an effective tumor suppressive role in both subcutaneous and orthotopic GB mouse models [112]. These data are extremely promising and can be exploited for the development of novel visionary therapeutic pipelines which could involve the isolation of TDEVs directly from the patients, their oncogenic disarm through cargo elimination protocols, the loading and /or functionalization with anticancer molecules, and the final administration to the patient. Finally, even though TDEVs preferentially target (and are uptaken by) the cells of the same tumor type, TDEV surface can be functionalized with specific molecules involved in EV-cell interaction to improve or alter natural tumor tropisms. Geng and coauthors [113], have shown that GDEVs are more easily uptaken by GB cells than other type of tumor cells such as pancreatic cancer (PC) cells and viceversa, but EV functionalization with cyclic arginine-glycine-aspartic acid-tyrosine-cysteine (cRGDyC), a ligand for the integrin $\alpha v \beta 3$ enriched in GB cells, is able to enhance ability of PC cells (and also Gb cells themselves) to target and be internalized into GB cells [113].

Other cells that naturally show a GB tropism are the neural stem cells (NSCs) and the teratocarcinoma cells. Both NSCs, from which GB might derive [48,49], and NTERA2 human teratocarcinoma cells, which resemble NSCs being able to differentiate into both glia and neurons [114,115], show GB tropism *in vivo* and were proposed for cell-based delivery systems in anti-GB therapy [116–118]. EVs released by NSCs and engineered with antisense oligonucleotides (ASOs) targeting oncogenic and tolerogenic signal transducer and activator of transcription 3 (STAT3) were effective after intracranial injection at distant site, in reaching glioma microenvironment, targeting and activating microglia to inhibit tumor growth [119]. Even more interestingly, native NTERA2-derived EVs, naturally carrying the oncodevelopmental factor Cripto [120–122], have been recently shown to impair GB cell migration, without inducing undesirable effects such as increased GB cell proliferation or enhanced TMZ drug resistance [123]. Cripto is a membrane-bound glycosylphosphatidyl inositol-anchored protein, that can be also cleaved and released as a soluble factor [124], and only recently found associated to EVs [123,125]. With respect to neural fate, mouse *Cripto* gene targeting was associated to neural differentiation in embryonic stem cell cultures, and specification of anterior neural identities *in vivo*, with *Cripto* null embryos mostly constituted by anterior neuroectoderm, including NSCs [126–128]. Cripto has been mostly identified associated to oncogenic features, also in GB, but it is also able to act as an antioncogene [129–132]. Even though these data have not yet been confirmed *in vivo*, they open the mind to new unobvious and even paradoxical paradigms in GB and more in general tumor progression and, subsequently, therapies.

First, EV sorting and delivery might be an alternative route for regulating the spreading and activity of soluble and/or membrane-bound signaling molecules, modulating their final impact on target cells and then on cancer development and progression [55]. Second, specific subsets of TDEVs might possess an antitumoral potential to be used per se or eventually empowered through ad hoc modifications [55]. Therefore, molecularly identifying and isolating, or bio-mimicking, or even inducing these specific subsets of TDEVs is certainly a novel ambitious and promising therapeutic direction to explore.

6. Mitigating the pitfalls and realizing the potential of EV exploitation in GB treatment

Although these extremely promising results, no EV-based GB clinical trial is ongoing, even though EV-based therapies are under evaluation in clinical trials for treatment of other tumors. In this paragraph we will try to schematize the main issues and directions to explore to find the optimal solutions and realize the full EV potential as therapeutic advanced platform for GB treatment. The analysis of Strengths, Weaknesses, Opportunity and Threat (SWoT) of EV exploitation in novel GB therapeutic strategies is reported in Figure 3.

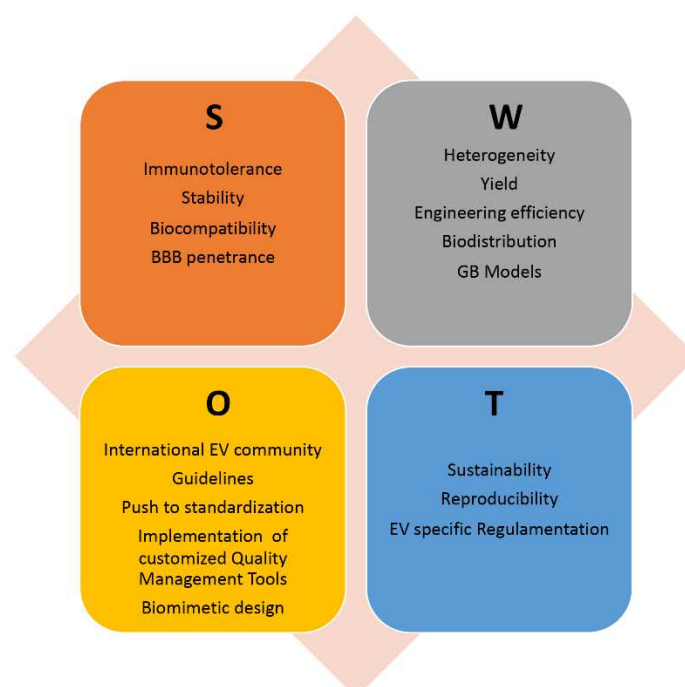


Figure 3. SWOT analysis of the EV exploitation in GB treatment, highlighting the relative Strengths (S), Weaknesses (W), Opportunities (O), and Threats (T).

6.1. EV source

We have described above the main EV sources that have been used for GB therapeutic assays both *in vitro* and *in vivo*. They are all derived by mammalian cells, including cell lines of brain endothelial cells, tumor cells (GB but also teratocarcinoma), and stem cells (both neural and mesenchymal). To be exploited in patient therapy, EVs must be produced in high quantity and with an extremely high quality, satisfying compliance with good manufacturing practice (GMP), pharmaceutical biologics' regulation and ongoing specific regulation for EV therapeutics [93]. The process needs careful scaling up and out, standardization, and quality assurance of the experimental pipeline, as well as quality control of the final products, requiring high commitment in terms of cost, resources and time. Alternative sources of EVs, such as plants, microalgae and bacteria have also been identified and their biocompatibility and biodistribution in animal models has been performing [93]. These alternative EV sources have lower cost of production, but unfortunately, until now no brain tropism has been proved. Therefore, the production pipeline must be extended with the

insertion of an EV surface functionalization phase and successive isolation and characterization of the loaded EVs. A careful evaluation of the advantage and disadvantages of each strategy must be performed, in terms of therapeutic efficacy and efficiency as well as of cost and sustainability.

6.2. EV handling procedures

Different techniques have been used until now for EV isolation, including differential Ultracentrifugation (dUC) that is still the gold standard, different commercial kits, exclusion chromatography, tangential flow filtration [133], and loading, usually subdivided into endogenous or indirect (manipulation of the parental cell) and exogenous or direct (EV engineering), the latter one subdivided into passive (co-incubation of molecules and EVs) and active (i.e. electroporation, sonication) [134]. Standardized procedures should be identified to guarantee low inter batches variability and high reproducibility of the results. The international Society for EVs (ISEV) is strongly committed in this directions since years, providing the EV scientific community with the minimal information for studying EVs and several position papers dedicated to specific aspects of EV research and exploitation [135–139]. More recently, it is increasingly evident how quality management tools already used in other scientific context [140–142] can help in solve main issues in EV research and development [143,144]. In particular, application of the Failure Mode and Effect Analysis for risk assessment and management of scientific procedures [145] to the single steps of the EV production pipeline might help in avoiding and preventing pitfalls and failures in EV bioprocessing and manipulation [144,146], whereas application of the Design of Experiments mathematical model [147,148] might be a valid model to identify and optimize key factors in multivariable processes such as production as well as engineering or functional assays of the EVs [144,146,149]. More recently, we have proposed a new tool, inspired to multicriteria decision making, that is a multi-criteria EV decision-making grid (EV-DMG) as a novel, customizable, efficient and easy-to-use tool to support responsible EV research and innovation [150]. All together these instruments and guidelines can greatly improve the choice, optimization, standardization, quality assurance and control, and ultimately the reliability of the EV landscape.

6.3. Biodistribution

Assessment of EV biodistribution mainly detected as preferential sites spleen, liver, lungs, kidneys and gastrointestinal tract [151]. Moreover, as already discussed TDEVs have a high tropism towards tumor cells, especially of the same type. The source and handling of EVs can influence the final biodistribution, as well as the “ad hoc” cell surface functionalization [152]. EV size is also an important variable to consider: smaller EVs are easily eliminated by blood circulation, whereas larger EV can pass through the BBB with difficulty, but seem to transfer their cargo content more efficiently [30,89]. Several EVs have shown to be able to penetrate the BBB and deliver therapeutic agents into the brain, being suitable drug-delivery vectors to be administered intravenously. Identifying the amount of EVs to inject and the frequency of administration requires the assessment of the amount of EVs which passes the barrier and enter to the brain and the amount of EV cargo that is effectively delivered to the GB cells. However, since GB therapy often comprises as first step surgical tumor resection, concomitant intracranial injection at tumor site of therapeutic EVs able to counteract reoccurrence would also be a suitable strategy.

6.4. Designing Biomimetic particles

Very recently, another important research direction is exploring the possibility to obtain biomimetic nanocarriers, joining the advantages of both artificial and natural delivery vectors through the development of technological advanced ad hoc nanoplatforms. Recently, a nanoplatform termed EV-DNs, in which doxorubicin loaded heparin nanoparticles (DNs) were attached to the surface of native grapefruit EVs, was shown to penetrate the BBB *in vivo*, reducing the GB cell proliferation and increasing animal model survival [95]. Another biomimetic system, named RGE-Exo-SPION/Cur was implemented by loading curcumin (Cur) and superparamagnetic iron oxide

nanoparticles (SPIONs) into EVs further functionalized by click chemistry to carry on the surface the neuropilin-1-targeted peptide (RGERPPR, RGE). RGE-Exo-SPION/Cur were efficient in targeting GB cells in orthotopic GB-bearing nude mouse models, accumulating in correspondence of the tumor, as well as in reducing the GB size, being relevant for simultaneous GB MRI-based diagnosis and therapy [153].

6.5. GB models

One of the main reasons for the limited success of novel therapeutic molecules entering clinical trials is the poor correlation between their efficacy in *in vitro*, *ex vivo* or *in vivo* tumor models and the patient treatment scenarios. More and more sophisticated *ex vivo* models able to mimic the GB microenvironment have been developed to fill this gap, starting from glioma slice cultures directly derived from biopsies, neurospheres, hydrogel-coated scaffolds of different biomaterials, and arriving to complex 3D models such as GB organoids, and tumor-on-chips [7]. GB organoids are produced resembling brain organoids, show tumor heterogeneity, and can be extremely use to study drug sensitivity. Recently, a biobank of patient-derived glioblastoma organoids, each one showing the parental tumor heterogeneity has been developed, with a high value for identification of personalized target and treatment strategies [154]. However, to assess also the ability to cross the BBB and deliver efficiently the therapeutic molecule, without inducing immune reaction or other undesired effects, *in vivo* studies are absolutely needed. Several studies have shown how orthotopic xenotransplanted immunocompetent mouse models, in which human GB cells are injected intracranially into brain parenchyma are able to recapitulate the patient GB-microenvironment interactions [155,156].

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