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# Identification of selective aptamer ligands for Glioblastoma Stem Cells as new therapeutic tools for Glioblastoma


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# “Identification of selective aptamer ligands for Glioblastoma Stem Cells as new therapeutic tools for Glioblastoma”

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## List of Abbreviations

Age-Related Macular Degeneration (AMD)
Alpha-Ketoglutarate (α -KG)
Blood Brain Barrier (BBB)
Cancer Stem Cells (CSCs)
Central Nervous System (CNS)
Computed Tomography (CT)
D-2-Hydroxy-Glutarate (D-2HG)
Epidermal Growth Factor Receptor (EGFR)
Extreme Limiting Dilution Analysis (ELDA)
Glial Fibrillary Acidic Protein (GFAP)
Glioblastoma (GBM)
Glioblastoma Stem Cells (GSCs)
Isocitrate Dehydrogenase (IDH)
Loss of Heterozygosity (LOH)
Magnetic Resonance Imaging (MRI)
Multiple Drug Resistance (MDR)
Peripheral Nervous System (PNS)
Phosphatase and Tensin (PTEN)
Phosphate Buffered Saline (PBS)
Platelet-Derived Growth Factor (PDGF)
Poly Ethylene Glycol (PEG)
Positron Emission Tomography (PET)
Radiotherapy (RT)
Selective Evolution of Ligands by Exponential Enrichment (SELEX)
Slow Off-rate Modified Aptamer (SOMAmer)
Temozolomide (TMZ)
World Health Organization (WHO)

# Abstract

Glioblastoma (GBM) is the most frequent and aggressive primary brain tumour in adults. Standard treatments for GBM patients consist of tumour resection, radiotherapy (RT) and chemotherapy with alkylating agent Temozolomide (TMZ). However, despite advances in surgical and medical treatment, prognosis for GBM patients remains dismal, with a median survival of 14–15 months. A small population of cancer stem cells (glioblastoma stem cells, GSCs), that retains stem cell properties including self-renewal and multipotency, has been implicated as responsible for the frequent relapse of glioblastoma and its resistance to conventional therapeutic. Thus, the identification of new molecules that can specifically target these GSCs is a fundamental challenge for the development of effective glioma therapies.

In this study, we developed a differential whole cell-SELEX, an in vitro evolution-based approach, which allowed us to generate RNA-ligands with high affinity and specificity for GSCs, named aptamers. These nucleic acids were obtained by means the iterative evolution of a random pool of sequences using human primary GSCs as target.

The obtained aptamer, 40L had been proved to be selective for GSCs distinguishing them from tumor differentiated cells, obtained from the stem cells induced to differentiate. Moreover, 40L revealed to be functionally active on target cells and able to inhibit stemness, cell growth and migration in vitro as well as in vivo.

In conclusion, our results indicate that 40L and its short form A40s can selectively target GSCs and, given the crucial role of these cells in GBM recurrence and multi-drug resistance, 40L and A40s represent innovative drug candidates with a great potential in the GBM treatment.

# 1. Background

## 1.1. Central Nervous System and its tumours

The Nervous System consists of all the nerve cells of our body which enable us to communicate with the outside world. The nervous system allows us to receive information through our senses, it is able to processes it and to trigger a reaction to external stimuli, such as making your muscles move or causing you to feel pain. Also, metabolic processes are controlled by the nervous system.
Basic working units of nervous system are specific nerve cells, also called neurons. The brain alone has about 100 billion neurons in it. Neuron made up of a cell body and various extensions. Dendrites are the shorter extensions, they extend from the neuron cell body and they receive signals from, for example, other neurons and transmit them on to the cell body which contains the nucleus and cytoplasm. The signals are then transmitted on via a long extension, called axon, which can be long up to one meter and often gives rise to many smaller branches before ending at nerve terminals. Synapses are the contact points where one neuron communicates with another.
Due to their location in the body, we can distinguish two different nervous systems, one called the central nervous system and one the peripheral nervous system. The central nervous system (CNS) includes the nerves in the brain and spinal cord. All of the other nerves in the body are part of the peripheral nervous system (PNS).

### 1.1.1. World Health Organization Classification of Tumours of the Central Nervous System

A classification and grading concerning human tumours that is recognised and used worldwide is still today an important aim to reach, considering that, studies and clinical trials could not cross national and institutional boundaries without clear diagnostic criteria.
In this respect, the World Health Organization (WHO) is drawing up international classifications of human tumours from 1957 until now.
Zülch published in 1979 the first edition regarding the histological typing of cancers of the nervous system. Thanks to contributions made by the immunohistochemistry introduction into diagnostic pathology, a second edition was edited by Kleihues et al[1]. in 1993. In the year 2000 Kleihues and Cavenee, defined brain tumours including genetic profiles as supplementary aid[2].
In 2016, WHO gives form to a new way to define CNS tumours in the molecular era, classifying them, for the first time, using molecular parameters in addition to histology[3]. Until this version the classification of brain tumours was mainly based on histogenesis, classifying the tumours according to their microscopic similarities with various putative cells of origin and their supposed grades of differentiation.
As shown in Table 1, following the 2016 World Health Organization Classification of Tumours of the Central Nervous System, diagnosis of these tumours is made up of a histopathological name followed by a comma and the genetic features as adjectives, such as "Anaplastic Astrocytoma, IDH-mutant". For those tumours which have more than one genetic determinant, all the molecular features have to be included in the name (e.g. Anaplastic Oligodendroglioma, IDH-mutant and 1p/19q-codeleted). The term "wildtype" is used for those tumours that luck a genetic mutation (e.g. Diffuse Astrocytoma, IDH-wildtype), instead the term "positive" can be used if a specific genetic alteration is present (e.g. Ependymoma, RELA fusion–positive). Finally, for some tumour types is possible the diagnostic designation "not otherwise specified" (NOS) for those sites which do not have any access to molecular diagnostic testing.

| | |
|---|---|
| **Diffuse astrocytic and oligodendroglial tumours** | |
| Diffuse astrocytoma, IDH-mutant | 9400/3 |
| Gemistocytic astrocytoma, IDH-mutant | 9411/3 |
| *Diffuse astrocytoma, IDH-wildtype* | *9400/3* |
| Diffuse astrocytoma, NOS | 9400/3 |
| Anaplastic astrocytoma, IDH-mutant | 9401/3 |
| *Anaplastic astrocytoma, IDH-wildtype* | *9401/3* |
| Anaplastic astrocytoma, NOS | 9401/3 |
| Glioblastoma, IDH-wildtype | 9440/3 |
| Giant cell glioblastoma | 9441/3 |
| Gliosarcoma | 9442/3 |
| *Epithelioid glioblastoma* | *9440/3* |
| Glioblastoma, IDH-mutant | 9445/3* |
| Glioblastoma, NOS | 9440/3 |
| Diffuse midline glioma, H3 K27M–mutant | 9385/3* |
| Oligodendroglioma, IDH-mutant and 1p/19q-codeleted | 9450/3 |
| Oligodendroglioma, NOS | 9450/3 |
| Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted | 9451/3 |
| *Anaplastic oligodendroglioma, NOS* | *9451/3* |
| *Oligoastrocytoma, NOS* | *9382/3* |
| *Anaplastic oligoastrocytoma, NOS* | *9382/3* |
| **Other astrocytic tumours** | |
| Pilocytic astrocytoma | 9421/1 |
| Pilomyxoid astrocytoma | 9425/3 |
| Subependymal giant cell astrocytoma | 9384/1 |
| Pleomorphic xanthoastrocytoma | 9424/3 |
| Anaplastic pleomorphic xanthoastrocytoma | 9424/3 |
| **Ependymal tumours** | |
| Subependymoma | 9383/1 |
| Myxopapillary ependymoma | 9394/1 |
| Ependymoma | 9391/3 |
| Papillary ependymoma | 9393/3 |
| Clear cell ependymoma | 9391/3 |
| Tanycytic ependymoma | 9391/3 |
| Ependymoma, *RELA* fusion–positive | 9396/3* |
| Anaplastic ependymoma | 9392/3 |
| **Other gliomas** | |
| Chordoid glioma of the third ventricle | 9444/1 |
| Angiocentric glioma | 9431/1 |
| Astroblastoma | 9430/3 |
| **Choroid plexus tumours** | |
| Choroid plexus papilloma | 9390/0 |
| Atypical choroid plexus papilloma | 9390/1 |
| Choroid plexus carcinoma | 9390/3 |
| **Neuronal and mixed neuronal-glial tumours** | |
| Dysembryoplastic neuroepithelial tumour | 9413/0 |
| Gangliocytoma | 9492/0 |
| Ganglioglioma | 9505/1 |
| Anaplastic ganglioglioma | 9505/3 |
| Dysplastic cerebellar gangliocytoma (Lhermitte–Duclos disease) | 9493/0 |
| Desmoplastic infantile astrocytoma and ganglioglioma | 9412/1 |
| Papillary glioneuronal tumour | 9509/1 |
| Rosette-forming glioneuronal tumour | 9509/1 |
| *Diffuse leptomeningeal glioneuronal tumour* | |
| Central neurocytoma | 9506/1 |
| Extraventricular neurocytoma | 9506/1 |
| Cerebellar liponeurocytoma | 9506/1 |
| Paraganglioma | 8693/1 |
| **Tumours of the pineal region** | |
| Pineocytoma | 9361/1 |
| Pineal parenchymal tumour of intermediate differentiation | 9362/3 |
| Pineoblastoma | 9362/3 |
| Papillary tumour of the pineal region | 9395/3 |
| **Embryonal tumours** | |
| Medulloblastomas, genetically defined | |
| Medulloblastoma, WNT-activated | 9475/3* |
| Medulloblastoma, SHH-activated and *TP53*-mutant | 9476/3* |
| Medulloblastoma, SHH-activated and *TP53*-wildtype | 9471/3 |
| Medulloblastoma, non-WNT/non-SHH | 9477/3* |
| *Medulloblastoma, group 3* | |
| *Medulloblastoma, group 4* | |
| Medulloblastomas, histologically defined | |
| Medulloblastoma, classic | 9470/3 |
| Medulloblastoma, desmoplastic/nodular | 9471/3 |
| Medulloblastoma with extensive nodularity | 9471/3 |
| Medulloblastoma, large cell / anaplastic | 9474/3 |
| Medulloblastoma, NOS | 9470/3 |
| Embryonal tumour with multilayered rosettes, C19MC-altered | 9478/3* |
| *Embryonal tumour with multilayered rosettes, NOS* | *9478/3* |
| Medulloepithelioma | 9501/3 |
| CNS neuroblastoma | 9500/3 |
| CNS ganglioneuroblastoma | 9490/3 |
| CNS embryonal tumour, NOS | 9473/3 |
| Atypical teratoid/rhabdoid tumour | 9508/3 |
| *CNS embryonal tumour with rhabdoid features* | *9508/3* |
| **Tumours of the cranial and paraspinal nerves** | |
| Schwannoma | 9560/0 |
| Cellular schwannoma | 9560/0 |
| Plexiform schwannoma | 9560/0 |

**Table 1.** Continue

| | |
|---|---|
| Melanotic schwannoma | 9560/1 |
| Neurofibroma | 9540/0 |
| Atypical neurofibroma | 9540/0 |
| Plexiform neurofibroma | 9550/0 |
| Perineurioma | 9571/0 |
| Hybrid nerve sheath tumours | |
| Malignant peripheral nerve sheath tumour | 9540/3 |
| Epithelioid MPNST | 9540/3 |
| MPNST with perineurial differentiation | 9540/3 |
| **Meningiomas** | |
| Meningioma | 9530/0 |
| Meningothelial meningioma | 9531/0 |
| Fibrous meningioma | 9532/0 |
| Transitional meningioma | 9537/0 |
| Psammomatous meningioma | 9533/0 |
| Angiomatous meningioma | 9534/0 |
| Microcystic meningioma | 9530/0 |
| Secretory meningioma | 9530/0 |
| Lymphoplasmacyte-rich meningioma | 9530/0 |
| Metaplastic meningioma | 9530/0 |
| Chordoid meningioma | 9538/1 |
| Clear cell meningioma | 9538/1 |
| Atypical meningioma | 9539/1 |
| Papillary meningioma | 9538/3 |
| Rhabdoid meningioma | 9538/3 |
| Anaplastic (malignant) meningioma | 9530/3 |
| **Mesenchymal, non-meningothelial tumours** | |
| Solitary fibrous tumour / haemangiopericytoma** | |
| Grade 1 | 8815/0 |
| Grade 2 | 8815/1 |
| Grade 3 | 8815/3 |
| Haemangioblastoma | 9161/1 |
| Haemangioma | 9120/0 |
| Epithelioid haemangioendothelioma | 9133/3 |
| Angiosarcoma | 9120/3 |
| Kaposi sarcoma | 9140/3 |
| Ewing sarcoma / PNET | 9364/3 |
| Lipoma | 8850/0 |
| Angiolipoma | 8861/0 |
| Hibernoma | 8880/0 |
| Liposarcoma | 8850/3 |
| Desmoid-type fibromatosis | 8821/1 |
| Myofibroblastoma | 8825/0 |
| Inflammatory myofibroblastic tumour | 8825/1 |
| Benign fibrous histiocytoma | 8830/0 |
| Fibrosarcoma | 8810/3 |
| Undifferentiated pleomorphic sarcoma / malignant fibrous histiocytoma | 8802/3 |
| Leiomyoma | 8890/0 |
| Leiomyosarcoma | 8890/3 |
| Rhabdomyoma | 8900/0 |
| Rhabdomyosarcoma | 8900/3 |
| Chondroma | 9220/0 |
| Chondrosarcoma | 9220/3 |
| Osteoma | 9180/0 |
| Osteochondroma | 9210/0 |
| Osteosarcoma | 9180/3 |
| **Melanocytic tumours** | |
| Meningeal melanocytosis | 8728/0 |
| Meningeal melanocytoma | 8728/1 |
| Meningeal melanoma | 8720/3 |
| Meningeal melanomatosis | 8728/3 |
| **Lymphomas** | |
| Diffuse large B-cell lymphoma of the CNS | 9680/3 |
| Immunodeficiency-associated CNS lymphomas | |
| AIDS-related diffuse large B-cell lymphoma | |
| EBV-positive diffuse large B-cell lymphoma, NOS | |
| Lymphomatoid granulomatosis | 9766/1 |
| Intravascular large B-cell lymphoma | 9712/3 |
| Low-grade B-cell lymphomas of the CNS | |
| T-cell and NK/T-cell lymphomas of the CNS | |
| Anaplastic large cell lymphoma, ALK-positive | 9714/3 |
| Anaplastic large cell lymphoma, ALK-negative | 9702/3 |
| MALT lymphoma of the dura | 9699/3 |
| **Histiocytic tumours** | |
| Langerhans cell histiocytosis | 9751/3 |
| Erdheim–Chester disease | 9750/1 |
| Rosai–Dorfman disease | |
| Juvenile xanthogranuloma | |
| Histiocytic sarcoma | 9755/3 |
| **Germ cell tumours** | |
| Germinoma | 9064/3 |
| Embryonal carcinoma | 9070/3 |
| Yolk sac tumour | 9071/3 |
| Choriocarcinoma | 9100/3 |
| Teratoma | 9080/1 |
| Mature teratoma | 9080/0 |
| Immature teratoma | 9080/3 |
| Teratoma with malignant transformation | 9084/3 |
| Mixed germ cell tumour | 9085/3 |
| **Tumours of the sellar region** | |
| Craniopharyngioma | 9350/1 |
| Adamantinomatous craniopharyngioma | 9351/1 |
| Papillary craniopharyngioma | 9352/1 |
| Granular cell tumour of the sellar region | 9582/0 |
| Pituicytoma | 9432/1 |
| Spindle cell oncocytoma | 9290/0 |
| **Metastatic tumours** | |

The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) [742A]. Behaviour is coded /0 for benign tumours; /1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in situ and grade III intraepithelial neoplasia; and /3 for malignant tumours. The classification is modified from the previous WHO classification, taking into account changes in our understanding of these lesions. *These new codes were approved by the IARC/WHO Committee for ICD-O. *Italics*: Provisional tumour entities. **Grading according to the 2013 *WHO Classification of Tumours of Soft Tissue and Bone*.

**Table 1**. **WHO classification of tumours of the CNS.** This reference classification is used worldwide as an indispensable guide, providing an international standard for oncologists and pathologists.

| WHO grades of select CNS tumours | |
|---|---|
| **Diffuse astrocytic and oligodendroglial tumours** | |
| Diffuse astrocytoma, IDH-mutant | II |
| Anaplastic astrocytoma, IDH-mutant | III |
| Glioblastoma, IDH-wildtype | IV |
| Glioblastoma, IDH-mutant | IV |
| Diffuse midline glioma, H3 K27M–mutant | IV |
| Oligodendroglioma, IDH-mutant and 1p/19q-codeleted | II |
| Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted | III |
| **Other astrocytic tumours** | |
| Pilocytic astrocytoma | I |
| Subependymal giant cell astrocytoma | I |
| Pleomorphic xanthoastrocytoma | II |
| Anaplastic pleomorphic xanthoastrocytoma | III |
| **Ependymal tumours** | |
| Subependymoma | I |
| Myxopapillary ependymoma | I |
| Ependymoma | II |
| Ependymoma, *RELA* fusion–positive | II or III |
| Anaplastic ependymoma | III |
| **Other gliomas** | |
| Angiocentric glioma | I |
| Chordoid glioma of third ventricle | II |
| **Choroid plexus tumours** | |
| Choroid plexus papilloma | I |
| Atypical choroid plexus papilloma | II |
| Choroid plexus carcinoma | III |
| **Neuronal and mixed neuronal-glial tumours** | |
| Dysembryoplastic neuroepithelial tumour | I |
| Gangliocytoma | I |
| Ganglioglioma | I |
| Anaplastic ganglioglioma | III |
| Dysplastic gangliocytoma of cerebellum (Lhermitte–Duclos) | I |
| Desmoplastic infantile astrocytoma and ganglioglioma | I |
| Papillary glioneuronal tumour | I |
| Rosette-forming glioneuronal tumour | I |
| Central neurocytoma | II |
| Extraventricular neurocytoma | II |
| Cerebellar liponeurocytoma | II |
| **Tumours of the pineal region** | |
| Pineocytoma | I |
| Pineal parenchymal tumour of intermediate differentiation | II or III |
| Pineoblastoma | IV |
| Papillary tumour of the pineal region | II or III |
| **Embryonal tumours** | |
| Medulloblastoma (all subtypes) | IV |
| Embryonal tumour with multilayered rosettes, C19MC-altered | IV |
| Medulloepithelioma | IV |
| CNS embryonal tumour, NOS | IV |
| Atypical teratoid/rhabdoid tumour | IV |
| CNS embryonal tumour with rhabdoid features | IV |
| **Tumours of the cranial and paraspinal nerves** | |
| Schwannoma | I |
| Neurofibroma | I |
| Perineurioma | I |
| Malignant peripheral nerve sheath tumour (MPNST) | II, III or IV |
| **Meningiomas** | |
| Meningioma | I |
| Atypical meningioma | II |
| Anaplastic (malignant) meningioma | III |
| **Mesenchymal, non-meningothelial tumours** | |
| Solitary fibrous tumour / haemangiopericytoma | I, II or III |
| Haemangioblastoma | I |
| **Tumours of the sellar region** | |
| Craniopharyngioma | I |
| Granular cell tumour | I |
| Pituicytoma | I |
| Spindle cell oncocytoma | I |

**Table 2. Grading of selected CNS tumours according to the 2016 CNS WHO.** A synthesized classification of select of central nervous system tumours is shown.

For concerning grades, as in the past, they are written in Roman numerals (e.g., I, II, III and IV; not 1, 2, 3 and 4) (Table 2), considering grade I tumours as the least aggressive, while grade IV as the most aggressive ones. Multiform Glioblastoma are Grade IV tumours (Table 3).

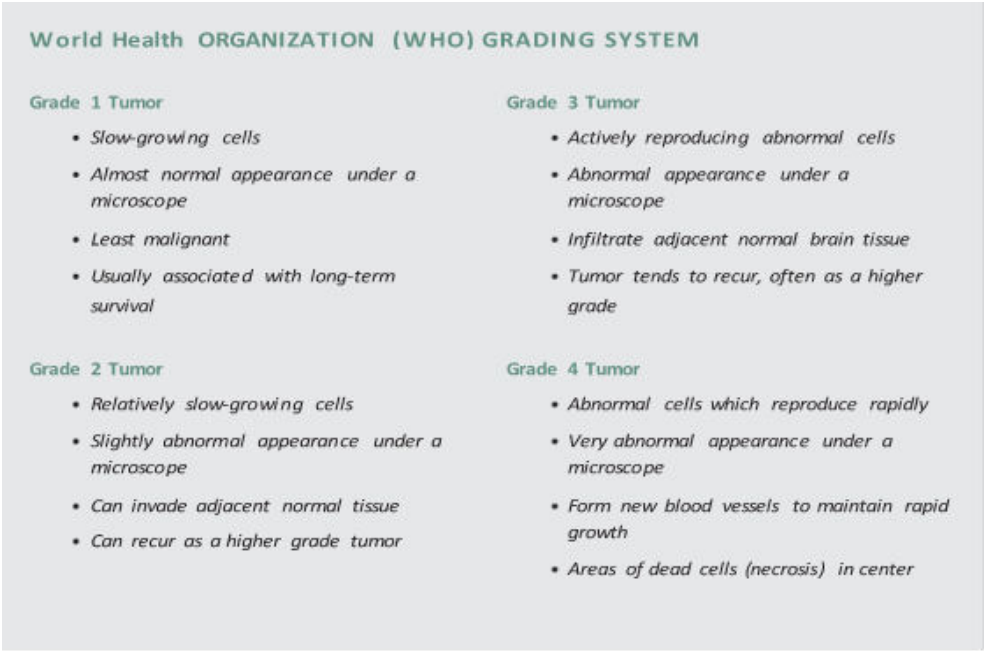


**Table 3. WHO Grading System of gliomas.** Grading System of gliomas defines progressive malignancy of gliomas. This more accurately predicts outcomes, considering hypercellularity, mitosis rates, presence of necrosis, and vascular proliferation. Picture source: http://www.abta.org/brain-tumor-information/tumor-grade/

# 2. Glioblastoma

Glioblastoma (formerly glioblastoma multiforme; GBM) is the most aggressive form of glioma and corresponds to grade IV based on WHO Classification[3]. About 90% of GBM cases grow de novo from glial cells, as primary tumours, instead the remaining percentage can be attributed to secondary cancers, caused by low-grade tumours progression (diffuse or anaplastic astrocytomas). Glioblastoma multiforme especially the primary form, grows very fast, within 3 months, and has a very high rate of recurrence[4]. Surgery is among the first GBM treatments. The first operation on a patient suffering from this disease was conducted in Vienna in 1904. Given the high capacity to invade normal brain tissue, GBM is still particularly difficult to be completely surgically removed. Furthermore, the surgery is often limited by the tumour location and by the patient ability to tolerate it. Despite the high invasiveness GBM rarely metastasizes beyond the brain.

## 2.1. Epidemiology

### 2.1.1. Incidence, risk factors and survival

Glioblastoma Multiforme is a rare tumor and it is the most common astrocytic tumour. In Europe, about 27,000 new cases of malignant astrocytic tumours are diagnosed per year with an annual incidence rate, for the period 2000-2007, of 5 per 100,000 . GBM represents more than 50% of astrocytic tumours[5] and it constitutes around the 25% of all the malignant cancers concerning the central nervous system. In Italy they are recorded around 7.000 new cases every year.
GBM does not have an identifiable cause, generally it occurs spontaneously. Despite this, certain factors have been linked to an increased risk of developing the disease:

- **Age-** The risk that GBM can arise increases with age, although it can occur at any moment of life, including in infants and children. The median age of diagnosis is 64 years. Age of 50 years is identified as cut-off for the clinical subdivision of GBM patients. An older age is associated to a more negative prognosis. The patients with an age range between 70 and 74 years have a higher risk of death than those 65–69 years old. This low chance of survival has been attributed to the decreased

ability to bear the neurological insults caused by the tumor and to tolerate surgery and / or other therapies.

- **Gender-** GBM is more frequently diagnosed in men. Its incidence is 1.6 times higher in males as compared to females with a higher frequency of primary GBMs men and secondary GBMs in women[6]. The survival rate, both in the man and in women, is greater in the first year after the diagnosis (36.7% and respectively of 32,8%). In the second year, it decreases notably with 13.7% in both the sexes. It gradually reduces subsequently, with a rate of survival after 5 years from the diagnosis of only, respectively, 4.7% and 4.6%. Therefore, men have a significant advantage of survival in comparison to women in the first year post-diagnosis, but the difference it is not considerable hereinafter[7].

- **Race/Ethnicity-** Analyzing GBM incidence in US from 2006-2010, it has been shown that white people have higher incidence comparing to blacks, Asian/Pacific Islanders and American Indian/Alaska Native (about two times higher), and non-Hispanics have higher incidence as compared to Hispanic people. Nevertheless, studies on population do not prove a different race-based GBM survival rate. However, there is bound to be some correlations among race, therapy surgical received and survival of GBM patients[7].
  These differences in incidence linked to race ethnic groups and gender, suggest the presence of identifiable, both biologic and environment based, causes.

- **Site-** Although GBM tumours can rise anywhere in the brain, they are more commonly located in frontal, temporal, parietal and occipital lobes (supratentorial region of the brain). GBMs occur rarely in the cerebellum and in very rare cases they are present in the spinal cord. Cerebellar GBMs occur in younger patients compared to the tumours in the supratentorial area, (median age of 50–56 years in contrast to 62–64 years for patients with supratentorial GBM). Glioblastoma multiforme has a high tendency to spread aggressively and to generate tumours in other areas of the brain, named “satellite tumours”, which make them difficult to treat. Among supratentorial GBMs, frontal lobe tumors have better survival as compared to other sites[8].

- **Other factors-** Ionizing radiations are risk factor for GBM. Indeed, previous therapeutic radiation or working in the nuclear industry may

increase the risk of developing GBM. On the contrary, there is no evidence which suggests a correlation between GBM incidence and cigarette smoking, alcohol consumption and use of drugs. Many studies have found conflicting results about the linking of brain tumours with mobile phone use. The long-term risks of mobile phone use remain unknown[9].

### 2.1.2. Prognostic Molecular Markers in GBM

Glioblastoma is the most common and malignant form of glioma. Current treatments are only palliative and they only provide a survival benefit of some weeks or, at most, few years. Therefore, it is critically important to perfectly understand GBM biology and to give the best treatment to the patient, resolving in a better survival.
TCGA, acronym for The Cancer Genome Atlas, is a project to create a catalogue of genetic mutations responsible for cancer. In 2008 it published a new classification of the GBM tumours, which organizes them into four different subclasses, Classical, Mesenchymal, Neural and Proneural, according to some molecular markers. This classification was widely used to predict survival time and treatment response kicking off new studies to better understand the molecular markers of GBM[10].

**MGMT methylation**
Alkylating agents like Temozolomide (TZM) are specific drug used to treat GBM and some other cancers. They function by transferring alkyl groups to guanine bases causing DNA damage that can result in cellular death through apoptosis. MGMT is a DNA repair protein that is involved in cellular defence against DNA damage caused by alkylating agents. The protein removes alkyl groups from the O6 position of guanine in DNA, catalysing the transfer of the methyl groups resulting in the repair of the toxic lesions and preventing apoptosis. Thus, MGMT mediates resistance to TZM. Methylation of the gene promoter has been associated with several cancer types, including glioblastoma. Expression of MGMT is regulated by methylation of the promoter. Methylation causes the reduction of the expression of this protein and, therefore, it increases TZM sensitivity. In contrast, unmethylated MGMT promoter, increases protein expression given chemotherapy resistance.
Thus, patients with a high level of methylation of the promoter survive longer than those with a low level[11].

**Isocitrate dehydrogenase mutation**
IDH mutation is generally observed with low grade glioma. Conversely, it is only rarely associated with genetic alterations that we get used to see with high rates in primary GBMs (e.g., EGFR). This suggests that this is not a driver mutation in these GBM subtypes. On the contrary IDH mutation seems to be a driver mutation in low grade gliomas, likely through 2-HG production[12]. The most common mutation both in IDH1 and in IDH2 is a single residue substitution of a histidine with an

arginine, representing a gain-of-function genetic change. The enzyme converts alpha-ketoglutarate (α-KG), the normal product, to D-2-hydroxy-glutarate (D-2HG), a possible oncometabolite, altering cellular metabolism and response to hypoxic and oxidative stress.
IDH mutated high grade gliomas arise from lower-grade gliomas and generally have a less aggressive clinical course compared to the primary GBM. Thus, these mutations are selective molecular markers of secondary GBMs differentiating them from primary GBM. Therefore, IDH mutated high grade gliomas have a more favourable prognosis than the ones without IDH1 mutation.
Consequently, IDH mutations can influence the prognosis and may also predict tumour response to radiation and/or alkylating chemotherapy[12].

**EGF Receptor**

EGFR or "epidermal growth factor receptor" is a tyrosine kinase receptor forming part of the ErbB family. This receptor is a cell surface protein able to bind epidermal growth factor. After binding, receptor dimerizes. This induces tyrosine auto-phosphorylation and determines the activation of a several signalling pathways and physiological responses which result in range of cellular activities, including growth, migration, survival and tumorigenesis. Studies demonstrated that in GBMs, EGFR signalling promotes cell division, tumour invasiveness, and resistance to radiotherapy and chemotherapy[13]. However, clinical studies have proved to be inconclusive to demonstrate the link between EGFR expression and poor prognosis. EGFRvIII (variantIII) is the most common mutation among EGFR amplified GBMs. About 50% of patients with EGFR amplification has this mutation due to deletion of exons 2–7, that results in a constitutively active form of the protein[10].
EGFRvIII over-expression has been shown to strongly predict poor prognosis in presence of EGFR amplification. Moreover, EGFR could be used as predictor for response to receptor tyrosine kinase (RTK) inhibitors, but it should be considered that EGFR-amplified tumours only initially respond to RTK inhibition becoming often resistant to the treatment.

**TP53 mutation**

The TP53 gene is a well-known tumour suppressor. It is involved and mutated in many cancers, playing a crucial role in tumour suppression. p53 is also a wide transcription factor, regulating more than 2500 genes, most of which affects tumorigenesis and tumour development[14]. Mutation of this gene has been reported in 60-70% of secondary GBMs and 25-

30% of primary GBMs[15]. Apart from deletion of this gene, p53 pathway is generally modulated by a number of factors (MDM2, MDM4, and p14ARF as well as ATM and ATR, and so on), the alteration of which is, its own, involved in GBM development. According to TCGA data, 78% of GBM have mutations somewhere within this pathway[14]. Secondary GBMs have generally direct mutations of p53 gene. On the contrary, primary GBMs have more often an alteration in p53 regulators. Upstream of MDM2 is an important regulator of the p53 pathway. It negatively controls p53 by the use of two mechanisms: a transcriptional inhibition by direct binding, and the degradation through its E3 ligase activity.
Primary GBMs often have a loss of INK4A/ARF (CDKN2A) gene locus. ARF (p14ARF) is a tumour suppressor protein which regulates p53 directly binding to MDM2 and, thereby, inhibiting its E3 ubiquitin ligase activity. INK4a (p16INK4A), as well, plays a crucial role in growth control influencing pRB pathway.
However, since p53 pathway influences several cellular responses, the prognostic and predictive role of this protein continues to be studied.

## Genetic losses of chromosomes

### Losses on chromosome 10

One of the most common alterations in GBM patients is the loss of heterozygosity (LOH) for chromosome 10[16]. These losses can concern the entire chromosome or only one of its arms (short or long), so they could involve until 800 genes which are contained in it and encode proteins that plays different roles. Phosphatase and tensin (PTEN), is located at 10q23.3 and it was the first tumour suppressor gene identified on this chromosome. Deletion of this gene occurs in 20-40% of GBMs, and they generally affect with higher frequency primary glioblastomas[6]. LOH for chromosome 10 seems to be an independent, adverse prognostic variable in high-grade glioma. Complete LOH for this chromosome are more commonly reported in grade 4 than grade 3 which, on the contrary, shows a higher frequency of partial loss compared to grade 4[17].
Thus, considering high grade tumours, this kind of genetic alteration was correlated with an overall survival decrease.

### 1p/19q status

Co-deletion of the short arm of chromosome 1 and the long arm of chromosome 19 (1p/19q) is a genetic event found in 80% of oligodendrogliomas[10]. This happens when there is an unbalanced whole-

arm translocation between chromosomes 1 and 19. It often occurs in IDH 1/2 mutated GBMs, whereas it is mutually exclusive with TP53 mutation. According to TCGA, patients with grades II/III gliomas with an IDH mutation and 1p/19q-co-deletion lives longer than patients with an IDH mutation and no 1p/19q-co-deletion or compared to wildtype IDH[18].
This co-deletion predict response to chemotherapy and give better prognosis in anaplastic oligodendrogliomas, proving to be an independent prognostic marker associated with increased survival in both diffuse low-grade and anaplastic tumours[19].

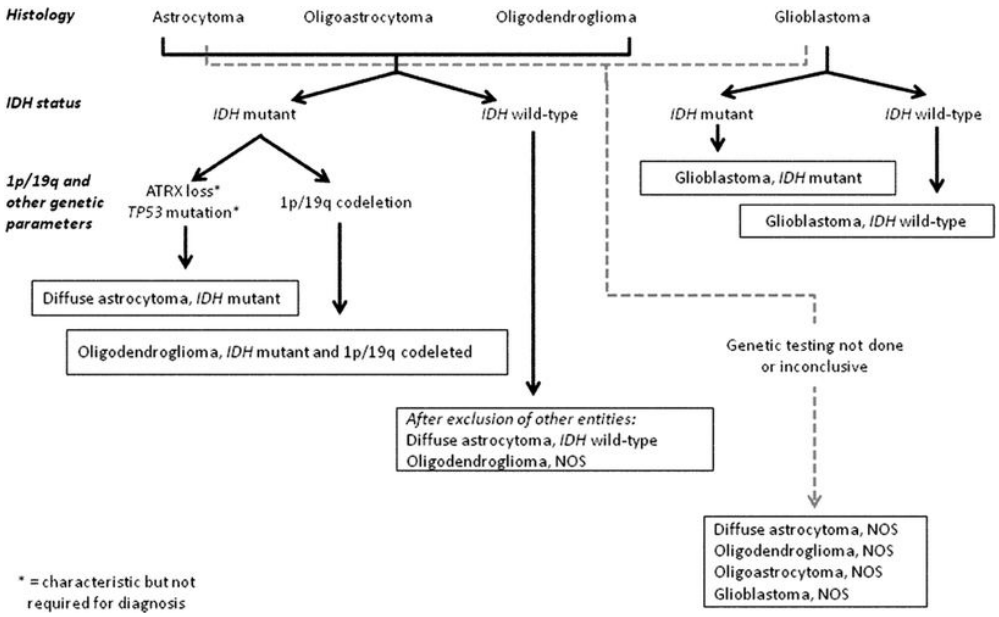


**Figure 1. A schematic algorithm for classification of the diffuse gliomas**. It provides a dynamic classification based on both phenotypic and genotypic features[3].

## 2.2 GBM Symptoms

According to where the tumour is localized, GBM symptoms can affect any of the brain's functions since, growing up, it can press up against nerves or other CNS structures damaging them. This can interfere with a lot of brain functions such as memory, thought, emotion, movement, vision, hearing and touch. Thus, the most common symptoms of this disease are the following:

- Frequent headaches, which make you up at the night or expand early in morning
- Crisis or convulsions, which appear as sudden unintentional muscle contraction
- Unexplained and intense nausea (feeling sick) with or without vomiting
- Abnormal gait
- Vision problems
- Memory loss
- Change in personality and irritability
- Impaired concentration
- Loss of vision, memory, impairment of speech and movement.

Considering that there is no treatment for GBM, symptom management and palliative care remains the only option representing an essential aspect of the cure.

### 2.3. GBM Diagnosis

Diagnosis generally initiates with a medical history, followed by some neurological examinations inter alia:

- Reflexes
- Coordination
- Feeling
- Pain response
- Muscle strength

These examinations are generally followed by imaging tests including:

- **Magnetic resonance imaging (MRI).** MRI is the primary and preferred test used to find a brain tumour and it uses potent magnetic fields, radio waves, and field gradients to generate detailed pictures of the brain and spinal cord. Before scanning, a special dye called contrast medium is injected or given to the patient as pill[20].

- **Computed tomography (CT) scan.** A CT scan is an imaging procedure that employs special x-ray measurements taken from different angles thanks to the rotation around the head. This technique produces cross-sectional (tomographic) images (virtual "slices") of specific areas of scanned cranium, letting the doctor to see inside the object without cutting it. A CT scan can also be useful in tumour size measurement. Here too a contrast medium can be given before the scan to provide better detail on the image.

- **Positron emission tomography (PET) scans.** PET observes metabolic processes in the brain or other body parts, using gamma rays emitted indirectly by a positron-emitting radionuclide (tracer), which is introduced into the body on a biologically active molecule. Then a computer generates three-dimensional images of tracer concentration in the brain. Recently PET and CT scanners work together in the same machine during an only session.

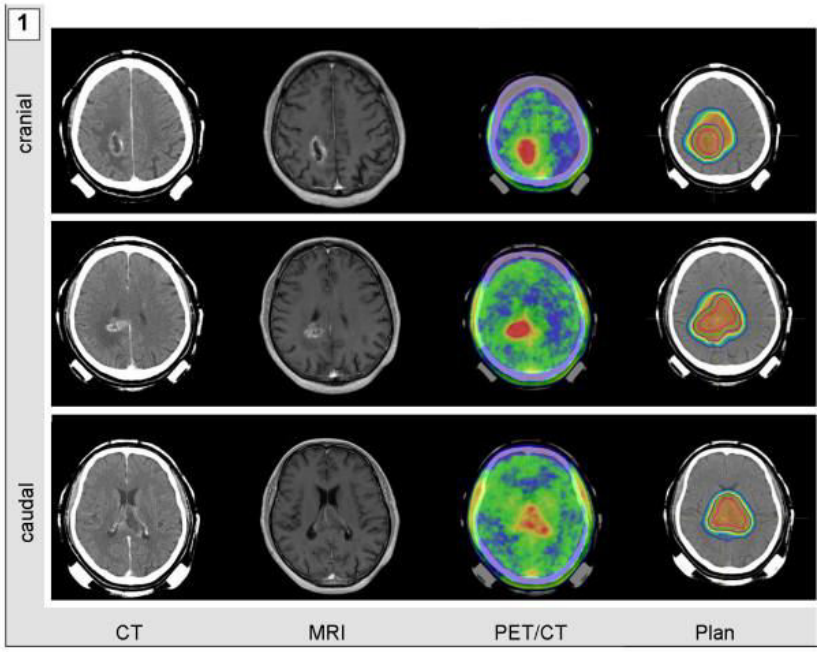


**Figure 2. Extensive glioblastoma multiforme in a 62-year-old man.** Contrast-agent enhanced CT and MRI scan were fused with a FET-PET/CT examination and used to decide the right radiotherapy plan[21].

Unlikely, to look directly at the tumour tissue still remains the only way to be sure that a cancer is a glioblastoma, thus, biopsy from the tumour have to be taken and examined under the microscope. Consequently, these tests can only suggest that a GBM is present, but only a biopsy can give a definitive diagnosis.

## 2.4. GBM treatments

Sadly until today, there is not cure for GBM. Thus, the treatment can only affect symptomatic aspects which aim to increase the survival rate and simultaneously to improve quality of life.

Treatment depends on the patient's medical situations. Because of the extremely aggressive nature of glioblastoma, tumours are typically treated simultaneously by two or more methods (Table 4). Thus, in the beginning, treatment consists of an immediate maximal-safe surgical resection (if and when possible); this medical procedure is generally followed by RT and TMZ chemotherapy. Additional six rounds of chemo with TZM are used as maintenance treatment.

Therefore, GBM cure includes surgery, radiation, and chemotherapy.

- **Surgery.** Surgery is, in most cases, the first treatment step. It consists of the complete removal of the tumour, wherever possible, and sometimes a preventative surrounding healthy tissue resection. This helps to relieve symptoms, reducing tumour pressing on the nervous system structures and allows the definitive diagnosis. In certain cases, the tumour cannot be completely removed, because cancer cells grow into surrounding brain tissue, making surgery difficult. This is the reason why the treatment includes additive therapy to kill the remaining cells. In some instances, surgery can damage part of the brain resulting serious consequences in the movement, breath, swallowing or consciousness. Furthermore, some GBM, named inoperable or unresectable, cannot be surgically removed because of their location and in these cases other treatments have to be given.
- **Radiation.** Radiations are the use of high-energy x-ray beams to destroy tumour cells stopping or slowing tumour growth. They kill cancer cells by damaging their DNA directly or creating charged particles (free radicals). The most common type of radiation treatment is named external-beam radiation therapy which aims high-powered x-rays at the tumor and surrounding tissues from outside the body. Interstitial radiation or brachytherapy is another radiotherapy that employs implants; in this case radioactive material is placed directly into the tumor. Proton therapy, called also proton beam therapy, is a further type of radiation treatment used for brain tumours; it uses protons to treat cancer. Here, radiations are delivered with no pain through the skin from a machine outside the body. This approach provides pinpoint focusing of the radiation beam to the tumor, by reducing damage to surrounding normal brain tissue. Unfortunately, radiation therapy can also damage normal cells, leading to

several side effects. Some of the main side effects are the following: tiredness and weakness, sore skin, loss of hair, dry mouth, infertility, nausea and vomiting.

- **Chemotherapy.** Chemotherapy is the use of drugs to destroy or to stop the growth of cancer cells. The doctor can give a single drug or a combination of different drugs simultaneously which can be taken by mouth (oral administration), injected into a vein (intravenous administration) or in a muscle (intramuscular injection). Chemotherapeutic drugs can also be given directly into the spinal canal, in a procedure named intrathecal chemotherapy; this allows treating cells on the surface of the brain and spine. This procedure is still an experimental treatment and cannot be available everywhere. TZM is the most used chemotherapeutic drug for GBM, and it is an alkylating agent. Another alkylating agent is Carmustine, it also applied as infused biodegradable discs, implanted after glioma surgery resection (Gliadel®)[22]. As well as radiotherapy, chemo can lead to several side effects. Some of the main side effects are: fatigue, risk of infection, nausea and vomiting, hair loss, loss of appetite, and diarrhoea.

| Type of Tumor | Therapy |
|---|---|
| Newly diagnosed tumors | |
| Glioblastomas (WHO grade IV) | Maximal surgical resection, plus radiotherapy, plus concomitant and adjuvant TMZ or carmustine wafers (Gliadel)† |
| Anaplastic astrocytomas (WHO grade III) | Maximal surgical resection, with the following options after surgery (no accepted standard treatment): radiotherapy, plus concomitant and adjuvant TMZ or adjuvant TMZ alone† |
| Anaplastic oligodendrogliomas and anaplastic oligoastrocytomas (WHO grade III) | Maximal surgical resection, with the following options after surgery (no accepted - standard treatment): radiotherapy alone, TMZ or PCV with or without radiotherapy afterward, radiotherapy plus concomitant and adjuvant TMZ, or radiotherapy plus adjuvant TMZ†‡ |
| Recurrent tumors | Reoperation in selected patients, carmustine wafers (Gliadel), conventional chemotherapy (e.g., lomustine, carmustine, PCV, carboplatin, irinotecan etoposide), bevacizumab plus irinotecan, experimental therapies‡ |

**Table 4. Summary of current therapy for Malignant Gliomas in adults.** The table indicates standard therapy for newly diagnosed malignant gliomas, it involves surgical resection when possible, radiotherapy, and chemotherapy[23].

## 2.5. Remission and recurrence

Remission is the situation in which the cancer is undiagnosable or there are no symptoms. This situation can also be named having “no evidence of disease” or NED.

Two different type of remission exist:

- **Partial remission.** A partial remission is when the tumour is still there, but it is smaller as a treatment result, so the patient can reduce or suspend the treatment as long as the cancer doesn’t begin to grow again.
- **Complete remission.** Complete remission, named also NED, means that the cancer cannot be detected by tests, physical exams, and scans. The term Complete remission or NED is preferred to the word “cure” because the remission may be also just temporary thus, unlikely, cancer cells can come back.

  If the tumour comes back, it commonly happens within 5 years following the first treatment. Thus, the patient should continue to check for signs of cancer periodically after a complete remission.

  Recurrent tumour is a tumour which returns after the original treatment. Understanding the risk of a recurrence is very important to feel yourself more prepared if the tumour does return. Cancer that comes back in the same place is named local recurrent tumour, similarly if it comes back nearby or in another place it is called regional or distant recurrent tumour respectively. The treatment plan often includes the same therapy described above, but they can be used in a different combination.

  If treatment fails, so if the cancer cannot be cured or controlled, the tumour is called advanced or terminal cancer.

# 3. Stem Cells

Stem cells are progenitor of more than 200 cell types forming adult body. They are unspecialized cells contributing to tissue generation, maintenance and repair.
Stem cells possesses two main features: Self renewal and Unlimited potency. Self renewal is process by which stem cells divide by maintaining their undifferentiated state, thus giving rise to more stem cells with basically the same non-specialized phenotype and replication potential. It occurs through a symmetric division to generate two identical cells. Instead, potency refers to their ability to produce daughter cells able to differentiate into different types of specialized cells. It occurs through an asymmetric cell division to generate one cell that is identical to the mother cell and one different cell, named progenitor cell, that will generate through differentiative steps the specialized cell.
Stem cells can have several grades of potential. **Totipotent** cells are cells capable of differentiating into all the specialized body cells, including extraembryonic tissues[24]. **Pluripotent** stem cells can produce any of the three different types of human embryonic leaflet, giving endoderm, mesoderm or ectoderm cells. Thus, these cells can produce any type of body cells except extra-embryonic tissue. **Multipotent** cells are able to differentiate into a limited number of other cell types.
Normal adult stem cells can be find in many tissues. As demonstrated by scientists, adult stem cells are also present in brain and heart, two locations where they were not previously supposed to reside. Now stem cells are increasgly becoming therapeutic tools for regenerating tissues[25].
Systemic or local signals regulate cell division of stem cells influencing senescent or damaged cells replacing and ensuring organs correct functions[26]. Thus, self renewal and differentiation processes are tightly controlled in order to maintain and repair adult tissues by responding to environmental changes. Researchers showed that failure of this sophisticated control mechanism underpins tumour occurrence and development[27].

## 4. Cancer Stem Cell Theory

Survey after survey shows that tumours contain a small population of cells, named cancer stem cells (CSCs)[28,29], which is responsible for tumor initiation, growth, and recurrence and is liable for cancer chemotherapy resistance[30]. Thus, cancer is now recognized as a heterogeneous group of cells showing several differentiation phenotypes.

CSCs are a very little population representing about 0.05-1% of the cancer cells, despite this they result to be crucial for chemoresistance and recurrence[31,32]. These cells share with the normal stem cell several characteristics, the most important of which are self-renewal and differentiation. Unlikely, they gain also numerous additional abilities including:

- **Self-sufficiency in growth signals**, it is the ability to proliferate aberrantly.
- **Insensitivity to anti-growth signals**, they resist to anti-proliferative growth signals.
- **Evading apoptosis**, ability to resist to programmed cell death.
- **Limitless replicative potential**, they are able to proliferate indefinitely.
- **Sustained angiogenesis**, they can stimulate the growth of blood vessels to supply nutrients that they need.
- **Tissue invasion and metastasis**, it refers to the ability to invade local tissue and spread to distant sites.

CSCs origin is still unclear, they could derive from normal adult stem cells after accumulating of oncogenic mutations or from differentiated cancer cells which undergo a reprogramming-like process acquiring stem-like properties. It has been showed that many embryonic stem cell signalings are reactivated in stem cells involved in several cancers[33]. For instance, Nanog, an embryonic stem cell-specific transcriptional factor, is highly expressed in several CSCs implicated in hepatic, colorectal, and brain tumours[34-36].

CSC subpopulations can be sorted on the basis of their cell surface marker profiles[37] or identified by tumorsphere culture, using their ability to grow in the absence of serum and without attachment to culture plates, so in condition in which differentiated cells cannot survive[38]. It appears clear that CSCs contribute to treatment failure[39], moreover only a small number of these cells is enough to initiate a tumour.

In summary, one of the greatest limitations of the currently therapeutic approaches is that they are failing to eliminate CSCs, as CSCs result to cause a multiple drug resistance (MDR) of the tumour[40]. Since CSCs

survive conventional therapies, they often lead to tumour recurrence (Figure 3). Therefore, many studies have focus on selection of new tools and novel therapies aiming to eliminate CSCs and strongly reduce cancer recurrence.

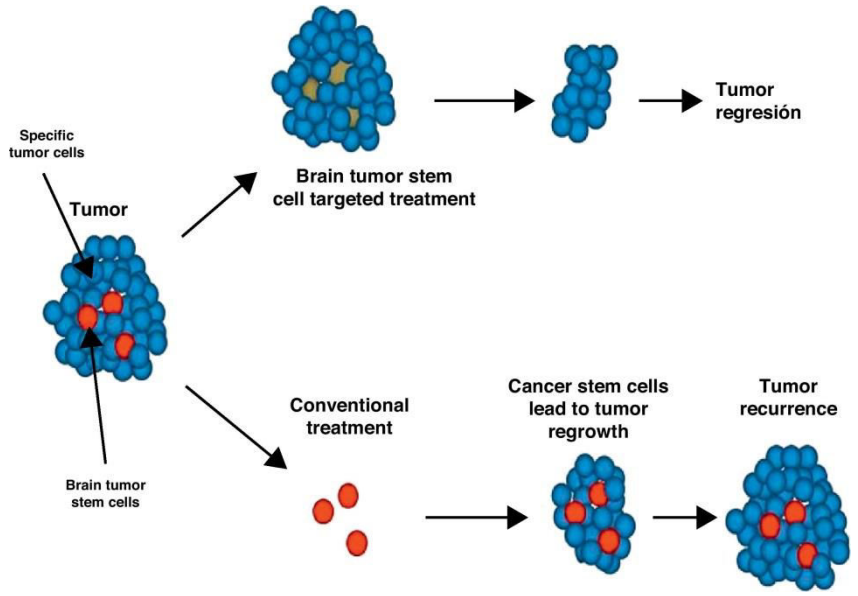


**Figure 3. Schematic representation of Cancer Stem Cells Theory.** Here it is illustrated the effect of current clinical treatments on tumour cell populations. Unlikely, current radiotherapy and chemotherapies target only highly proliferative cells, but they appear to be unable to target that small population of quiescent cells (GSCs) which, over time, are responsible for tumor recurrence.

GBM as well is a heterogeneous tumour, consisting of normal cells and a small population of CSCs; thus, identifying a selective therapy against GSCs is of the utmost importance in the fight against GBM.

# 5. Aptamers

The term “Aptamer” was coined by Andy Ellington in 1990. It stems from the greek terms “aptus,” meaning to fit, and “meros,” meaning part. Aptamers are single-stranded oligonucleotides, DNA or RNA molecules, able to bind tightly to a target molecule cause the “fit" to their target acting directly by binding the protein target with high affinity[41]. Thereby, thanks to their three-dimensional conformation, aptamers are known to recognize their specific target with high affinity and selectivity.
Aptamers are selected by using a technique named SELEX. Up to now, from the SELEX we achieved more than 900 aptamers[42]. The most common targets are proteins, although aptamers can bind a wide range of targets such as inorganic ions, small organic ligands, amino-acids, nucleotides and derivatives, oligonucleotides, antibiotics, peptides, proteins, sugars, parasites, virus, cells and tissues.
Especially in oncology there has been a noticeable increase in aptamer field. In a medicine which needs to be more and more specific to selectively destroy cancer cells sparing healthy cells, along with the necessity of personalizing the treatment according to the different type of tumours, highly specific tools, such as aptamers, are becoming more and more attractive instruments in fighting cancer. In addition, their specific binding to cell surface receptors will allow not only molecular imaging but also the direct delivery of drugs into cancer cells expressing the target.

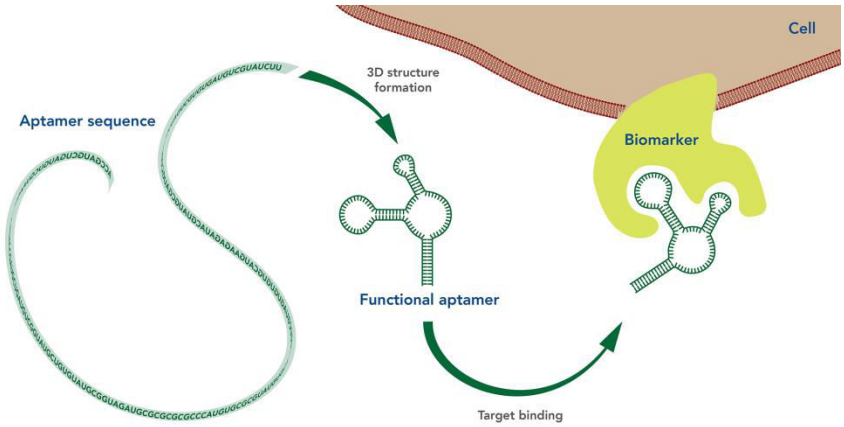


**Figure 4. Engineering aptamers binding specific targets.** Aptamers are single-stranded DNA or RNA sequences able to tightly bind to target molecules thanks to their 3-dimensional structures. Picture source: http://www.idtdna.com/pages/decoded/decoded-articles/core-concepts/decoded/2016/03/15/planning-to-work-with-aptamers

Moreover, aptamers possess several advantages over antibodies: there are synthetically created reducing the production costs; they are more stable both at room temperature and at elevated temperature (after which they are able to self-refolding) compared to antibodies; moreover, they are smaller than antibodies and thus, they have a higher tissue penetration; furthermore, aptamers have no toxicity or immunogenicity and they can easily be chemically modified. Another very important aspect of aptamers is also their ability, in some cases, to cross the blood brain barrier (BBB) making them attractive instruments for brain cancer[43,44].
Aptamers can be distinguished in ssDNA and RNA aptamers. The first ones are more stable and cheaper than second ones, but their preparation during the SELEX is more challenging than RNA aptamers which give also more diverse three-dimensional structures than ssDNA aptamers.

### 5.1. Aptamer modification

Being aptamers nucleic acids, they have two major drawbacks. One of them is their nuclease sensitivity, especially regarding RNA aptamers; this would lead rapid elimination from the blood[45] resulting in pharmaceutical development difficulties. The second one is that aptamers, with their only 4 nucleotides, can have a lower chemical diversity compared to 20 amino acids based library. Fortunately, aptamers prove to be very easily chemically modifiable; these modifications allow overcoming these two aptamers limits. Generally, the nucleophilic cleavage occurs on the 2'-OH group; thus, modifying this group, nuclease sensitivity is greatly reduced. 2'-OH group of pyrimidines can be easily replaced with 2'-fluoro (2'-F) or 2'-amino (2'-NH2) group. Other modified nucleotides can be 2'-O-methyl nucleotides, 2'-thio (2'-SH), 2'-azido (20-N3), locked nucleic acid (LNA) or hexitol nucleic acid (HNA). Modifications can be incorporated via an efficient engineered mutant T7 RNA polymerase, embedded in the SELEX library or, in case of they do not rearrange aptamers shapes, can be introduced post-selection.
In order to expand the chemically diversity of aptamers Gold et al[46]. created a new class of aptamer, the Slow Off-rate Modified Aptamer (SOMAmer), by the incorporation of four modified nucleotidentriphosphate analogs: 5-benzylaminocarbonyl-dU(BndU), 5-naphthylmethylaminocarbonyl-dUn(NapdU), 5- tryptaminocarbonyl-dU (TrpdU), and 5-isobutylaminocarbonyl-dU (iBudU); these modifications

enhance binding affinity and enables a slower complex dissociation rate. Thus, SOMAmers are currently employed to discover and validate biomarkers by using SOMAscan™ assay.
Another way, used by the company NOXXON Pharma AG (Berlin, Germany), to prevent aptamers enzymatic degradation consists of using spiegelmers ('spiegel' means 'mirror' in german). Spiegelmers are synthetic RNAs made of L-ribose instead of D-ribose units, this chemical modification give an increased resistance to nuclease degradation and more stability in vivo. In this case, the SELEX is performed with D-RNA library on the mirror image of the protein (artificially synthesized) with D-aminoacids. Once the aptamer is selected, it is converted to L-RNA, understandably this approach can be applied only by using synthetically targets.
Additional modifications may concern the improvement of aptamers half-lives. Therefore, ssDNA and RNA aptamers are often conjugated to bulky groups, such as poly (D, L-lactic-co-glycolic acid) (PLGA), poly ethylene glycol (PEG), liposomes, streptavidin or cholesterol, which can strongly reduce systematic clearance[47].

## 5.2. SELEX technology

SELEX is the technique through which aptamers are selected; SELEX is an acronym which stands for 'selective evolution of ligands by exponential enrichment'. It represents a chemistry technique used in molecular biology to select single strand nucleic acids able to specifically bind target molecules. The selection starts from a very large oligonucleotides library consisting of $10^{14}$-$10^{15}$ ssDNAs or RNAs which have a central randomly degenerated sequence flanked by constant 5' and 3' regions, containing binding sites for primers (and T7-RNA polymerase binding site for RNA-SELEX). For a degenerated region of length n, $4^n$ are the possible obtainable sequences. The sequences are placed in contact with target; during the partitioning step, nucleic acids able to bind the target are recovered instead sequences which do not bind are washed away. Then, thanks to the fixed regions, binding molecules are amplified by PCR or RT-PCR according to whether ssDNA or RNAs are respectively used as oligonucleotides. A round of SELEX consists of three steps:

1. Selection, sequences are exposed to the target
2. Partitioning, binding sequences are selected but the unbound sequences are washed away.

3. Amplification, binding sequences are amplified and used for the next SELEX round.
   The number of rounds can be decided according to personal needs.
   Rounds can be made more complex by adding negative selection to environmental elements (such as supports like filters or beads) and/or by the addition of a counter selection step against not target molecules (like negative cells or related proteins). These additional steps can be inserted either before or after the positive selection to remove non-specific binding aptamers.
   The choice of what to use as positive-, negative- or counter-selection is of highly important for the selection.
   As shown in figure, two are the main methods used to identify aptamers, one is the classical protein-SELEX approach and another one is the more accurate cell-based SELEX procedure.

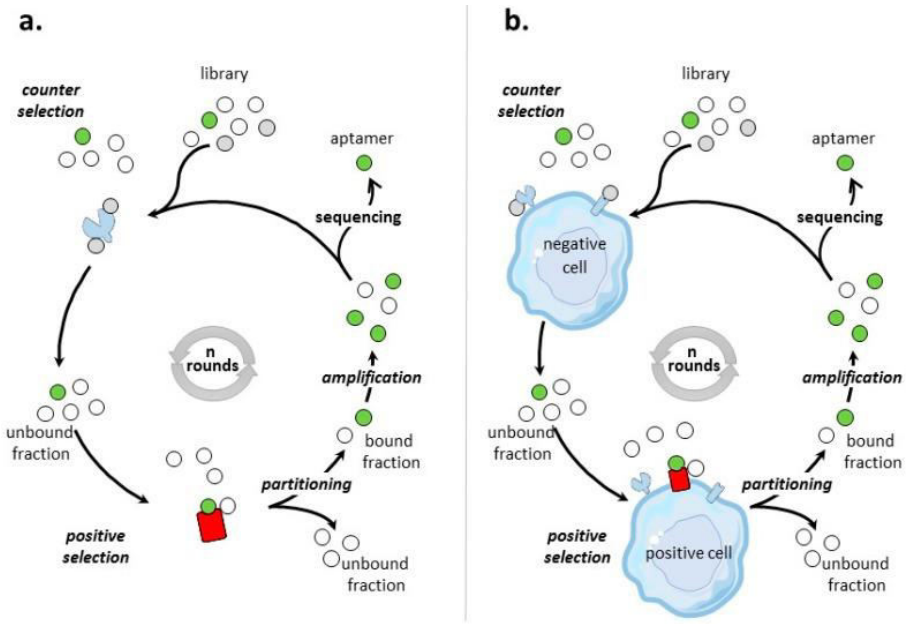


**Figure 5 Scheme of protein- and cell-based SELEX processes.** Continue.

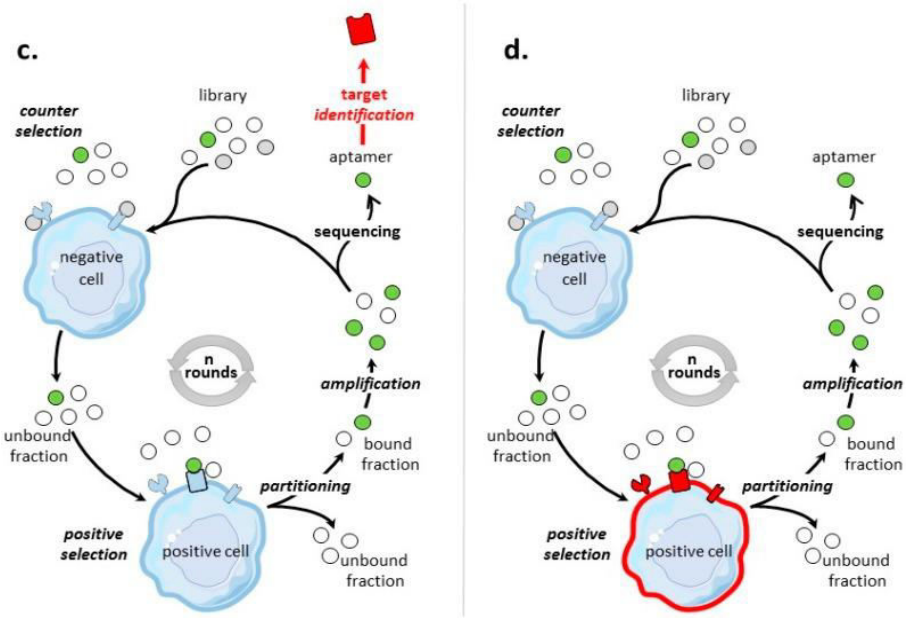


**Figure 5 Scheme of protein- and cell-based SELEX processes.**[47] Briefly, the process of SELEX involves three steps: selection, partitioning and amplification. The selection shall be carried out by using the target cells. During the partitioning nucleic acids able to bind are recovered. Finally, they are amplified and used for the next SELEX cycle. In the end of SELEX, the obtained nucleic acid molecules are cloned and sequenced. Individual sequences are aptamers. (a) Protein-based SELEX. The pre-identified purified protein is used as target for this kind of SELEX. It is visible in red. (b) Cell-based SELEX on a pre-identified tumor cell-surface biomarker colored in red. (c) Cell-based SELEX on a post-identified tumor cell-surface biomarker. The target is shown in red, it is identified at the end of the SELEX process. (d) Cell-based SELEX to a tumor cell type. In this case, no particular cell-surface biomarkers are identified and aptamers are specific to the cell's molecular signature. The whole cell used for positive selection is colored in red.

### 5.3. Protein-SELEX technique

Protein-SELEX represents the easiest form of SELEX. Full-length or truncated versions of full length proteins can be used during this technique. Proteins are generally coupled to tags (His-tags, Fc fragments of antibody or GST), in order to make it easier to purify and select by affinity. This technique presents various limits; some of them are the difficult to extract, purify and solubilize membrane protein from the lipid membrane or often large amount of proteins are required for a whole protein-SELEX. Furthermore, some membrane proteins expressed in prokaryotic or lower eukaryotic systems may not have or have different post-translational modifications, compared to eukaryotic cells; these differences (phosphorylation, glycosylation, ubiquitination, methylation, myristylation, acetylation) can significantly alter protein structure reducing or completely eliminating aptamer binding[48].
Broadly, the major downside of protein-SELEX is that these purified and solubilized proteins have a very different structure which is a long way from the physiological conformation. Therefore, some aptamers selected with protein-SELEX failed to recognize their cell-surface targets in its endogenous environment. Moreover, some biomarkers need co-receptors for their appropriate folding. This limitation can be overcome by using a cell-based SELEX.

### 5.4. Cell-Based SELEX

As shown in Figure. b-d, the whole cell represents the target of cell-based SELEX. Here, differently than protein-SELEX, target is in its native environment on the cell surface and, because of that, it is in its physiological conformation by enabling the right selection of aptamers able to bind it also *in vivo.*
Many papers have been published on cell-SELEX approach, especially linked to cancer.
In internalized cell-SELEX, aptamers are selected on the basis of their ability to be internalized after target binding. According to the target of the cell-SELEX, we can have:

- **Pre-Identified Tumor Cell-Surface Biomarkers,** when the objective of the SELEX is to select aptamers for an identified and known biomarker expressed on cell surface (Figure. b). This technique may be combined to a protein-SELEX generating a hybrid-SELEX; thus, the first rounds of the selection are performed on cells and last SELEX cycles are made on

purified protein. A reverse hybrid-SELEX couples first protein-SELEX and later cell-SELEX.

- **Post-Identified Tumor Cell-Surface Biomarkers,** when the target is identified after the cell-SELEX. This kind of SELEX is very important because it allows identification of new biomarkers (Figure c). Thus, there is first aptamers selection against a tumor cell type followed by target identification which could represent a new biomarker. The identification results often to be challenging, it is generally shall be affected by affinity purification followed by mass-spectrometry. The first post cell-SELEX identification was realized by Daniels et al[49].in 2003. They identified Tenascin-C as target of GBI-10 aptamer, selected using U251 glioblastoma cell line as positive selection. It is not a cell-surface receptor, but an extracellular matrix protein.
- **Undetermined Targets,** when aptamers are selecting against a tumour cell type without pre- or post-identification of targets. In this instance, aptamers result to be specific for a molecular signature (Figure d).
  Targets, during a cell-based SELEX, are certainly closer to their natural distributions and conformations; this gives more assurance about a good selection of the aptamers, moreover contrary to a protein-SELEX, cell-based SELEX, offers the opportunity to identify a new tumor protein biomarker. However, this type of selection is more complex than protein-based SELEX; a high number of cells are needed, therefore sell lines have to be available, cultivable and stable. In addition, it is generally difficult to select aptamers specific for a less expressed target on cells and sometimes cells have to be modifying over- and/or under-expressing cell-surface protein target for selection and/or counter-selection and this may lead modifications of the cell line which can do not represent the disease cellular context anymore. Furthermore, it has been proved that cell-SELEX needs more selection/counter-selection rounds than protein-SELEX to achieve selective aptamers for the targets. Finally, aptamer-mediated target identification is often not an easy procedure.

## 5.5. Applications of Aptamers in Oncology

### 5.5.1. Aptamers as Detection and Imaging Reagent

A timely detection of biomarkers indicating disease is of great importance, especially in the cancer context, where the timeliness is essential. Aptamers, being a class of bio-recognition molecules, may be

used as diagnostic tool. Biomarker detections is made possible by using aptasensors (aptamers used as biosensors), by converting the aptamer conformational change, caused by the binding to the target, into measurable signals. Sun et al. in 2016 showed the detection of soluble tumour biomarkers through the use of aptamer-based analytical platforms with low limits of detection[50]. Aptamers can also be utilized, in the contest of circulating tumours, in sensitive diagnostic assays[51,52]. Bukari et al[53]. in 2017, developed a method by which aptamers are used in histopathological diagnosis.
A very important aptamers issue concerns their application as imaging agents; in this case, they are combined with radionuclide or fluorescent labels, bioconjugates or nanoparticles, representing therefore a non-invasive manner for defining the tumor tissue border. This is a very tempting opportunity in cancer, especially in brain cancer in which perfectly defining tumour border could allow to completely eliminate the tumour and, simultaneously, do not eliminate healthy tissue.

### 5.5.2. Aptamers in Therapy

Aptamers, after interaction with their targets, can act as agonist or antagonist by activating or blocking target cellular functions respectively. Several aptamers, such as CD28, OX40 and 4-1BB, have been proved to have immunomodulatory activity if they are used both alone and in combination with first-line treatments in cancer therapy[54].
Given the ability of some aptamers to be internalized following the binding to the target, they can be used as a delivery tool of therapeutic agents. Thus, aptamers able to be internalized are cargoes which carry therapeutic agents inside the cytosol of specific targeted cells[55]; this constitutes a very pioneering aspect considering that current anticancer treatments are definitely not selective, affecting both cancerous and healthy cells and consequently generating very strong adverse effects.
Therapeutic agents can be coupled to aptamers both in a non-covalent and covalent manner, by forming aptamer chimeras which are composed of an aptamer and therapeutic molecules (such as small interfering RNA, micro RNA, anti-microRNA and small hairpin RNA or anti-tumor drugs or toxins etc.)[56]. Therefore, aptamer chimera appears to have a very promising role in a more selective medicine.

## 5.6. Aptamers approved or in Clinical Trials

Currently eleven are the aptamers approved or under examination during clinical trials. Three of them are involved in eye disorders, four in coagulation, two concern inflammation and two are implicated in cancer treatment response[57]. Generally, aptamers target are proteins which are on cell surface; this means that aptamer activity can be reversed by using of an oligonucleotide antidote, as in the case of pegnivacogin. Pegnivacogin is an antithrombotic aptamer, whose action can be reversed by anivamersen, a 15-nucleotide long 2'-O-methyl RNA.
Almost all the aptamers, listed in Figure 6, have a PEG-modification. As mentioned before in 4.2, this modification can strongly limit renal clearance and enhance aptamer stability, increasing its molecular weight and prolonging its circulating half-life. Unlikely, Pegnivacogin clinical trials was stopped because of 3 patients on 640 who presented allergic reaction (two anaphylactic and one dermal); however, it was observed that pegnivacogin or its degradation was not responsible for these allergic reaction, as well as inflammation response or histamine release, but these reactions were caused, in the three patients, by their high levels of antibody to PEG.

### 5.6.1. Aptamer therapeutics for age-related macular degeneration (AMD)

**Pegaptanib sodium or Macugen®**
Macugen® (Eyetech Inc and Pfizer) is a 27 ribonucleotide aptamer, it was the first aptamer drug approved by Food and Drug Administration, in December 2004. This disease appears as a vision loss in older adults due to retinal damage. It has been demonstrated that Macugen® is able to block macular degeneration by inhibiting vascular endothelial growth factor (VEGF), a signal protein that stimulates vasculogenesis and angiogenesis[58]. Thus, a 0.3 mg/eye Macugen® dose, administered once every 6 weeks by intravitreal injections, is sufficient as anti-angiogenic medicine to treat wet age-related macular degeneration. Moreover, Macugen® has also potential therapeutic effects to deal with solid cancers characterized by extensive angiogenesis.
**E10030 (Fovista™)**
Studies have proved that the AMD treatment is more effective if anti-VEGF agent is combined to an aptamer able to bind to platelet-derived growth factor (PDGF). Fovista™ is a 29 nucleotide-long DNA aptamer, developed by Ophthotech Corp, capable to bind PDGF; it is standing by

to enter in phase III. Studies are testing 0.03, 0.3, 1.5, 3 mg of E10030 in combination with an anti-VEGF agent, once a month for 3 months; for the time being, this appears to enhance visual outcome[57].

**ARC1905**

ARC1905 (Ophthotech Corp) is a 39 ribonucleotide aptamer whose target is complement component 5 (C5), which is a pro-inflammatory protein forming part of the innate immune system; it is found in crystalline deposits under the retina in AMD. Inhibition of C5 prevents the progression of the macula, being therapeutic in both in wet and in dry AMD.

### 5.6.2. Therapeutic aptamers for hemostasis

**RB006**

RB006 is a 34 nucleotide RNA aptamer which binds to factor IXa; along with complementary active control oligonucleotide, RB007; it forms the anti-coagulant system named REG1. This system inhibits an important step in prothrombin assembly and thrombin generation by blocking the factor VIIa/IXa catalyzed conversion of factor X to factor Xa[57]. A single subcutaneous dose of 1 mg $kg^{-1}$ has proved to achieve high plasma concentrations and maintain near complete inhibition of factor IX activity for several days after administration. Currently, REG1 system is waiting to enter in phase III.

**ARC1779**

ARC1779 (Archemix Corp) is the second aptamer involved in coagulation disease. It is formed by 39 ribonucleotides, and it is able to bind the A1 domain of activated von Willebrand factor (vWF). This binding is responsible for decreasing platelet adhesion and aggregation, and thereby for reducing thrombus growth in arterial beds. Thus, by inhibiting vWF factor, it can be used to treat acute coronary syndromes, von Willebrand disease, vWF-related platelet disorders such as thrombotic thrombocytopenic purpura (TTP) and other thrombotic microangiopathies[59]. Currently, phase II trial is ongoing.

**NU172**

NU172 is a DNA aptamer composed by 26 unmodified nucleotides; it was developed by Nuvelo and ARCA Biopharma. It inhibits blood protein thrombin, preventing its cleavage by the fibrinogen. Phase II is ongoing also for this NU172, which is proving to can be used as a rapid anticoagulant for invasive medical procedures[60].

**BAX499**

BAX499 or ARC19499 is an RNA aptamer which is, right now, subjected to a Phase I clinical trial. It is the last of the four aptamers involved in coagulation disorders. It treats hemophilia. Hemophilia is a rare genetic blood clotting disorder; it subdivides into hemophilia A and B, according to factor VII or factor IX is implicated. This disease results in deficient blood coagulation via the intrinsic pathway. BAX499, allow clot initiation via the extrinsic coagulation pathway, by binding to tissue factor pathway inhibitor (TFPI), which negatively affects this pathway[61].

### 5.6.3. Therapeutic aptamers for cancer

**AS1411**

AS1411 or AGRO100 is an DNA quadruplex aptamer which binds the extracellular domain of what everyone knew better as a nuclear protein. Its target is nucleolin, a multifunctional protein located in the nucleolus but overexpressed at the membrane of several cancer cells. This protein is implicated in cell survival, growth and proliferation, nuclear transport, and transcription. It, in fact, is able to interact with key oncogenes (bcl-2, Rb, p53, Akt-1) and to transfer specific extracellular ligands into the cells. Nucleolin inhibition by AS1411 binding acts on several signaling pathways, including NF-κ-B[62] and Bcl-2[63]. AS1411 was the first aptamer approved by FDA for phase I clinical to test different type of cancer and the first-in-class drug known to specifically target nucleolin. Clinical trials show promising signs of activity, thus AS1411 is currently under phase II trial to treat acute myeloid leukemia[64,65] and metastatic renal cell carcinoma[66].

**NOX-A12**

NOX-A12 (Spiegelmer®, NOXXON Pharma AG, Berlin, Germany) is a 45 nucleotide-long spiegelmer which targets and blocks stroma cell-derived factor 1 (SDF-1 or CXCL12)[67]. This protein is a chemokine intermediate which, via interaction with the receptors CXCR4 and CXCR7, is involved in stem cell migration to the bone marrow and induces vasculogenesis, tumor growth, and metastasis. As mentioned above, having L-ribose units, NOX-A12 is highly resistant to degradation by abundant nucleases. NOX-A12 is currently under phase II trial to treat chronic lymphatic leukemia and multiple myeloma, both alone and in combination with chemotherapeutic agents[68].

### 5.6.4. Therapeutic aptamers involved in inflammation

**NOX-E36**

NOX-E36 (Emapticap pegol) is also a Spiegelmer aptamer created by the company Noxxon; its length is 40 nucleotide-L-RNA. NOX-E36 targets and inhibits the pro-inflammatory chemokine C-C motif-ligand 2 (CCL2), also named MCP-1 or Monocyte Chemoattractant Protein 1. NOX-E36 is in clinical development for the treatment of type II diabetes mellitus, nephropathy, and lupus nephritis. Studies, carried out to date, have proved that NOX-E36 is generally safe and well tolerated, reduces the urinary albumin/creatinine ratio and has beneficial effects on glycated haemoglobin in type 2 diabetic patients with albuminuria[69].

**NOX-H94**

NOX-H94 (lexaptepid pegol) is a specific spiegelmer for hepcidin. Hepicidin is a peptide hormone which plays a key role in the iron metabolism reducing iron concentrations in the blood. Thus, NOX-H94 is under phase II trial to treat anemia of chronic disease[70]. This anemia, known also as anemia of chronic inflammation, is a form of anemia seen in chronic infection, chronic immune activation, and malignancy; it is generally characterized by impaired erythropoiesis due to functional iron deficiency, often caused by excessive hepcidin. Hepcidin in fact degrades ferroportin which is the iron cellular channel reduce available iron in the blood for hemoglobin production required for red blood cells formation. Therefore, NOX-H94, offering a hepcidin-specific approach, causes iron release from the storage cells providing a new therapeutic tool to treat patients with anemia who do not respond properly to current therapies.

| Therapeutic Purpose | Name | Target | Form | Modification | Status |
|---|---|---|---|---|---|
| Macular degeneration | Pegaptanib | Vascular endothelial growth factor (VEGF) | RNA | 2′-fluoro pyrimidines, 2′-O-methyl purines, 3′-inverted dT, PEGylated | Approved for age-related macular degeneration (wet AMD) |
| | ARC1905 | Complement component 5 | RNA | 3′-inverted dT, PEGylated | Phase I completed |
| | E10030 | Platelet-derived growth factor (PDGF) | DNA | 2′-fluoro pyrimidines, 2′-O-methyl purines 3′-inverted dT | Phase III await |
| Cancer | AS1411 | Nucleolin | DNA | G-rich, PEGylated | Phase II on-going |
| | NOX-A12 | The chemokine (C–X–C motif) ligand 12 (CXCL-12) | L-RNA | L-form, PEGylated | Phase II on-going |
| Coagulation | RB006 | Coagulation factor IXa | RNA | 3′-inverted dT, PEGylated | Phase III await |
| | ARC1779 | von Willebrand factor (vWF) A1 domain | DNA | 3′-inverted dT, PEGylated | Phase II on-going |
| | NU172 | Thrombin | DNA | Unmodified DNA | Phase II on-going |
| | BAX499 | Tissue factor pathway | RNA | 3′-inverted dT, PEGylated | Phase I on-going |
| Inflammation | NOX-H94 | Hepcidlin | L-RNA | L-form, PEGylated | Phase II on-going |
| | NOX-E36 | The chemokine (C–C motif) ligand 2 (CCL2) | L-RNA | L-form, PEGylated | Phase II on-going |

**Figure 6. Therapeutic aptamers.** Here aptamers in on-going or completed clinical trials for therapeutics are shown[71].

## 6. Aim of the study

Glioblastoma is the most common primary brain tumour of adulthood and the most malignant glioma. The median overall survival of patients is very low, standing at around 15 months. Unlikely, despite many studies aimed to improve treatment efficacy, the overall survival has not increased in a significant way in the last years. This poor prognosis is strongly caused by the almost universal recurrence of glioblastoma tumour. Thus, recurrences seem to be inevitable event for GBM patients showing up within 6-9 months after treatment. The prognosis of a recurrent patient is poorer than the first one, with a median survival of only 3-6 months. Glioblastoma Stem Cells appear to be closely involved in tumour development, metastasis and recurrence. Therefore, it is clear how important it is to target GSCs in order to increase GBM patient overall survival. Accordingly, the present work aims to identify selective aptamer ligands for glioblastoma stem cells as new therapeutic tools for glioblastoma. It can be strongly assumed that a specific therapy against GSCs can considerably reduce GBM malignancy with an impressive prolonged patient survival.

# 7. Materials and methods

**Glioblastoma stem-cell isolation and differentiation**

GBM tissue samples were obtained from the Institute of Neurosurgery, School of Medicine, Università Cattolica, Rome, Italy after craniotomy of adult patients (as described by Pallini et al.) from which, before surgery, informed consent was obtained. Stem cells were isolated through mechanical dissociation of GBM tumor specimens and cultured in a serum-free medium supplemented with EGF and bFGF as previously described. To induce differentiation, cells were plated on flasks coated with BD Matrigel™ Basement Membrane Matrix (BD Biosciences) in presence of 10% serum and absence of EGF and bFGF for 2 weeks.

**Whole-Cell SELEX**

The SELEX cycle was performed essentially as described by Fitzwater T et al. 1996. Given the resistance to degradation against seric nucleases provided by the fluoropyrimidine, transcription was performed in the presence of 1 mM 2′-F pyrimidines and a mutant form of T7 RNA polymerase (2.5 u/µl T7 R&DNA polymerase, Epicentre Biotechnologies, Madison, WI.) was used to improve yields. The complexity of the starting pool was roughly $10^{14}$. Before each incubation with the cells, the 2'F-Py RNAs were heated at 85°C for 5 min, snap-cooled on ice for 2 min, and allowed to warm up to 37°C.

Selection step. In order to sort aptamers able to selectively bind GSCs, a selection step was performed incubating the pool with $10^7$ GSCs cells at 37°C for 30 min up to $14^{th}$ round or for 15 min in the last two rounds of SELEX. The bound aptamers were recovered after washings (one for the first two cycles and two for the others cycles) with 5 ml of DMEM-F12 serum free.

Counterselection step. To select sequences recognizing specifically the GSCs cells, before the selection step, a counterselection against glioblastoma differentiated cells was performed in order to not select aptamers which identify also the glioblastoma differentiated cell surface. In this case the pool was first incubated for 30 min (one time up to $13^{th}$ round and two times in the last three rounds) with $10^7$ GSCs (150-mm cell plate), and unbound sequences were recovered for the selection phase.

During the selection process, we increased number of counterselections or of washings and decreased incubation time to progressively raise the SELEX selective pressure. The use of poly inosinic acid as competitor has been introduced to minimize non-specific binding.

At the end of SELEX, before sequencing, sequences of the pools were cloned with TOPO-TA cloning kit (Invitrogen Life Technologies). Afterwards, they were compared by Clustal and their structure predictions were obtained by the RNAstructure or DNASIS software.

**Binding and internalization Analysis**

200000 cells were treated with 200 nM of individual aptamers (or the starting pool as a negative control) for 30 minutes at 37°C in the presence of 100 µg/ml polyinosine used as a nonspecific competitor (Sigma). Following two washes with PBS, to remove unbound RNA, bound RNA was recovered by TRIzol (Life Technologies) containing 0.5 pmol/ml of a non-related aptamer used as a reference control (at each experiment, the obtained data were normalized to the reference control). The amount of bound RNAs was determined by performing RT-qPCR, as reported, with the following primers for the long sequences: P10(Forward):

5'-TAATACGACTCACTATAGGGAGACAAGAATAAACGCTCAA-3', P20 (Reverse): 5'-GCCTGTTGTGAGCCTCCTGTCGAA-3', Scrambled aptamer and A40s were amplified respectively with the following primers Scrambled (Forward): 5'-TTCGTACCGGGTAGG-3', Scrambled (Reverse): 5'-TGACACGTTCTATGTGCA-3', A40s (Forward): 5'-CATCCCTGTTGTTCG-3', A40s (Reverse) 5'-CAGGCCTGTTGTGAC-3'.To check internalization, cell surface bound aptamers were removed washing three times the cells before recovering with cold PBS 0.5M NaCl, and internalization rate was expressed as percentage of internalized aptamer compared to total bound aptamer.

**Western blot analysis**

After washing cells twice in ice-cold PBS, protein extracts were prepared by incubating cell pellets in JS buffer (50 mM HEPES pH 7.5 containing 150 mM NaCl, 1% Glycerol, 1% Triton X100, 1.5mM MgCl2, 5mM EGTA, 1 mM Na3VO4, and 1X protease inhibitor cocktail). Protein concentrations was determined by Bio-Rad Protein Assay reagent, and equal amounts of proteins were separated by SDS-PAGE (10% polyacrylamide gel). The separated proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked for 1 hr with 5% non-fat dry milk in Tris Buffered Saline (TBS) containing 0.1% Tween-20. Primary antibodies were incubated at 4°C over night, peroxidase-conjugated secondary antibodies were used to perform an enhanced chemiluminescence (ECL Star, Euroclone Milan, Italy) reaction according to the manufacturer's protocol in order to identify target proteins. Primary antibodies used were: anti-β3tubulin,

anti-GFAP, anti- Sox2 (Santa Cruz Biotechnologies, MA), anti-βactin (Sigma, Milan Italy).

***In vitro* limiting dilution assay**

A number of 1, 5 or 10 cells per well were seeded in stem cell medium into a 96-well plate. Two weeks after seeding, the number of wells containing spheroids for each cell plating density was counted, and Extreme limiting dilution analysis was performed using software available at http://bioinf.wehi.edu.au/software/elda. Given the long period of treatment, aptamers were renewed in wells two times a week at a concentration of 100nM.

**Cell viability**

Dissociated tumor spheres were counted and $1 \times 10^5$ cells/point were pretreated with the aptamer or the starting pool, as a negative control, at a concentration of 400nM. Following 72 h, cells were seeded ($1 \times 10^3$ cells/well in 96-well plates) and treated with the aptamer or the starting pool at 400nM. For long treatments, aptamers were renewed two times a week at 100nM. Cell viability was assessed by using CellTiter 96H AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) measuring the absorbance at 492 nm with Multiskan FC Microplate Photometer (Thermo Fischer Scientific).

**Transwell migration assay**

Dissociated tumour spheres were counted and $1.5 \times 10^5$ cells/point were pretreated with the aptamer or the starting pool, as a negative control, at 400nM. Following 72 h, $1 \times 10^5$ cells were seeded in the upper chamber of transwell (Corning, Corning, NY, USA) in serum-free DMEM-F12. 10% FBS was used to induce cells migration towards the transwell lower chamber. Migrated cells were visualized after 24 h after seeding by staining with 0.1% crystal violet in 25% methanol. The percentage of migrated cells was evaluated by eluting crystal violet with 1% sodium dodecyl sulfate (SDS) and reading the absorbance at 594 nm wavelength.

**RNA extraction and real-time PCR**

After treating cells with aptamers or chimera, total RNAs (miRNA and mRNA) were extracted using EuroGOLDTriFast (EuroClone, Milan, Italy) according to the manufacturer's protocol. All the RNAs was reverse trascribed as described by Iaboni et al[72]. Therefore, reverse transcription of total mRNA was performed starting from equal amounts of total RNA/sample (500 ng) using SuperScript® III Reverse Transcriptase

(Invitrogen, Milan, Italy). By contrast, reverse transcription of total miRNA was performed starting from equal amounts of total RNA/sample (500 ng) using miScript reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative analyses of GFAP, NANOG and β-ACTIN (as an internal reference) were performed by real-time PCR using specific primers (IDT, Bologna, Italy) and iQ™ SYBR Green Supermix (Bio-Rad). Quantitative analysis of miRNAs and RNU6B (as an internal reference) was performed by real-time PCR using specific primers (Qiagen) and miScript SYBR Green PCR Kit (Qiagen). All reactions were run in duplicate. To amplify genes of interest we used the following primers:
β-ACTIN fw:5′-TGCGTGACATTAAGGAGAAG-3′, β-ACTIN rv:5′-GCTCGTAGCTCTTCTCCA-3′;
NANOG fw:5′-CAAAGGCAAACAACCCACTT-3′, NANOG rv:5′-TCTGGAACCAGGTCTTCACC-3′; GFAP fw: 5'-CTGCGGCTCGATCAACTCA-3'; GFAP rv: TCCAGCGACTCAATCTTCCTC-3'.

**Immunofluorescence analysis**
For immunofluorescence, cells were treated with 500nM Alexa488-A40s or Alexa488-unrelated aptamer (Scrambled) at 37°C. Subsequently, cells were washed two times with phosphate buffered saline (PBS) and they were forced to adhere on glass polylysine coated coverslips for 15 minutes, then cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Coverslips were washed three times in PBS, mounted with Invitrogen Gold antifade reagent with DAPI and finally cells were visualized by confocal microscopy. Images were captured at the same setting enabling direct comparison of staining patterns.

**Aptamer-miRNA chimera**
For the chimera production, we used RNAs synthesized by TriLink Biotechnologies (San Diego, CA). Below, we provide sequences used for chimera conjugate:
miR-34c passenger strand sticky: 5′
miR-34c guide strand: 5′
A40s sticky: 5′
All RNAs have 2′-fluoropyrimidine and UU in bold are 3′-overhang.
To prove that miR-34c is selectively delivered through the aptamer, the negative control is made up of the singles portions of the chimera not annealated (miR-34c guide strand, miR-34c passenger strand sticky and not sticky A40s).

To prepare A40s/miR-34c, 10 µM of passenger RNA strand and 10 µM of the guide strand, in the appropriate binding buffer 10x (200 mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid, pH 7.4, 1.5 M NaCl, 20 mM $CaCl_2$) were firstly denatured at 95 °C for 15 minutes, subsequently brought at 55 °C for 10 minutes and finally warmed up to 37 °C for 20 minutes. The annealed passenger and guide strand therefore obtained, is lastly combined whit A40s sticky and kept 30 minutes at 37 °C.

***In vivo* experiments**

Housed athymic CD-1 nude mice (nu/nu) in a highly controlled microbiological environment, were injected subcutaneously with 2 x $10^6$ BTSC #1-GFP on both flaks. To assess the A40s ability to inhibit in vivo tumor growth, mice were intravenously treated by caudal vein with 1,600 pmol in 100 µl/injection of A40s or unrelated aptamer (named scrambled), one injection a week. Tumor growth was measured with calipers, tumor volume was calculated as follows: L*(W^2)*3,14/6 (W is the shortest dimension and L is the longest dimension). Animals were sacrificed following 11 weeks of treatment.

**Histology and immuno-immunohistochemistry**

Xenograft formalin fixed tissue were embedded in paraffin block and were cut in sections of 5um thickness. The human KI67 (Antigen clone MIB-1 IR62; Dako UK Ltd.) Staining was performed with an automatic Benchmark XT staining machine (Ventana Medical Systems Inc.,Tucson, AZ, USA) according to manufacture procedure. KI67 nuclear staining intensity was evaluated by one expert pathologist. For H&E staining, 2.5 µm sections of all fixed samples were mounted on superfrost slides and performed using standard methodology.

**Statistical analysis**

Continuous variables are given as mean ± 1 standard deviation. Statistical values were defined using GraphPad Prism 6 (San Diego, CA, USA) software, by student's *t*-test (two variables) or one-way ANOVA (more than two variables). $P$ value $< 0.05$ was considered significant for all analyses.

# 8. Results

## 8.1. Results of SELEX selection

In order to identify new specific ligands able to affect glioblastoma malignancy, we aimed to isolate aptamers able to distinguish within the tumor mass the rare population of glioma cells growing as stem-like non-adherent spheres.
To this end, we used a differential cell SELEX approach using primary glioma stem cell lines derived from two patients **(Fig.7)**. The line BTSC #1 was derived from a patient with a diagnosis of "neuronal glioblastoma", the line BTSC #83 from a patient with a diagnosis of "mesenchymal glioblastoma".
Cell were propagated as non-adherent spheres in minimal F12 medium supplemented with growth factors (epidermal growth factor and basic fibroblast growth factor) as previously described (Pallini R. et al.) and used as targets in the SELEX process. Stem phenotype was evaluated by assessing major stem cells markers (**Fig. 8**).
In order to select sequences exclusively able to recognize GSCs and discriminate them from differentiated glioblastoma cells, at each round, the selection was preceded by one (or two) counterselection step incubating the pool with adherent cells obtained from either BTSC.1 or BTSC.83 (**Table 1**).

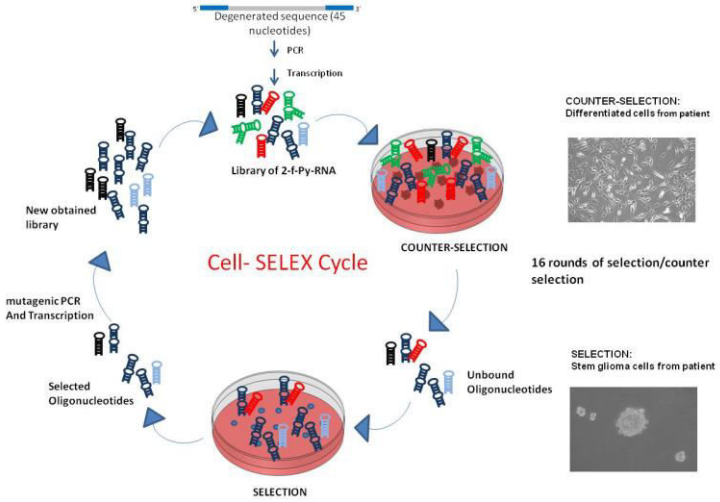


**Figure 7. Schematic representation of Cell-SELEX methodology.** 16 rounds of selection/counter selection were performed using GSCs or differentiated cells respectively, Cells were obtained from patient affected by glioblastoma undergoing craniotomy.

On the contrary, for counterselections, BTSC.1 or BTSC.83 cell lines were grown as adherent cells on matrigel substrate for two weeks in rich F12 medium in order to produce the differentiated counterparts (**Fig 8**).

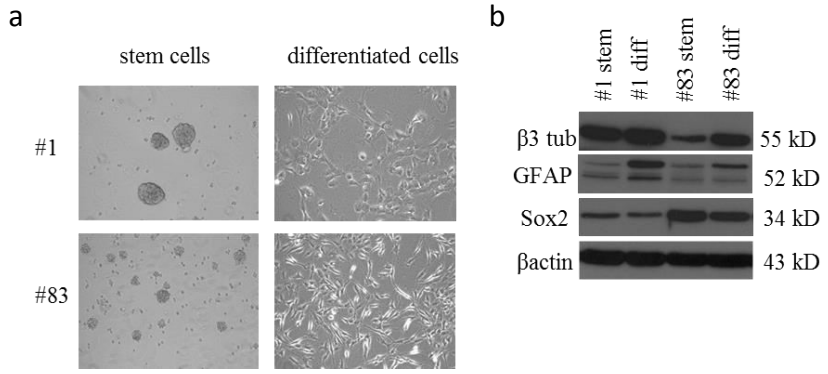


**Figure 8. Growth pattern of glioblastoma cultures established under different cell conditions.** a) Pictures show primary glioblastoma cells growing as suspension or adherent cell culture. b) WB illustrates different expression markers between stem cell cultures and adherent cell cultures obtained after two weeks of growth in 10% FBS media.

For selection steps, spheres have been dissociated and then incubated with the aptamer pool. Then, in order to eliminate non-selective aptamers, as mentioned previously, each selection step was preceded by one or, in the last cycles, two counterselection steps incubating the pool with adherent cells from either BTSC #1 or BTSC #83. As shown in Table, to progressively raise the SELEX selective pressure, we increased number of counterselections or of washings and decreased incubation time; moreover, we introduced the presence of a competitor to minimize non-specific binding.

| Cycle | n° counterselection | counterselection on differentiated cells | incubation temperature | n° selection | selection on stem cells | incubation temperature | incubation time | washes | competitor presence |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | pz 1 | 30 min | 1 | pz 1 | 37°C | 30 min | 1 | no |
| 2 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 1 | no |
| 3 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 4 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 5 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 6 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 7 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 8 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 9 | 1 | pz 83 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 10 | 1 | pz 1 | 30 min | 1 | pz1 | 37°C | 30 min | 2 | no |
| 11 | 1 | pz 83 | 30 min | 1 | pz 83 | 37°C | 30 min | 2 | no |
| 12 | 1 | pz 83 | 30 min | 1 | pz 1 | 37°C | 30 min | 2 | no |
| 13 | 1 | pz 83 | 30 min | 1 | pz 1 | 37°C | 30 min | 2 | no |
| 13 | 1 | pz 83 | 30 min | 1 | pz 83 | 37°C | 30 min | 2 | no |
| 14 | 2 | pz 83 | 30 min | 1 | pz 1 | 37°C | 30 min | 2 | no |
| 15 | 2 | pz 83 | 30 min | 1 | pz1 | 37°C | 15 min | 2 | no |
| 16 | 2 | pz 83 | 30 min | 1 | pz 1 | 37°C | 15 min | 2 | yes |

**Table 5. Schematic presentation of selective approach of cell-SELEX to identify specific aptamer ligands for GSCs.** Number of selection or counterselection, temperature and time of incubation, washes and presence of the competitor are shown for each SELEX round.

Upon sixteen SELEX rounds, 100 clones were sequenced and aligned for homology within their variable core region (**Fig.9**). Together three families dominated the pool with approximately 30% of sequences.

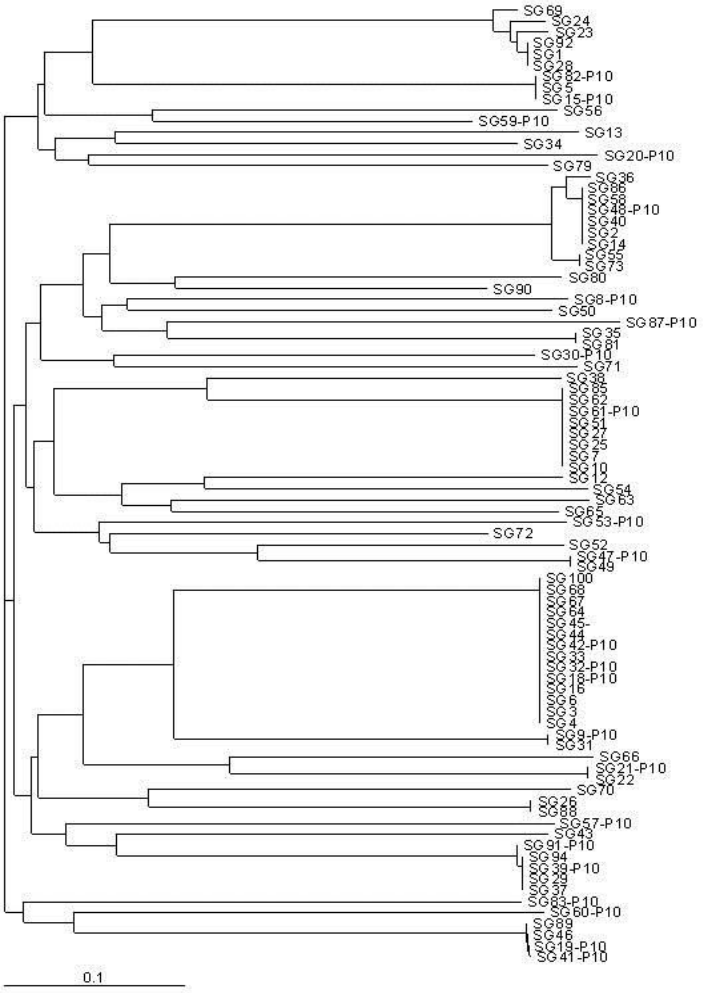


**Figure 9. Aptamer sequence alignments.** The random regions of all the sequenced aptamers were aligned using Clustal program. Dendogram shows visual classification of similarity among 100 individual sequences cloned after 16 rounds of selection.

In order to validate the information obtained by clustering, the enriched pools from rounds 10, 11, 13, 14, 15 and 16 were sequenced by HTS **(Fig.10)**.

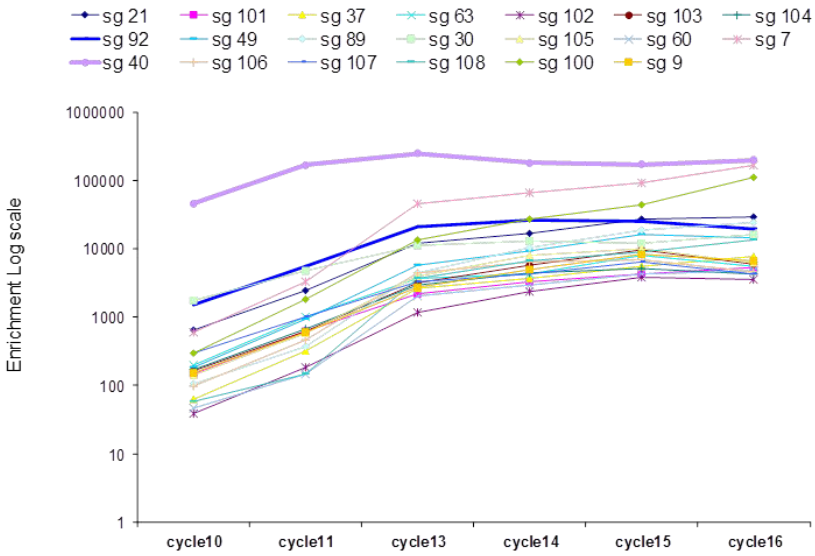


**Figure 10. Deep sequencing of last SELEX rounds.** The enriched pools from rounds 10, 11, 13, 14, 15 and 16 were sequenced by high-throughput sequencing (HTS).

As shown, the most enriched sequences identified by HTS belong to the four large clusters also found by conventional sequencing (Sanger). Indeed, based on the advantages provided by each technique the information obtained by coupling the two sequencing approaches may provide a reliable way to focus on the most promising sequences.

## 8.2. Binding assay

Given the good correlation between the two sequencing approaches, we determined the sequences that preferentially bind the GSCs tumor spheres as compared to cells induced to differentiate growing on adherent substrate. To this end, we analyzed by RT-qPCR binding at 200nM on the primary cell line BTSC1, the cell line used for the majority of selection rounds.

Analysis has been first performed for those aptamers that belong to the major clusters or that are rapidly enriched through the last six SELEX rounds, i.e. aptamer Sg. 7, 37, 40L, 89, 92, 38, (**Fig. 11a**). Sequences Sg.5, 37, 40L, 89, 92, showing binding to BTSC1 stem cells, specifically recognizes stem cells as shown by poor binding to the differentiated counterpart (**Fig. 11b**).

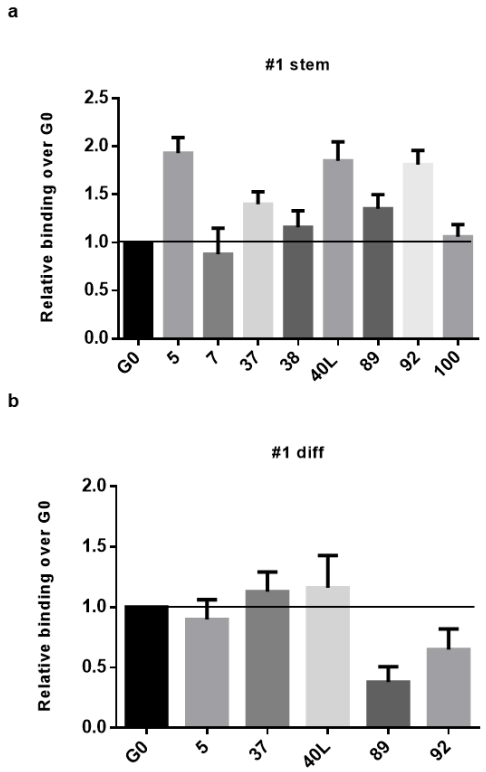


**Figure 11. Binding of the enriched sequences.** Binding was performed at 200nM on BTSC#1 (a) and adherent cells from the same patient (b); results are expressed relative to the background binding detected with the starting pool of sequences used for selection. Vertical bars indicate standard deviation values.

We then focused on one aptamer, 40L that was the more rapidly enriched during the SELEX rounds (at round 10). By RT-qPCR we first validated the binding of 40L on a broader set of patient's derived BTSC. We determined at 200 nM the relative binding on nine different primary cell lines (#1; 74; 23p; 83;169; 7; 163; 144p; 196). As shown in the panel a of Figure 12, 40L may bind to almost all the patients analyzed. Moreover, 40L shows no detectable binding for the differentiated counterparts of any cell lines (**Fig. 12**). This proved once again, over a larger number of patient lines, aptamer ability to selectively bind to GSCs and discriminate against differentiated cells obtained from the same patients.

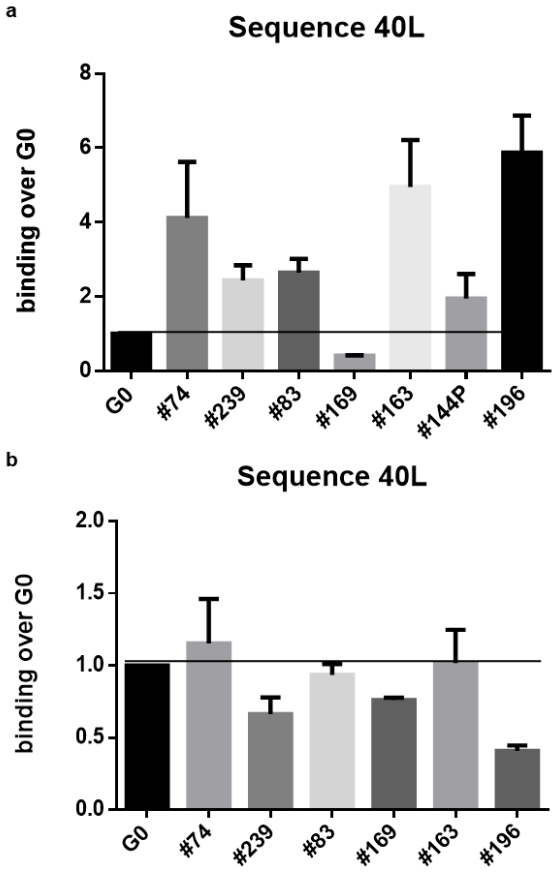


**Figure 12. Binding of 40L aptamer to several GBM stem cell lines.** Binding was performed at 200nM on several GBM cell lines obtained from patients undergoing craniotomy. Cells grew up in suspension (a) or adherent condition (b). Results are expressed relative to the background binding detected with the starting pool of sequences used for selection. In (a) and (b) vertical bars indicate standard deviation values.

We also tested 40L binding to the stem-like cells obtained from the GBM stable cell line U251MG and U87MG. As shown, 40L was able to bind only to U251 stem-like cells but not the adherent counterpart (**Fig 13a**). The 40L did not bind U87 stem-like cells (**Fig 13b**).

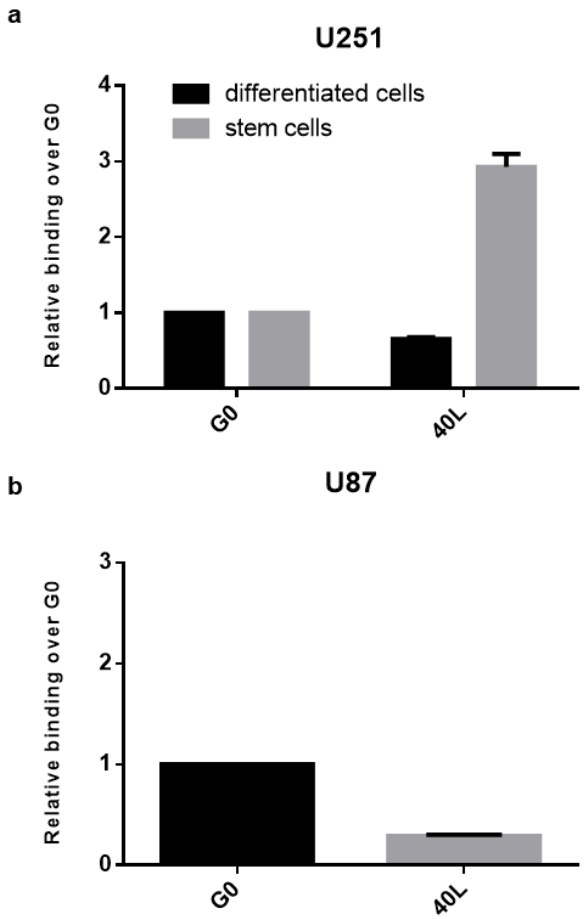


**Figure 13. Binding of 40L aptamer to human glioma cell lines U251 and U87.** a) RT-qPCR shows 40L binding on human glioma cell line U251 in suspension and adherent conditions. b) RT-qPCR illustrates 40L binding to U87 stem-like cell line. Results are expressed relative to the background binding detected with the starting pool of sequences used for selection. In (a) and (b) vertical bars indicate standard deviation values.

### 8.3. 40L *in vitro* functional inhibition

Considering that aptamer ability to bind specifically stem cells could affect crucial pathways that control stemness state, with the aim of determining the functional effects of aptamer binding to BTSCs, we decided to investigate the role of 40L on stemness regulation. In this respect, we performed a limiting dilution assay (LDA) in #83 primary stem cells. This assay is able to establish the frequency of cells having a particular function (in our case we focused on the stemness property) that are present in a mixed population of cells.
Data were analyzed using ELDA (Extreme Limiting Dilution Analysis) software. Cells were treated with 40L for 2 weeks showed an inhibition of spheroid frequency, compared to scrambled aptamer control (**Figure 14**).

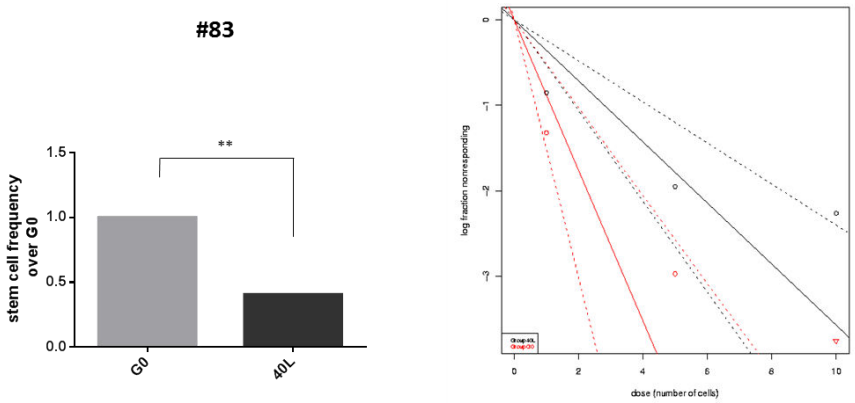


**Figure 14. Reduction of stem cell frequency resulting from 40L binding to BTSC.** LDA revealed a depletion of stem-like population in BTSC#83 after 40L treatment. Cells were treated with the aptamer and limiting dilution analyses were performed using Extreme Limiting Dilution Analysis (http://bioinf.wehi.edu.au/software/elda). Results are expressed in relation to the background effect detected by using G0, the starting pool of sequences employed during the SELEX. *P* value <0.05 is considered as statistically significant.

We also evaluated stem/differentiation markers expression following 40L incubation. As shown in figure 15, 40L is able to both increase GFAP, which is a differentiation marker in brain, and reduce one the transcription factor involved with self-renewal and essential in stemness maintaining, NANOG, in the two patient cell lines under investigation.

This finding underlined that aptamer can afflict both stem cell number and stemness state, reducing in this manner the malignancy of tumor.

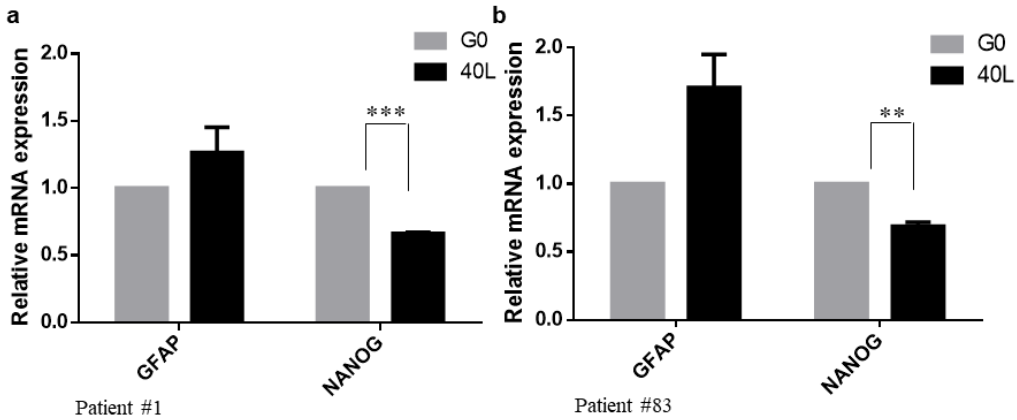


**Figure 15. 40L reduces stemness increasing differentiation of patients BTSC.** Real-time PCR was performed to analyze GFAP and NANOG levels in BTSC#1 (a) and BTSC#83 (b). Results are expressed in relation to the background effect detected by using G0, the starting pool of sequences employed during the SELEX. In (a) and (b) vertical bars indicate standard deviation values. *P* value <0.05 is considered as statistically significant.

Further, as determined by MTT analysis 40L aptamer inhibits stem cell proliferation of about 50% at 6 days of treatment (**Fig. 16a**) indicating that 40L has also an inhibitory effect on cell vitality.

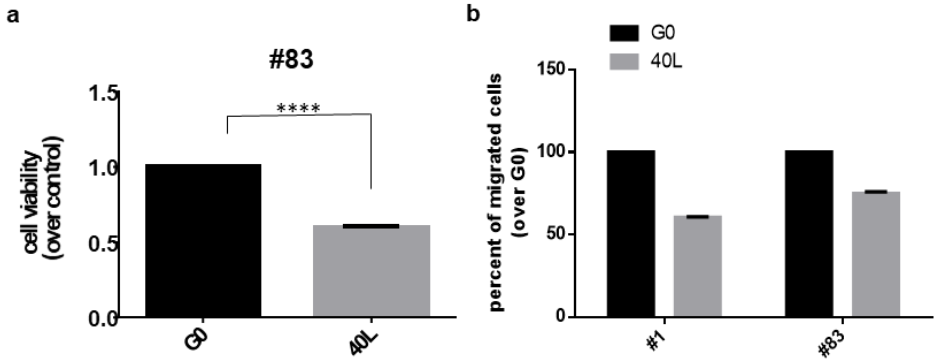


**Figure 16. 40L affects BTSC viability and migration.** Panels show cell viability reduction (a) and a decrease of cell ability to migrate (b). Results are expressed in relation to the background effect detected by using G0, the starting pool of sequences employed during the SELEX. In (a) and (b) vertical bars indicate standard deviation values. *P* value <0.05 is considered as statistically significant.

Moreover, by using a Boyden-chamber cell migration assay we assessed that 40L also interferes with the ability to migrate toward 10% FBS used as chemoattractant (**Fig. 16b**). Same results were obtained with stem cells derived from U251 cells (**Fig. 17**). The capability of 40L to reduce the migration can be considered as an interesting potential therapeutic function of 40L, since CSCs are now considered as the key element for tumor invasion and dissemination.

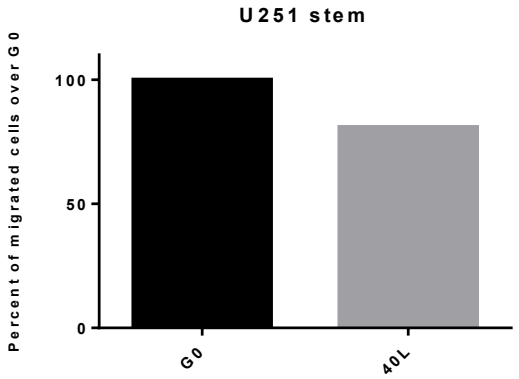


**Figure 17. 40L reduces U251 migration.** A reduction of cell migration has been seen treating U251 cells with 40L. Results are expressed in relation to the background effect detected by using G0, the starting pool of sequences employed during the SELEX.

Thus, we assessed that 40L can not only bind GSCs, but also inhibits several features that characterized cancer stem cells, the force drive of tumor.

## 8.4. Cell uptake efficiency

As previously reported, aptamer sequences for transmembrane cell surface receptors may be internalized together with the target protein. Thus, we determined if treating BSTC #1 and BSTC #83 cells with the 40L aptamer would result in rapid internalization. To this end, upon 30 min of binding, we treated the cells with acid wash to dissociate aptamers exposed on the cell surface and then extracted RNA. As determined by RT-qPCR, approximately 40% of total bound aptamer was recovered in the intracellular compartment of both GSCs (**Fig. 18**).

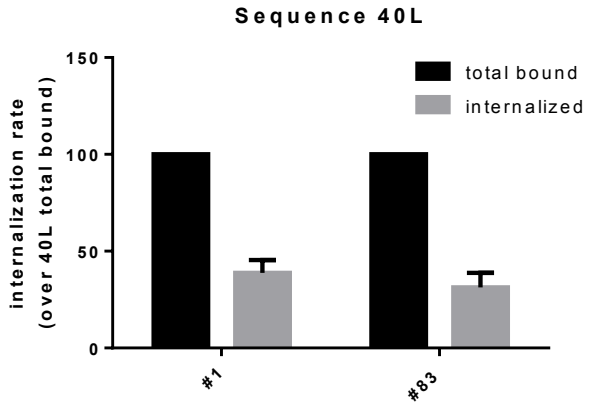


**Figure 18. Internalization assay of 40L.** 40L ability to be internalized into GSC is shown. Results are expressed as percentage of the total bound after 30 minutes of incubation. Vertical bars indicate standard deviation values.

## 8.5. Modification of the aptamer sequence

In order to obtain a shorter aptamer with best properties, such as a best tissue penetration and a considerable cost reduction, which, at the same time, shows comparable characteristics compared to the longer aptamer, we utilized a rational approach given by structure nucleic acid prediction programs (RNA structure and DNAasis) to design and then synthesize a 30bp sequence A40s, which represents the truncated version of 40L (**fig. 19**).

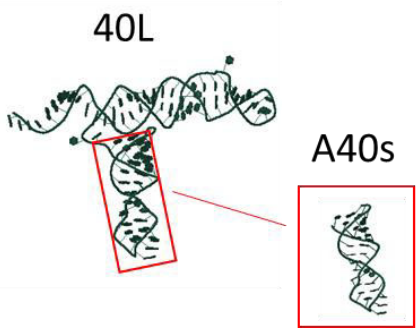


**Figure 19. 40L sequence post-SELEX modification.** 40L has been cut in order to have a shorter aptamer with best properties. Tridimensional shape prediction has been illustrated. The selected portion is shown in the red square.

After confirming that, A40s maintains the same tridimensional structure that this portion has in the long aptamer before cutting, A40s aptamer was tested for its ability to bind to BTSC. It showed to contain the active site of 40L since it preserves high binding affinity to GSCs.
Moreover, when it is compared to the negative control, A40s exclusively bind to BTSC (**Fig. 20a**) but not its differentiated counterpart (**Fig. 20b**) as well as 40L aptamer.

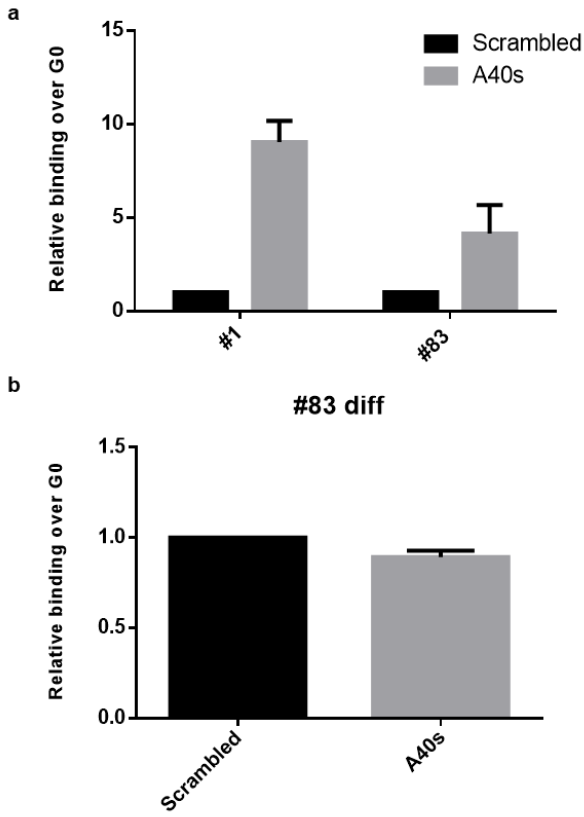


**Figure 20. A40s binding ability.** Binding assay was performed at 200nM on #83 cells grown as stem (a) or differentiated (b) cells. Results are expressed relative to the background binding detected with an unrelated aptamer of the same A40s length. In (a) and (b) vertical bars indicate standard deviation values.

In addition, we assessed A40s ability to be internalized into BTSC. As shown in figure 21, upon 30 min of incubation, almost 100% of the A40s aptamer is internalized into the cells. This suggests that A40s, compared to 40L, not only keeps the ability to bind to and discriminate GSCs, but it

presents also a higher skill to be internalized after binding. This ability confers to A40s the possibility to be used as therapeutic not only thanks to its functional aspects, but also and above all thanks to the opportunity to use it as a selective drug delivery system for GSCs.

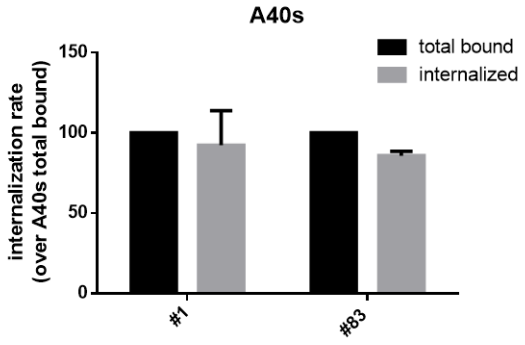


**Figure 21. Internalization assay of A40s.** A40s ability to be internalized into GSC is shown. Results are expressed as percentage of the total bound after 30 minutes of incubation. Vertical bars indicate standard deviation values.

A40s ability to bind BTSC is also assessed and confirmed through immunofluorescent assay. As shown in figure 22, Alexa488 labeled A40s is more capable to bind to #83 stem cells and to be internalized into them, compared to Alexa488 labeled unrelated aptamer (Scrambled) after 30 minutes of treatment.

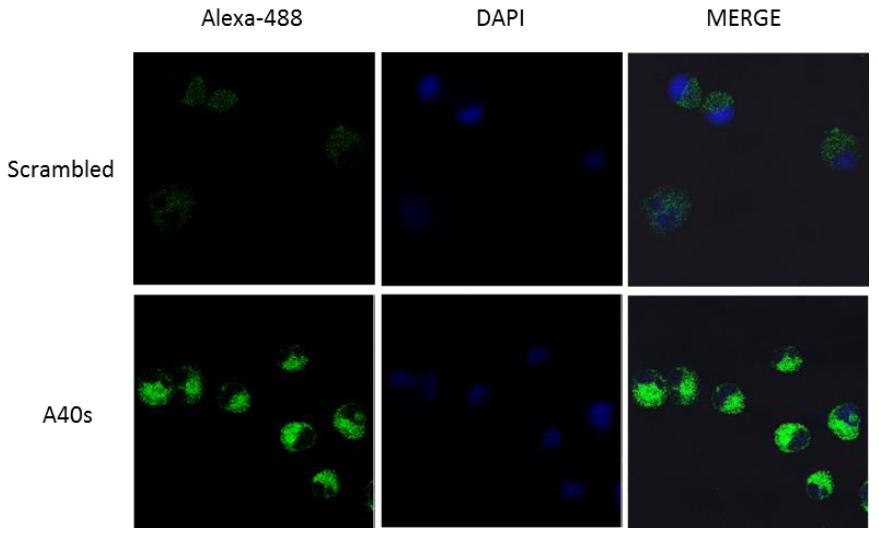


**Figure 22. A40s-Alexa488 binding ability.** Immunofluorescence assay was performed by treating #83 stem cells with A40s-Alexa488 or Scrambled-Alexa488 at 500nM for 30 minutes. All images were captured at the same setting enabling direct comparison of staining patterns.

## 8.6. A40s *in vitro* functional aspects

To assess whether A40s is able to maintain an active functionality likened to 40L, we evaluated the efficacy of A40s to reduce colony formation by limiting dilution assay, as described. Interestingly, as for the longer aptamer, A40s was able to reduce stem cells frequency of about 50% (**Fig. 23**). This indicates that A40s still preserves the functional aspect on stemness that Sg40 has.

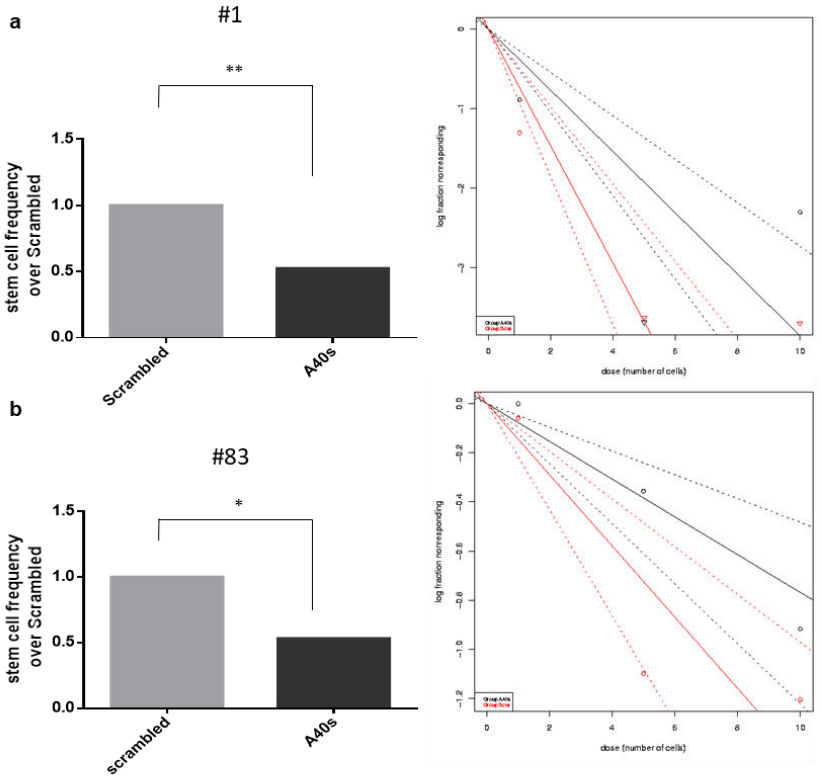


**Figure 23. Reduction of stem cell frequency in GSC by using A40s.** LDA revealed a depletion of stem-like population both in BTSC#1 (a) and in BTSC#83 (b) after A40s treatment. Cells were treated with the aptamer and limiting dilution analyses were performed using Extreme Limiting Dilution Analysis (http://bioinf.wehi.edu.au/software/elda). Results are expressed in relation to the background effect detected by using an unrelated aptamer of the same A40s length. *P* value <0.05 is considered as statistically significant.

We also assessed stem cells/differentiation markers expression upon A40S incubation on BTSC #83. As shown in figure 24, A40s preserves 40L ability to downregulate NANOG and up-regulate the differentiation markers GFAP (**Fig 24**) compared to negative control.

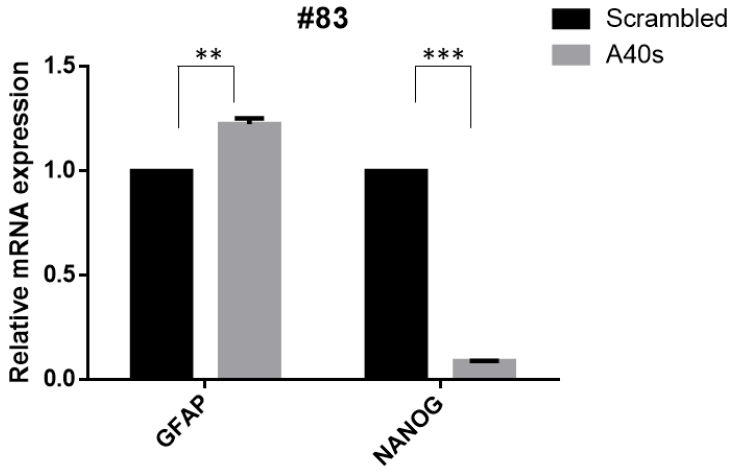


**Figure 24. A40s reduces stemness increasing differentiation in GSC.** Real-time PCR was performed to analyze GFAP and NANOG levels in BTSC#1 and BTSC#83. Results are expressed in relation to the background effect detected by using an unrelated aptamer of the same A40s length. Vertical bars indicate standard deviation values. *P* value <0.05 is considered as statistically significant.

## 8.7. Design and folding of an aptamer-miRNA conjugate

Since A40s has the ability to be internalized, it can be considered as a good candidate for the delivering of molecule able to repress stemness properties. For this reason, we wanted to generate, via stick-end annealing, a molecular chimera, consisting of a duplex miRNA cargo and a nucleic acid aptamer as delivery carrier. Our laboratory acquired much experience with aptamer annealings and miRNAs studies. A chimera conjugate, between Gl21.T aptamer and miR-34c has already been generated in our laboratory. Therefore, we decided to generate a similar conjugate by annealing A40s with miR-34c. We fused the passenger strand of miR-34c and A40s by the mean of complementary sticky ends elongated at the 3' end of the aptamer and at the 3' end of the miRNA passenger strand, respectively. Finally, we annealed the guide strand of miRNA to the template (**Fig. 25).** We verified the correct annealing of the conjugate by non-denaturating gel electrophoresis analysis (data not shown).

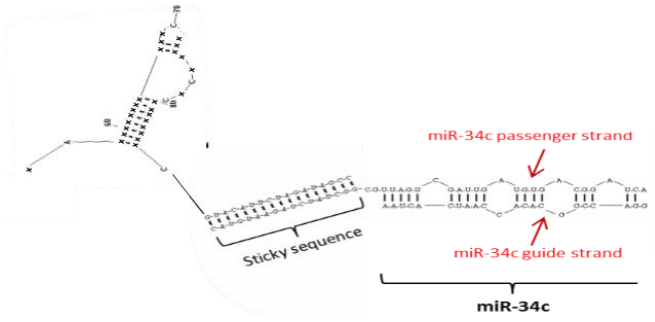


**Figure 25. Design and folding of an aptamer-miRNA conjugate.** A chimeric molecule has been generated by annealing A40s with miR-34c.

In order to investigate whether A40s aptamer acts as a selective carrier for delivering the conjugated miRNA, we performed *in vitro* experiments in BTSC #83 or differentiated cells treated with A40s/miR-34c for 24-48 hours. As shown in figure 27, A40s/miR-34c conjugate increased miR-34c levels; it was assessed by RT-qPCR and it occurred only in BTSC, but not in differentiated cells (**Fig. 26**).

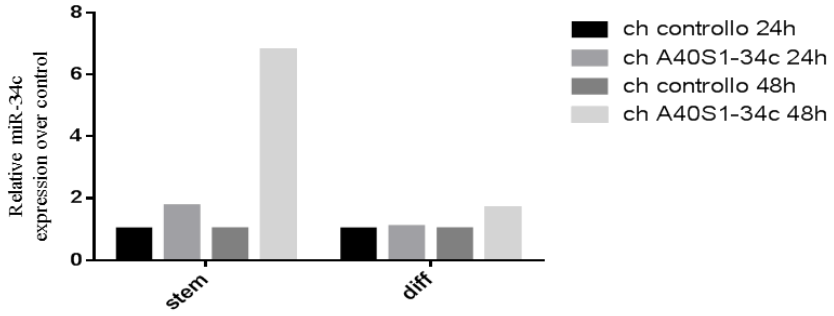


**Figure 26. miR-34c is delivered through A40s only in stem cells.** Relative miR-34c levels were assessed by using qRT-PCR at 24 and 48h after chimera A40s/miR-34c treatment. Chimera ability to deliver miR-34c was evaluated both in stem and in differentiated brain tumour cells.

### 8.8. A40s serum stability and i*n vivo* functional aspects

An important feature for the potential clinical translation of new therapeutics is represented by their in vivo stability. Therefore, we evaluated the A40s stability incubating the aptamer in high concentrated human serum solution for increasing times up to one week. Serum-RNA samples were recovered at the indicated time-points and analyzed by non-denaturing polyacrylamide gel electrophoresis (**Fig. 27a).** As shown, the aptamer was stable up to approximately 4-8 hours and then gradually degraded. This period of stability seems to be comfortable to reach the tumour mass, given that A40s needs only 30 minutes to bind and be internalized into the cells.
After confirming A40s sufficient stability in human serum, we tested *in vivo* effects of A40s by xenografts experiment with BTSC#1 by intravenous injection of 1600 picomoles of A40s or scrambled control aptamer. As shown in figure 27b, A40s induced a reduction of tumor growth strongly affecting tumor size. This is corroborated by histological analysis of tumour samples (**Fig. 27c**) showing a decrease of cell positivity to the proliferation marker Ki-67.

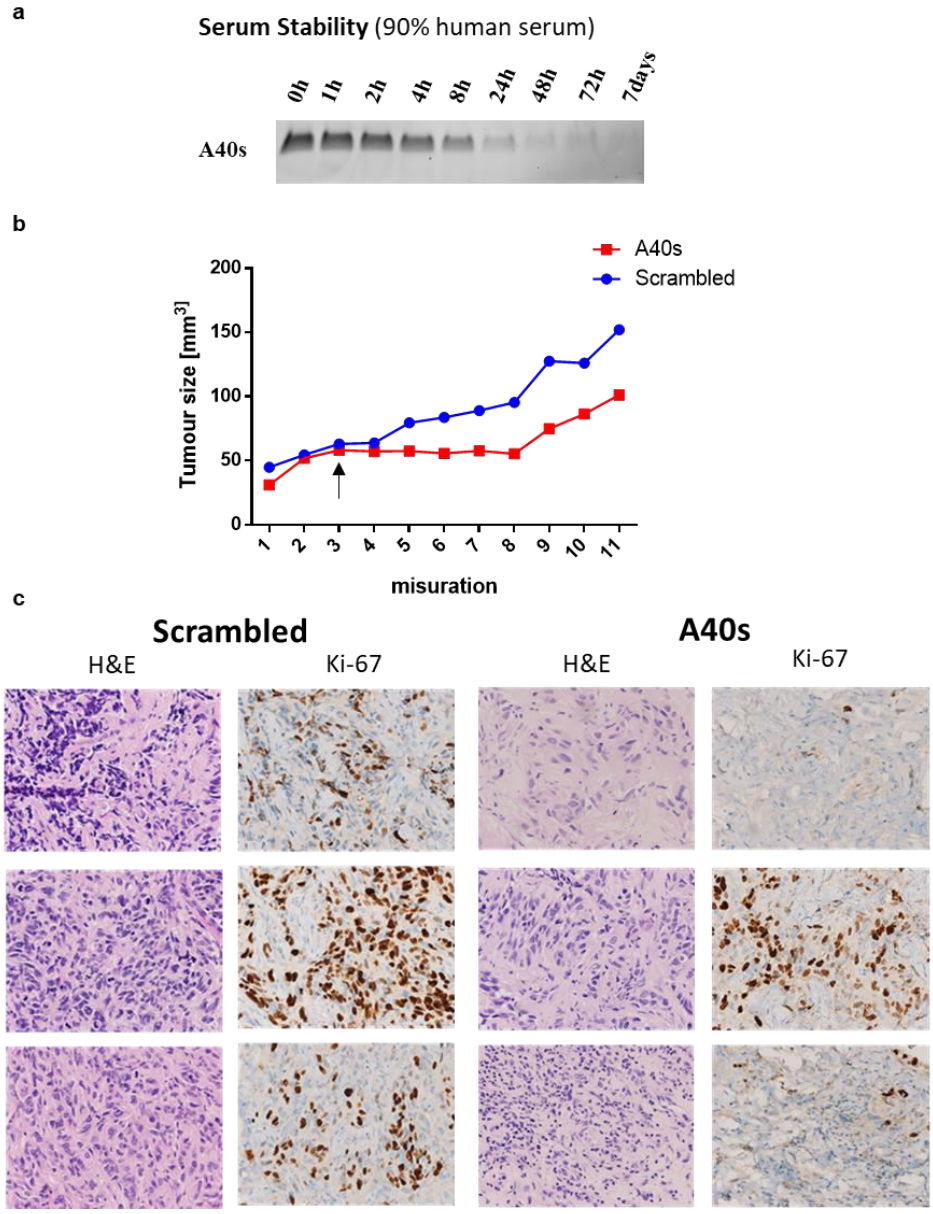


**Figure 27. A40s effect *in vivo*.** a) Non-denaturing polyacrylamide gel electrophoresis illustrates A40s stability in 90% serum. b) *In vivo* experiment was performed to asses A40s ability to reach and reduce tumour size. Tumour size is strongly reduces after A40s intravenous injection. The arrow indicates treatment initiation. c) Immuno-histochemistry analysis shows Ki-67 and H&E staining of superfrost slides using standard methodology. Results are expressed in relation to the background effect detected by using an unrelated aptamer of the same A40s length.

# 9. Discussion

Glioblastoma (formerly glioblastoma multiforme; GBM) is the most common primary brain tumour of adulthood and it is the most aggressive form of glioma and corresponding to grade IV based on WHO Classification. Glioblastoma multiforme, especially the primary form, grows very fast within 3 months, and has a very high rate of recurrence. Given the high capacity to invade normal brain tissue, GBM is still particularly difficult to be completely surgically removed. The median overall survival of patients is very low, standing at around 15 months. Unlikely, despite many studies aimed to improve treatment efficacy, the overall survival has not increased in a significant way in the last years. This poor prognosis is strongly caused by the almost universal recurrence of glioblastoma tumour. Thus, recurrences seem to be inevitable event for GBM patients showing up within 6-9 months after treatment. Sadly, until today, there is not any cure for GBM. Thus, the treatment can only affect symptomatic aspects which aim to increase the survival rate and simultaneously to improve quality of life.

Glioblastoma Stem Cells appear to be closely involved in tumour recurrence. Survey after survey shows that tumours contain a small population of cells, named cancer stem cells (CSCs), which is responsible for tumor initiation, growth, and recurrence and is liable for cancer chemotherapy resistance. Thus, cancer is now recognized as a heterogeneous group of cells showing several differentiation phenotypes. GBM, as well, is a heterogeneous tumour, consisting of normal cells and a small population of CSCs; thus, it is clear how important it is to target GSCs in order to increase GBM patient overall survival. Therefore, identifying a selective therapy against GSCs is of the utmost importance in the fight against GBM. It can be strongly assumed that a specific therapy against GSCs can considerably reduce GBM recurrence with an impressive prolonged patient survival.

Aptamers are short, single-stranded oligonucleotides that are high affinity ligands for disease-associated proteins expressed on the cell surface. Aptamers, as such, appear as excellent candidates able to bind a specific target on GSCs cell surface. Moreover, their characteristics (e.g. short development and synthesis time, high stability and shelf life, low size and cost, ease to be modified, good tissue penetration and high affinity and specificity) make aptamers a new class of therapeutic and diagnostic molecules comparable or even better then monoclonal antibodies[73].

Furthermore, the aptamer-mediated targeted delivery of therapeutics has been proven many times and, nowadays, many studies have shown the great aptamers ability to be internalized when conjugated with other molecules[74]. Consequently, their capacity to be used as a drug delivery system has been demonstrated in several occasions[75].

Accordingly, the present work has focused on the identification of selective aptamer ligands for glioblastoma stem cells usable as new therapeutic tools for glioblastoma. Currently, many are the aptamers approved or under examination during clinical trials, Macugen® is the first aptamers approved by FDA for age-related macular degeneration therapy[58]. As mentioned, above, two aptamers are already in clinical trials to treat cancer. Here, we took advantage of a panel of primary cultures of GSCs isolated from human patient's tumours to select 40L, starting from a library pool. We chose to use primary cells in order to obtain data that was more related to the "physiological/pathological condition". For the same purpose, cell-SELEX approach was preferred to protein-SELEX technique. 40L aptamer had shown its effectiveness to selectively bind to GSCs, discriminating them from the differentiated adherent counterparts. Interestingly, 40L not only exclusively binds to GSCs, but it also exhibits a functionally activity on target cells. Indeed, it is able to reduce stemness, cell viability and migration, explicating the role as stemness regulator.

In order to improve the potential use of this aptamer as therapeutic molecule for glioblastoma treatment, we decided to reduce 40L structure obtaining a shorter form, able to bind GSCs as well as the longer 40L. This reduction has important clinical and economic consequences because it can strongly reduce manufacturing costs and improve aptamer tissue penetration.

We found that, similar to 40L, A40s is able to discriminate between GSCs and differentiated glioma cells and moreover, it remains functionally active on stemness.

40L and A40s demonstrated to have a high rate of penetration into the cells; thus, all their functions can be improved by creating chimeric molecules, conjugating 40L or A40s with cell toxic components which can be selectively delivered into the target cells trough the aptamer.

Moreover, the aptamer on its own has proved to be capable of affecting *in vivo* proliferation of GSCs. Indeed, *in vivo* experiment shows a very encouraging result with a good reduction of the tumour volume.

Both aptamers proved to be able to affect cell stemness features, but unfortunately, much needs to be done to understand the mechanism by which it happens. Moreover, the aptamer target is still unidentified as well

as its affinity for the target which remains undefined at this stage. For this reason, elucidation of pathway, which is governed by aptamer action, can improve aptamer potential application in glioblastoma therapy. Thus, other aspects still have to be investigated.

Future therapeutic approach, which targets key pathways regulating the stemness state of glioma stem cells, is a rapidly emerging request for glioblastoma treatment. Therefore, targeting the stemness-like properties of cancer cells with aptamers as A40s may represent a promising strategy for cancer therapy in the future. Considering that A40s is able to modify stemness properties, its use in therapy could strongly contribute to reduce the GSCs number and to increase their sensitization to chemotherapy, greatly impeding tumor relapse and metastasis.

# 10. Conclusion

In this work, we identified and characterized a new tool to target glioblastoma stem cells. We described the potential use of aptamer 40L (and A40s) as a therapeutic molecule able to selectively bind GBM stem cells and discriminate them from differentiated cells.

Despite the aspects which have yet to be considered, such as target identification, it can be easily concluded that 40L is able to reduce viability, migration and stemness. Likewise, A40s, the truncated form of 40L, is able to bind exclusively to GSCs, distinguishing GBM stem cells from differentiated cells, and it too is capable of reducing stemness. Furthermore, both the aptamers, especially A40s, can be easily internalized into target cells. This confers the additional opportunity to use them as drug delivery system.

In addition, *in vivo* experiment using A40s has been proved its ability to reduce GBM stem cells proliferation by decreasing tumor size in mice, showing a very encouraging result.

In summary, 40L and A40s demonstrate to be, at least potentially, a very useful tool in the GBM treatment, being able to selectively act on GSCs on which currently therapies fail.

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## 13. List of publications

1. **An anti-PDGFRβ aptamer for the selective delivery of small therapeutic peptide to cardiac cells.** Alessandra Romanelli; **Alessandra Affinito**; Concetta Avitabile; Silvia Catuogno; Paola Ceriotti; Margherita Iaboni; Geroloma Condorelli; Daniele Catalucci. Under revision

2. **Cancer-associated fibroblasts release exosomal microRNAs that dictate an aggressive phenotype in breast cancer.** Donnarumma E, Fiore D, Nappa M, Roscigno G, Adamo A, Iaboni M, Russo V, **Affinito A**, Puoti I, Quintavalle C, Rienzo A, Piscuoglio S, Thomas R, Condorelli G. Oncotarget. 2017 Mar 21;8(12):19592-19608. doi: 10.18632/oncotarget.14752.

3. **RYK promotes the stemness of glioblastoma cells via the WNT/ β-catenin pathway.** Adamo A, Fiore D, De Martino F, Roscigno G, **Affinito A**, Donnarumma E, Puoti I, Ricci Vitiani L, Pallini R, Quintavalle C, Condorelli G. Oncotarget. 2017 Feb 21;8(8):13476-13487. doi: 10.18632/oncotarget.14564.

4. **MiR-24 induces chemotherapy resistance and hypoxic advantage in breast cancer.** Roscigno G, Puoti I, Giordano I, Donnarumma E, Russo V, **Affinito A**, Adamo A, Quintavalle C, Todaro M, Vivanco MD, Condorelli G. Oncotarget. 2017 Mar 21;8(12):19507-19521. doi: 10.18632/oncotarget.14470.

5. **miR-340 predicts glioblastoma survival and modulates key cancer hallmarks through down-regulation of NRAS.** Fiore D, Donnarumma E, Roscigno G, Iaboni M, Russo V, **Affinito A**, Adamo A, De Martino F, Quintavalle C, Romano G, Greco A, Soini Y, Brunetti A, Croce CM, Condorelli G. Oncotarget. 2016 Apr 12;7(15):19531-47. doi: 10.18632/oncotarget.6968.