UNIVERSITÀ DEGLI STUDI DELL'AQUILA

DIPARTIMENTO DI MEDICINA CLINICA, SANITA' PUBBLICA, SCIENZE DELLA VITA E DELL'AMBIENTE

Dottorato di Ricerca in **SCIENZE DELLA SALUTE E DELL'AMBIENTE**

Curriculum ***Medicina Traslazionale***

XXXV ciclo

Titolo della tesi

PIVOTAL ROLE OF CYCLOOXYGENASE 2(COX-2) IN TEMOZOLOMIDE-RESISTANCE OF GLIOBLASTOMA CELL LINES

SSD MED/04- *Patologia Generale*

Dottorando

Dr.ssa Mitilda Gugu

Coordinatore del corso

Chiar.ma Prof.ssa M.Grazia Cifone

Tutor

Chiar.ma Prof.ssa Paola Palumbo

a.a. 2021/2022

To my Parents and my Family

Leonard, Leonis & Luis

SUMMARY

ABSTRACT

Glioblastoma multiforme (GBM) is one of the most lethal brain cancers worldwide since it is a highly infiltrating and aggressive central nervous system tumor representing about 50% of all adult malignant primary brain tumors. Nowadays, treating malignant GBM is multidisciplinary since it is characterized by a high degree of genetic and cellular heterogeneity. Surgical resection is usually followed by radiotherapy and chemotherapy with alkylating agent temozolomide (TMZ). However, TMZ resistance remains a main limitation in GBM treatment. The cytotoxicity of TMZ is modulated by O6-methylguanine-DNA methyltransferase (MGMT), whose expression is determined by MGMT gene promoter methylation status. Despite continuous efforts to ameliorate the GBM therapeutic approaches, GBM often relapses due to the presence of GBM stem cells (GSCs), a small population of tumor-initiating cells which remain after surgical exeresis and show stem cell-like properties such as self-renewal and differentiation ability and can promote tumor progression, therapy resistance, and metastasis.

GBM is characterized by the presence of an inflammatory milieu, and inflammation, a key component of the tumor microenvironment, is emerging as a target for new treatment modalities. Cyclooxygenase-2 (COX-2), an inflammation-associated enzyme, has been implicated in GBM tumorigenesis, progression, and stemness. The poor survival of GBM was mainly associated with the presence of GSCs and the markedly inflammatory microenvironment. To deeply explore the involvement of COX-2 in GBM biology, our first aim was to study the effects of NS398, a selective COX-2 inhibitor, on GSCs derived from COX-2-expressing GBM cell lines showing intrinsic diversity and individual genetic features, i.e., U87MG and T98G, assessing the neurospheres' growth, autophagy induction, and extracellular vesicle (EVs) release. The previous results demonstrated that neurospheres derived from both GBM cell lines resulted highly influenced by NS398 exposure, showing remarkable morphological changes, a reduced growth rate, and a relevant level of autophagy. In addition, it has been verified that COX-2 inhibition induced a functional modification of the EVs released by neurospheres. In particular, EVs derived from neurospheres pre-treated with NS398 strongly reduced the migration ability and significantly triggered the autophagic process in the adherent U87MG and T98G cells, thus causing effects similar to those observed following NS398 direct addition.

Recently, COX-2 inhibitors are increasingly considered an add-on treatment to improve GBM sensitiveness to traditional chemo- and radiotherapy, increasing apoptosis and reducing tumor migration and stemness potential. In this scenario, the effect of TMZ, as a single agent and in combination with NS398, on COX-2 expression in GBM cell lines showing different COX-2 levels and TMZ sensitivity (T98G and U251MG) was here investigated. Surprisingly, COX-2 expression resulted in dose-dependently upregulated by TMZ in T98G, COX-2 positive cells, while no influence was recorded in U251MG, COX-2 negative cell line. TMZ was also able to upregulate Wnt/β-catenin signaling, MGMT expression, and stemness potential, all crucial components implicated in the GBM-chemoresistance. Moreover, the combined effects of NS398 and TMZ (NS398+TMZ) were studied in two GBM cell lines. COX-2 inhibition by NS398 was able to overcome TMZ-induced overexpression of COX-2, β-catenin, MGMT, and SOX-2 in T98G, to significantly enhance the dead cell percentage, was able to induce apoptosis and to inhibit the clonogenic potential in T98G cells dramatically. These findings strongly support the key and the hierarchically superior role played by the COX-2/PGE2 system in the cascade of pathways activated by TMZ and implicated in chemoresistance.

1. INTRODUCTION

1.1 Overview of glioma

Gliomas are the most common neuroepithelial tumors originating from the glial cells in the Central Nervous System (CNS). Glial cells provide a structural role within the nervous system and are crucial for brain development and homeostasis. Traditionally, gliomas were thought to originate from neural stem cells, glial progenitors, including oligodendrocyte progenitor cells, or astrocytes (Zong et al., 2012). Gliomas are locally invasive cancers that seldom metastasize (Cuddapah et al., 2014) and exhibit the classic hallmarks of cancer including significant anaplasia, malignancy, and proliferation (Hanahan et al., 2011); besides, they are highly chemo-resistant and radio-resistant, leading to tumor recurrence after surgical resection (Birzu et al., 2021). Diffuse gliomas are highly heterogeneous at the histological, cellular, and molecular level as indicated by a broad spectrum of genomic alterations (Wenger et al., 2022). Although glial tumors account for fewer than 2% of newly diagnosed malignancies, diffuse gliomas are a leading cause of death and morbidity (Molinaro et al. 2019). Gliomas constitute ~30% of all primary tumors, 80% of all malignant ones, and most of the deaths caused by primary brain tumours. Generally, gliomas comprise a complex heterogeneous group of primary central nervous neoplasms that are divided into circumscribed gliomas and diffuse gliomas; commonly, after a complete surgical resection, the first group being benign is curable and the second one, being more malignant, is unable to be cured (Yang et al. 2022). Each year, approximately 100,000 people worldwide are diagnosed with diffuse gliomas, and the incidence rate rises with increasing age, male gender, white race, and non-Hispanic ethnicity (Molinaro et al., 2019). The incidence of gliomas in adults varies between 1.9 and 9.6 per 100,000 persons depending on age, gender, ethnicity, and geographic region (Ostrom et al., 2014; Śledzińska et al., 2021). The incidence rates of gliomas increase in males (5.51 per 100,000 population) than in females (3.65 per 100,000 population), except for diffuse midline gliomas, which are higher among females (0.324 versus 0.288) (Patil et al., 2021). Moreover, the incidence rate is about 2 times higher in American and northern European populations than in the Asian population. Epidemiological studies have shown a different incidence trend based on geographic regions (Pellerino et al., 2022). Incidence peaks between ages 55 to 60 and the prevalence is higher in males, with a relative sex ratio of 1.66 in England and 1.56 in the USA (Esemen et al., 2022). Diffuse midline glioma represents 31.1% of all childhood gliomas, followed by pilocytic astrocytoma (18.3%), diffuse astrocytoma, and anaplastic astrocytoma (5.3%), and glioblastoma (2.6%) (Pellerino et al., 2022). Ostrom and colleagues reported that most of the cases with gliomas occurred in the supra-tentorium (62% of gliomas occur in the supratentorial compartment: 27.0% in the frontal lobe, 20.2% in the temporal lobe, 11.6% in the parietal lobe, and 2.8% in the occipital lobe) and only a small proportion may occur in other CNS sites, including the brainstem (4.3%), spinal cord and cauda equina (4.0%), the cerebellum (2.8%), and other brain sites (20.0%) (Ostrom et al., 2020).

1.2 Glioma classification

Before 2016, gliomas were classified by the World Health Organization (WHO) grade criteria (I to IV) into multiple specific histologic subtypes, based on the cell type of origin and molecular characteristics (Louis et al., 2016). In 2021 the WHO published the fifth revision of the classification system based on the latest advances in molecular genetics and the development of epigenetic profiles. The WHO CNS5 groups together tumors into more biologically and molecularly defined entities, and introduce new tumor types and subtypes, especially in the paediatric population. Then, the 2021 WHO classification of CNS tumors, more strictly defining the biological behaviour of GBM as a highly aggressive tumor with an unfavourable outcome, updates the oldest one integrating molecular with histological data to better define a diagnosis and treatments (Louis et al., 2021). In particular, in the revised classification, some molecular biomarkers, based on their role in clinical role, have been included: the isocitrate dehydrogenase 1 (IDH1) mutation status, histone variant H3F3A alterations, 1p/19q codeletion, α thalassemia/mental retardation syndrome X-linked (ATRX) gene mutations, O[6]-methylguanine-DNA methyltransferase (MGMT) promoter methylation status, loss of cyclin-dependent kinase inhibitor 2A (CDKN2A), epidermal growth factor receptor (EGFR) amplification, combined gain of chromosome number 7 and loss of chromosome number 10 (7+/10−), and telomerase reverse transcriptase (TERT) promoter mutations (Sledzinska et al., 2021).

Other main novelties in the CNS5 classification regarding nomenclature and grading:

- names have been simplified as much as possible, and only location, age, or genetic modifiers with clinical utility have been used (e.g., *Extraventricular neurocytoma* vs *Central neurocytoma*).
- Terms like "*anaplastic*" have been eliminated (e.g., "astrocytoma, IDH-mutant, CNS WHO grade 3" now replaces "anaplastic astrocytoma").
- The traditional description of the CNS tumor grades in Roman numerals has been changed into Arabic numbers to conform with WHO grading in non-CNS tumor types (Komori et al., 2022).
- Neoplasms are graded within types (rather than across different tumor types).
- The term "type" replaces "entity," and variants are referred to as subtypes to harmonize the terminology with other organ systems (Osborn et al., 2022).
- The suffixes Not Otherwise Specified (NOS) and Not Elsewhere Classified (NEC) are used to point out the quality of diagnostic tests. Particularly, adding a NOS suffix indicates that the histological or molecular information necessary to define a WHO diagnosis is unavailable, warning the oncologist that a molecular work-up has not been undertaken or failed technically. An NEC suffix, on the other hand, indicates that the necessary diagnostic testing has been successfully performed but that the results do not readily allow for a WHO diagnosis. then, also an NEC suffix provides a warning to the oncologist describing that the tumor does not conform to a standard WHO diagnosis.

- Differentiation of diffuse gliomas into adult-type and the paediatric-type considered as clinically and biologically distinct groups. Thus, the latest WHO CNS 5 2021 classifies diffuse gliomas into four general groups 1) adult-type diffuse gliomas, 2) paediatric-type diffuse low-grade gliomas, 3) paediatric-type diffuse high-grade gliomas, and 4) circumscribed astrocytic gliomas (Louis et al., 2021).
- The methylome profiling, arrays used to determine DNA methylation patterns across the genome, has emerged as a powerful tool for CNS tumor classification, for this reason, it has been included as suitable criteria in WHO 2021 (Louis et al., 2021). Some of the new and rare types (e.g., high grade astrocytoma with piloid characteristics) can be diagnosed based on the methylation profile. However, due to limited availability, methylome profiling is still not recommended as a primary or routine diagnostic test for tumour classification. Integrating histology with signature genetic alterations remain the most useful and widely available technique to diagnose CNS tumor types and subtypes (Gritsch et al., 2022).
- 22 new glial tumors added (in particular, seven in the glioma category, three in the glioneuronal category, four each in the ependymal and embryonal groups, three sarcomas, and one new pituitary tumor (Gritsch et al., 2022) (Table 1).
- One of the most significant changes in the classification with the greatest practical implications involves GBM. Previous criteria for the placement of the diagnosis of GBM multiforme were exclusively histological based on high-grade features (necrosis and/or microvascular proliferation). Novelties included both *IDH-mutated* (10%) and *IDH wildtype* (90%) tumors with different biologies and prognoses (Wen and Packer 2021).

  Nowadays, the diagnosis of GBM requires the lack of isocitrate dehydrogenase 1 and 2 mutations (IDH-wildtype) as well as a lack of mutation in histone 3 (H3-wildtype) (Stoyanov et al., 2021). In addition, IDH wildtype diffuse astrocytic tumors without classical histological features of GBM but in the presence of one or more of 3 genetic parameters (TERT promoter mutation, EGFR gene amplification, or combined gain of entire chromosome 7 and loss of entire chromosome 10 [+7/−10]) can be considered as GBM. While all *IDH*-mutant diffuse astrocytic tumors are classified as a single type (astrocytoma, *IDH*-mutant) and are graded as 2, 3, or 4. Grading is no longer entirely histological (e.g., IDH-mutant astrocytomas with CDKN2A/B homozygous deletion having the worst prognosis are classified as WHO CNS grade of 4 tumors regardless of the absence of microvascular proliferation or necrosis (Wen and Packer 2021).

Table 4 WHO CNS5 classification of gliomas, glioneuronal and neuronal tumours

Tumour group	Types
Adult-type diffuse gliomas	- Astrocytoma, *IDH*-mutant - Oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted - Glioblastoma, *IDH*-wildtype
Paediatric-type diffuse low-grade gliomas	- Diffuse astrocytoma, MYB- or MYBL1-altered - Angiocentric glioma - Polymorphous low-grade neuroepithelial tumour of the young - Diffuse low-grade glioma, MAPK pathway-altered
Paediatric-type diffuse high-grade gliomas	- Diffuse midline glioma, H3 K27-altered - Diffuse hemispheric glioma, H3 G34-mutant - Diffuse paediatric-type high-grade glioma, H3-wildtype and *IDH*-wildtype - Infant-type hemispheric glioma
Circumscribed astrocytic gliomas	- Pilocytic astrocytoma - High-grade astrocytoma with piloid features - Pleomorphic xanthoastrocytoma - Subependymal giant cell astrocytoma - Chordoid glioma - Astroblastoma, MN1-altered
Glioneuronal and neuronal tumours	- Ganglioglioma - Desmoplastic infantile ganglioglioma/desmoplastic infantile astrocytoma - Dysembryoplastic neuroepithelial tumour - Diffuse glioneuronal tumour with oligodendroglioma-like features and nuclear clusters - Papillary glioneuronal tumour - Rosette-forming glioneuronal tumour - Myxoid glioneuronal tumour - Diffuse leptomeningeal glioneuronal tumour - Gangliocytoma - Multinodular and vacuolating neuronal tumour - Dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease) - Central neurocytoma - Extraventricular neurocytoma - Cerebellar liponeurocytoma
Ependymomas	- Supratentorial ependymoma - Supratentorial ependymoma (ZFTA or YAP1 fusion-positive) - Posterior fossa ependymoma - Posterior fossa ependymoma (PFA or PFB group) - Pinal ependymoma - Spinal ependymoma, MYCN amplified - Myxopapillary ependymoma - Subependymoma

Table 1: Types of gliomas according to the 2021 World Health Organization Classification of Tumors of the Central Nervous System WHO (2021) (Torp et al., 2022).

1.3 Glioblastoma, IDH-wildtype

Glioblastoma (GBM) is a grade IV glioma and is referred as the most lethal brain cancers worldwide characterized by a high degree of genetic and cellular heterogeneity. The main symptoms of GBM are advancing neurological deficits, persistent headaches, loss of appetite, double or blurred vision, vomiting, and seizures. Due to its highly infiltrating and aggressive behaviour, the complete surgical resection to eliminate GBM is almost impossible. GBM often relapses due to the presence of tumor-initiating cells, called GBM stem cells (GSC), promoting tumor recurrence, therapy resistance and metastasis (Nduom et al., 2012; Bischof et al., 2017; Mattei et al., 2021). Unfortunately, the development of personalized therapeutic approaches for GBM is limited, and no improvements in terms of overall survival have been achieved so far, confirming the chemoresistance issue as the main barrier to patient outcome. Histological examination shows typically astrocytic morphology with hypercellular proliferation of atypical glial cells, nuclear pleomorphism, and neoplastic cells with irregular enlarged hyperchromatic nuclei with fibrillary processes, associated with microvascular proliferation (Lopes

Abath Neto et al., 2021; Smith and Ironside et al., 2007). Other typical features of GBM are elevated mitotic activity and necrosis (Figure 1) (Perez et al., 2021).

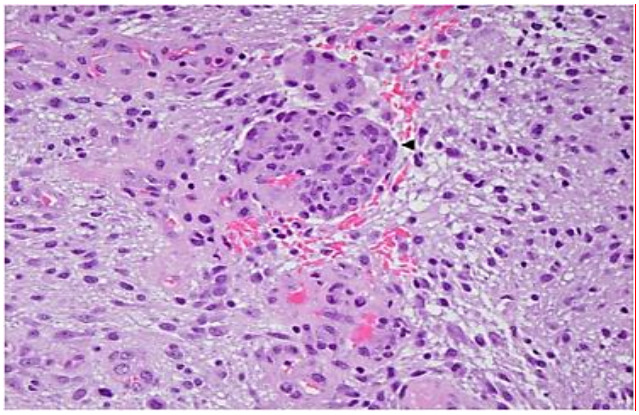


Figure 1: Glioblastoma, IDH-wild type. High-power field demonstrating classic glomeruloid microvascular proliferation (400 × magnification) (Perez et al., 2021)

WHO CNS 5 classification recognizes three morphological subtypes of GBM: giant cell GBM, epithelioid GBM and gliosarcoma with a wide spectrum of phenotypes. Giant cell GBM consist of extremely enlarged, bizarre, multinuclear cells, in addition to smaller spindled cells and focal ill-defined rosettes, in a reticulin network. They tend to have a more circumscribed architecture, to be more resectable, and consequently have a slightly better prognosis (Lopez et al., 2021; Orasanu et al., 2022). Epithelioid GBM, is also known as adenoid GBM or GBM with epithelioid metaplasia. It mainly consists of epithelioid, melanoma like, or rhabdoid cells with abundant cytoplasm, eccentric nuclei, and prominent nucleoli. Other common features are palisading and solid lamellar necrosis, high proliferative activity, more mitotic figures, and microvascular hyperplasia (Wang et al., 2020). Gliosarcoma (GS) is a rare histopathologic subtype of glioblastoma (GBM) characterized by a biphasic growth pattern composed both by glial and sarcomatous components (Frandsen et al., 2019).

1.4 Glioblastoma epidemiology, survival, and risk factors

The Central Brain Tumor Registry of the United States (CBTRUS) reports that GBM is the most common malignant tumor of the brain and CNS system, accounting 14.3% of all tumors, 49.1% of malignant tumors and the majority of gliomas (58.4%). GBM was more common in older adults (median age is 65) and incidence rates for GBM were approximately two times greater in whites than in blacks (Ostrom et al., 2021). The incidence of GBM varies depending on the report, from 3.19-4.17 cases per 100,000 person-years. While the incidence in the pediatric population (0–18 years) is 0.85 per 100,000, where paediatric GBM (p-GBM) accounts for 3–15% of primary brain tumors (Grochans et al., 2022). Despite advances in surgical and medical neuro-oncology, the median overall survival (OS) of GBM patients remains low, at only 15 months (Koshy et al., 2012; Tran et al., 2010; Cruz et al., 2022; Grochans et al., 2022). Only 6.8% of patients survived five years post-diagnosis and females compared to males had similar survival outcomes for glioblastoma (Ostrom et al., 2021). Different studies have

recognized age as the most significant variable among demographic and molecular prognostic factors affecting the incidence of GBM where most of the cases occur in people over 40 years of age (Weller et al., 2009; Stark et al., 2012; Gittleman et al., 2019; Ostrom et al., 2021; Grochans et al., 2022). IDH wild-type high-grade gliomas, which have a worse prognosis are typically common in older patients (Pellerino et al., 2022).

Factors associated with GBM risk are prior radiation, decreased susceptibility to allergy, immune factors and immune genes, and some nucleotide polymorphisms detected by genome-wide association (Tamimi et al., 2017). Recent results show that people with asthma and other allergic conditions have a lower risk of GBM, precisely the genotypes associated with an increased risk of asthma are at the same time associated with a reduced risk of GBM. A higher incidence of GBM has been described in subjects with high socioeconomic status. There is no proven correlation between lifestyles such as smoking, alcohol consumption, drug use, and nitrous compounds. The association of GBM with cell phone use is discussed. Survival is influenced by several factors such as the possibility of surgical resection, advanced age, comorbidities, and general patient conditions (Schwartzbaum et al., 2005).

1.5 Pathogenesis, molecular mechanisms, and glioblastoma markers

GBM represents the most aggressive and heterogeneous tumor type among gliomas, associated with poor outcomes due to its complexity and resistance to current therapeutic approaches. Different genetic and molecular abnormalities leading to significant modifications of the crucial signalling pathways can cause GBM progression. Over the past decade, modern advances in genomic sequencing technology have allowed the profiling of the gene expression patterns and mutations that drive tumorigenesis. Much effort has been invested in understanding the key molecular changes that trigger GBM to successfully predict patient response to therapy. Cancer Genome Atlas (TCGA) network outlines four distinct molecular subtypes of GBM: Neural, Proneural, Classical, and Mesenchymal, characterized by unique molecular and genetic aberrations. As previously discussed, molecular markers were integrated for the first time in the WHO 2016 classification and were better refined in the latest 2021 WHO classification of central nervous system tumors. The WHO 2016 CNS classification distinguished primary, or de novo, and secondary GBM which evolved from previously known low-grade gliomas (WHO grade II and III). Primary GBMs arise de-novo without precursor lesions in contrast to secondary GBMs, which progress slowly from lower-grade astrocytoma (Ramos et al., 2018). According to the recently published WHO classification, only 90% of primary GBM are IDH-wildtype and are designated as GBM, while the former IDH-mutated GBM lesions are now classified as IDH-mutated astrocytoma as they develop from lower-grade IDH-mutated astrocytomas (Louis et al., 2021). Thus, the WHO classification of CNS tumors now incorporates IDH status in the diagnosis of gliomas. The molecular markers described below carry prognostic and predictive information for GBM:

1. **ATRX (a-thalassemia/mental-retardation-syndrome-X-linked) mutation.** The ATRX gene located on the q arm of the X chromosome (Xq21.1) encodes a protein, part of the SNF2 (SWI/SNF2) familchromatin-remodelinglling proteins (He J., et al., 2017; Nandakumar et al., 2017), with two highly conserved domains that allow histone H3.3 to incorporate into heterochromatin. ATRX is currently recognized not only as a heterochromatin remodeler, but also as a protein involved in various essential mechanisms such as gene expression, replication, senescence, DNA repair, and stress responses. It has been hypothesized that in patients with the ATRX syndrome, both copies are inactivated, one by mutation and the other by X inactivation (He et al., 2017) ATRX mutations result in reduced protein expression. ATRX loss in gliomas has been demonstrated to promote the alternative lengthening of telomeres (ALT) phenotype. In almost 57% of secondary GBM cases occur ATRX mutations. Studies have shown that ATRX mutations co-occur with IDH1 and TP53 mutations and that ATRX loss in GBM patients is a prognostic factor (Grochans et al., 2022).
2. **TERT (Telomerase Reverse Transcriptase) promoter mutation.** Telomeres are nucleoprotein complexes that compose and protect the ends of each chromosome from being recognized as double-strand breaks and destroyed by the DNA damage response system. Telomeres shorten at every cell cycle, eventually leading to cell death or senescence. To overcome this mechanism, cells can activate telomere-maintenance mechanisms such as telomerase activation and alternative lengthening of telomeres (ALT) (Olympios et al., 2021). The TERT gene encodes telomerase, an enzyme that is responsible for the repair of telomeres to maintain their length, and protect chromosome ends. Telomere lengthening is required for chromosomal integrity and is essential for cancer growth to achieve the infinite proliferation of cancer cells. The two most common alterations in the TERTp region are C228T and C250T, located at base pairs 124 and 146, respectively, which encode this promoter (Nakahara et al., 2004). Different studies report that TERTp mutations increase TERT expression level by 6.1 times compared to that of wildtype tumors (Arita et al., 2013; Brennan et al., 2013; Ceccarelli et al., 2016). The role of TERT promoter mutation as a prognostic factor has not been established due to numerous confounding co-occurring factors (age, surgical intervention, IDH mutation, EGFR amplifications, and MGMT methylation status) (Olympios et al., 2021). IDH- wildtype GBM are characterized by a higher frequency of TERTp mutations (72%) than IDH-mutant GBM (26%) (Louis et al., 2016). Thus, additional prospective investigations on large cohorts of a homogenous patient population (e.g., IDH-wildtype and O6-methylguanine DNA methyltransferase (MGMT) promoter-unmethylated glioma) are required to independently evaluate the prognostic impact of TERT promoter mutations (Olympios et al., 2021).
3. **TP53 (Tumor protein P53) mutation:** The TP53 gene is a tumor suppressor gene located on human chromosome 17p13.1. Functional p53 protein is a homotetramer that plays a key role in maintaining cellular homeostasis (Grochans et al., 2022). Tp53 in glioblastoma: under normal circumstances suppresses tumor activity by modulating the expression of genes involved in the cell cycle, division and differentiation of the cells, and apoptosis (Jadoon et al., 2022). The presence of TP53 mutations

is associated with the progression of GBM (Wang et al., 2001). The inactivation of p53 leads to increased invasiveness (Djuzenova et al., 2015) and proliferation of cancer cells (England et al., 2013), and decreased cell apoptosis (Park et al., 2006). GBM cell lines carrying the inactivated mutant show greater resistance to DNA-damaging therapeutic drugs, such as cisplatin (Park et al., 2006; Zhang et al., 2018). Although Petitjean and colleagues correlated TP53 mutations with poor prognoses in breast and many other cancers (Petitjean et al., 2007) they are not considered independent markers of poor prognosis in GBM (England et al., 2013; Kraus et al., 2000; Rich et al., 2005). TP53 mutations are more common in IDH-mutant GBM than IDH-wildtype GBM (81% vs. 27%) (Louis et al., 2016). These mutant versions of p53 not only result in the loss of normal functions but surprisingly, also involve mutant proteins with novel abilities that provide cancer cells with key gain-of-function activities (GOF's) (Ortiz et al., 2021). Wang et al. reported that the gain of function (GOF) fuels tumor progression: enhanced proliferation, migration, invasion, and resistance to chemotherapy. Thus, GOF mutations are associated with worse OS, and they reduce GBM sensitivity to temozolomide by increasing MGMT expression (Wang et al., 2014).

4. **B-RAF V600E mutation:** B-RAF protein, a member of the RAF family of serine/threonine kinases, participates in the cascade of the RAS-RAF-MEK-ERK MAP pathway that affects cell growth, differentiation, proliferation, and apoptosis (Maraka et al., 2018). *BRAF*-V600E mutation, located in exon 15, nucleotide 1799, is the most common *BRAF* mutation in human cancers (90%), which involves the substitution of valine for glutamate at codon 600 (V600E) (Davies et al., 2002; Matallanas et al., 2011). The missense mutation produces the permanently activated serine/threonine kinase B-RAF, which activates extracellular signal-regulated kinase 1 and 2 (ERK1/2) and other mitogen-activated protein (MAP) kinases which leads to uncontrolled cell proliferation and tumorigenesis (Grochans et al., 2022) In the literature, the frequency of all B-RAF mutations in GBM is estimated at 2–6% (Behling F., et al., 2016). A meta-analysis of Vuong et al. reported that since *BRAF* V600E was associated with improved overall survival (OS) in glioma patients (HR = 0.60; 95% CI = 0.44–0.80) has a positive prognostic impact in gliomas and its prognostic value might depend on patient age and tumor grade. Further studies are required to clarify its prognostic value taking into account other confounding factors (Vuong et al., 2018). Targeted BRAFV600E inhibitors have been associated with better overall survival, especially within pediatric patients and young adults (17–35 years). Everolimus and sorafenib are two drugs in phases 1 and 2 clinical trials, respectively, for recurrent glioblastomas targeting BRAF-signaling-associated pathways (Senhaji et al., 2022). Published case reports have shown the clinical response to vemurafenib (a B-RAF kinase inhibitor) in three pediatric high-grade gliomas (Bautista et al., 2014).
5. **GATA4 (GATA-binding protein 4):** GATA4 is a transcription factor of the GATA6 family, considered a suppressor gene. Agnihotri et al. showed by using retroviral gene trapping on transgenic mouse glioma models that GATA4 is expressed in normal murine and human embryonic and adult neurons and glia of CNS. Despite that the reduced expression of the GATA 4 gene does

not alter cell growth, inhibiting the GATA4 gene and null-p53 status in mice demonstrated to induce transformation and lead to increased proliferation and resistance to chemotherapy or radiation-induced apoptosis (Agnihotri et al., 2009). Further studies by Agnihotri and colleagues showed that GATA4 expression was lost in most of human GBMs tumor cells; GATA4 inhibited transformation to GBM *in vitro* and *in vivo*, and the re-expression of GATA4 in GBM cells increased their response to temozolomide, regardless of the MGMT mutation status (Agnihotri et al., 2011). Further research is needed to confirm the GATA4 mutation status as a predictive biomarker (Grochans et al., 2022).

6. **FGFR1 (Fibroblast Growth Factor Receptor (1)):** the FGFR family of proteins is a group of transmembrane receptors with tyrosine kinase function which controls cell proliferation, survival, and cytoskeletal regulation It remains unclear to what extent signaling by FGFR contributes to the pathobiological aspects of individual cancers (Jimenez-Pascual et al., 2019). Yamaguchi and colleagues found that high-grade gliomas show an increased expression of FGFR1 and that α to β isoform switch is associated with increasing tumor stage and grade (Yamaguchi et al., 1994). Several studies (Gouazé-Andersson et al., 2016; Hale et al., 2019; Kowalski-Chauvel et al., 2019) have provided strong evidence supporting that FGFR1 contributes to a worse prognosis in GBM, and signaling through this pathway correlates with increased radioresistance, invasiveness, and stemness (Grochans et al., 2022).
7. **EGFR (epidermal growth factor receptor):** EGFR is a receptor with tyrosine kinase activity that is activated by EGF (epidermal growth factor). RTK-encoding genes including EGFR are found to be implicated in GBM development. Abnormal EGFR activity or function may engage different signaling pathways, including PI3K/Akt, Ras/Raf/Mek/ERK, signal transducer and the activator of transcription 3 (STAT3), and phospholipase C gamma which results in altering cellular functions such as proliferation and migration, angiogenesis, and resistance to apoptosis (Eskilsson et al., 2018). The EGFR gene is located at locus 7p12 and mutations that occur in about 50% of all GBM samples, of which more than 40% are gene amplification, and mutations, rearrangements, splicing site changes, etc. (Yang et al 2022). EGFR amplification is more common in IDH wildtype GBM than IDH-mutant GBM (35% vs. exceptional) (Louis et al., 2016). Although the results from different studies are inconclusive, the amplification of EGFR was found to be correlated with poor prognosis (Newcomb et al., 1998; Simmons et al., 2001; Shinojima et al., 2003; Heimberger et al., 2005; Cowppli-Bony et al., 2011). The EGFR most common gene mutation is EGFRvIII (deletion of exons 2–7) which has attracted research interest as a potential marker of treatment for GBM. The EGFRvIII mutation is never seen in secondary GBMs or healthy tissues. EGFR inhibitors and antibodies, vaccines, CAR-T, or other approaches are being investigated for the treatment of GBM with EGFR as a target. Peptide vaccine strategy targeting EGFRvIII (Rindopepimut) did not present effectiveness in a phase III trial (NCT01480479) (Weller et al., 2017). A first-in-human phase I study of CAR T cells directed to EGFRvIII demonstrated feasibility in patients with recurrent GBM

who had received multiple previous doses of temozolomide and completed a course of radiation (O'Rourke et al., 2017).

8. **MGMT (O6 -methylguanine DNA methyltransferase)** is an enzyme that performs DNA repair by removing an alkyl group from the O6 position of guanine, a crucial DNA alkylation site. The gene MGMT is located on chromosome 10q26. The antitumor effect of TMZ is reflected in the O6 -guanine methylation although TMZ-induced DNA methylation occurs at the N7 -guanine (>70%) and the N3 -adenine (>9%) to a greater extent than the O6 -guanine (5%). In the absence of effective base exchange repair and DNA mismatch repair, the aberrant pairing of the guanine with thymine rather than cytosine results in mutations which lead to cell death (Talhoui et al., 2017). The therapeutic activity, as well as the toxicity of TMZ, depends on the extent of methylation at the O6 position of guanine in DNA. Specialized enzymes like MGMT demethylate the O6 position of guanine and thus counteract the TMZ effect. The expression level of MGMT strongly depends on the methylation level of its promoter region. The lack of expression of MGMT can be caused by epigenetic silencing of MGMT gene promoter in the CpG-rich region, via methylation (Taylor et al., 2015). Hegi et al., recommended the value of identifying the MGMT promoter methylation status by methylation-specific PCR to find patients who would benefit from adding temozolomide to standard treatment rather than radiotherapy alone (Hegi et al., 2005). Karayan-Tapon L and colleagues assessed in a multicentre study, the significance of MGMT status for predicting overall survival in GBM patients. Multiple TMZ treatments were beneficial for 50% of GBM patients, and this efficacy is associated with the DNA methylation-induced silencing of MGMT on its promoter (Karayan-Tapon et al., 2010). Folate supplementation for the methylation of the MGMT promoter was shown to silence its expression and consequently increase TMZ efficacy in MGMT-expressing GBM (Cartron et al., 2012). Another paper in 2012 (Malmström et al., 2012) reported that in GBM cells with a methylated MGMT promoter, temozolomide was more effective (i.e., reduced MGMT expression). O6 -benzylguanine (O6 -BG), an MGMT inhibitor, restored temozolomide (TMZ) sensitivity in TMZ-resistant cell lines LN-18 and T98G (Kanzawa et al., 2003). Accordingly, MGMT appears to be the primary modulator of TMZ resistance in GBM, as expected from its biochemical role. Recently, *MGMT* promoter methylation has demonstrated the potential to be a prognostic factor for patients with GBM (Binabaj et al., 2018). Despite the survival benefit associated with TMZ among patients with a methylated MGMT promoter, MGMT methylation is not the unique factor determining the patient outcome (Grochans et al., 2022).
9. **WT1 (The Wilms tumor gene):** The WT1 gene is located at locus 11p13 and encodes a 4 zinc-finger transcription factor which is essential in the cell proliferation and differentiation (Pritchard-Jones et al., 1999) WT1 was first identified as a tumor suppressor gene observed in childhood renal neoplasm and Wilms tumor and then the overexpression of WT1 was found in leukemias and various solid tumors including breast and ovarian cancers (Call et al., 1990; Inoue et al., 1995; Miyoshi et al., 2002). Wilms tumor 1 (WT1) gene has shown a role in gliomagenesis,

making it a potential immunotherapy target in glioblastomas (Clark et al., 2010; Izumoto et al., 2008). Several studies have demonstrated the overexpression of WT1 in high-grade gliomas (Kijima et al., 2014; Kurdi et al., 2021). Nakahara et al. investigated the immunohistochemical expression of WT1 protein in glioblastoma cases and 94% of GBM samples showed positive staining for the WT-1 protein (Nakahara et al 2004). However, the utility potential of WT1 expression as a biomarker has not been sufficiently confirmed (Kurdi et al., 2021).

10. **PTEN (Phosphatase and tensin homolog deleted on chromosome 10):** the PTEN gene is identified as a tumor suppressor gene located on 10q23. PTEN is a protein with protein phosphatase and lipid phosphatase functions. Several groups have shown that its lipid phosphatase activity is responsible for most of the tumor-suppressive properties (Maehama et al., 1998; Knobbe et al., 2002). The inhibitory effect of LOH (loss of heterozygosity) or methylation mutation in PTEN disrupts the phosphatidylinositol-3'-kinase (PI3K)/Akt signaling pathway through dephosphorylation of phosphatidylinositol-(3,4,5)-triphosphate (Knobbe et al., 2002) resulting in loss of cell-cycle control, uncontrolled proliferation, and escape from apoptosis (Hopkins et al., 2014). Koul et al, found that LOH of PTEN or other PTEN mutations is present in at least 60% of GBM cases. The loss of PTEN expression is indicative of the progression of highly malignant tumors and is associated with a poor prognosis of GBM (Koul et al., 2008). Despite this, PTEN loss could be associated with a more favourable prognosis, since it leads to a better response to chemotherapy by compromising the homologous recombination of DNA, through the transcriptional regulation of Rad5. In Brito et al. study PTEN deletion was considered a factor of good prognosis in GBM IDH-wildtype samples. Further work should be undertaken to evaluate the mechanisms of the PTEN dual effect (Brito et al., 2019).

1.6 Standard of care: surgery, radiation, and chemotherapy

Great efforts devoted to exploring potential treatments through its molecular mechanisms have brought many novel therapies to light, however, with limitations (Grochans et al., 2022). Although recent advances in molecular profiling have led to identifying prognostic factors, it has not changed the therapy or survival and GBM remains incurable cancer (Hanif et al., 2017). The current treatment schedule consists of a multimodality approach combining neurosurgery with radiotherapy and chemotherapy. Karnofsky performance score, neurological function, and age need to be considered in clinical decision-making in neuro-oncology (Jiang et al., 2021). The standard of care for newly diagnosed IDH-wildtype GBM, WHO grade IV, is based on the Stupp protocol described in the pivotal phase 3 trial in 2005 which involved the addition of alkylating chemotherapeutic temozolomide (TMZ) to the treatment regimen. More specifically, the standard approach includes maximal safe resection as the first step in all patients followed by concomitant daily TMZ and radiotherapy and then six maintenance cycles of temozolomide. For younger patients aged 70, the surgery must be followed by involved-field RT (60 Gy in 1.8–2.0 Gy fractions) + TMZ (75 mg/m^2

daily throughout RT, including weekends) + 6 cycles of maintenance temozolomide (150–200 mg/m2, 5 out of 28 days) + Tumor-Treating Fields (TTFs). An alternative in MGMT promoter-unmethylated tumors is surgical resection and RT alone. For poor performance status (KPS < 70) hypo-fractionated RT (40 Gy in 15 fractions) + TMZ or TMZ alone or best supportive care (BSC) are considered (Rodríguez-Camacho et al., 2022).

Even though therapy is always followed by tumor recurrence and progression, recent advances in multimodality therapy have improved the median survival to approximately 15 months (14–21 months), the progression-free survival (PFS) to 10 + 1 months before recurrence, the one-year survival rate to 41.4%, and a five-year survival rate to 6.8% (Rodríguez-Camacho et al., 2022). Since 2011, the antimitotic treatment Tumor-Treating Fields, is a promising alternative to chemotherapy for recurrent GBM which require low intensity (1–2 V/cm) and intermediate frequency (100 kHz to 1 MHz) electric fields to induce cell death. Later in 2015, FDA approved it as an adjuvant TMZ maintenance therapy for newly diagnosed GBM. TTFs are applied via placing electrodes on the shaved skull, because of which mild dermatitis and local skin irritation are commonly reported side effects (Janjua et al., 2021). EF-14 trials showed that TTFields treatment after concomitant chemoradiotherapy increases the median OS to 20 months compared to 16 months in the group with TMZ alone. Moreover, it resulted in a longer median PFS, 6.7 months vs. 4.0 months (Fabian et al., 2019). As treatment of GBM is not curative, 90% of the patients will experience a recurrence of the disease at some point thereafter, which is considered a local tumor progression (McBain et al., 2021). There are no established guidelines for treating recurrent GBM (rGBM) (van Linde et al., 2017) and little evidence for any interventions that prolong OS. Indeed, a considerable proportion of patients may not even be eligible for second-line therapy. However, following recurrence after chemoradiotherapy, a proportion of people can undergo further treatment; however, elderly, and frail people are likely to receive palliative care only (McBain et al., 2021). Some of the treatment options for recurrent GBM include re-operation, re-irradiation, systemic therapies such as lomustine or bevacizumab, combined approaches, and best supportive care (Tan et al., 2020). The meta-analyses of McBain et al. found no good evidence that any of the treatments tested were better than lomustine. Other combination therapies showed a higher risk of serious side effects and did not improve overall survival compared with lomustine alone. Limited evidence suggested that a second operation with or without other treatments may be of value in selected individuals with a first recurrence.

Surgery

Before the Stupp et al. trial (Stupp et al., 2005), the initial gold standard of treatment was gross total resection (GTR) of tumor mass as safely as feasible without risking the patient's functional state (Rodriguez Camacho et al., 2022). The surgery's primary treatment goal is to achieve tumor volume reduction, histological diagnosis, and tumor genotyping which are essential factors in decision

treatment making. A stereotactic or open biopsy is suggested when surgical resection is not an option (Tan et al., 2020). Depending on the size and location of the tumor, patients typically undergo surgical resection of the tumour mass after glioblastoma diagnosis (Janjua et al., 2021). Aggressive surgical resection plays an important diagnostic and therapeutic role in the management of GBM: for accurate histological and molecular diagnosis, immediate relief of the tumor-related mass effect and its associated symptoms, and potential cytoreduction. Clinical reports suggest that a maximal surgical tumor resection improves patients' outcomes and quality of life (Louis et al., 2007; Chaichana et al., 2014). Various studies demonstrated that extensive surgical tumor debulking i.e., more than 1–2 cm from the tumor boundary increased the overall survival (OS) compared to those with less aggressive tumor resection. However, since GBM is characterized by high proliferative activity, the risk of postoperative neurologic deficits, and the lack of clear tumor boundaries, complete tumor mass resection is impossible (Janjua et al., 2021). Studies have shown that intraoperative fluorescence-guided surgery with 5-ALA, the navigation-guided fence post-procedure, and intraoperative MRI have facilitated maximal and almost complete resection of tumors (Rajaratnam et al., 2020).

Radiotherapy

The Stupp protocol includes radiotherapy as a significant modality in treating glioblastoma to improve local control and survival. The conventional radiotherapy after surgery delivers 60 Gy in 2-Gy fractions on weekdays for six weeks for first-time treated GBM, starting 3–5 weeks after surgery. RT usually starts 3–5 weeks after surgery. The inter-lapse between surgery and RT/CT is inversely related to PFS and OS (Rodriguez Camacho et al., 2022). Up to the present, none of the novel techniques to deliver radiation have demonstrated superior efficacy over standard fractionated radiotherapy (Tan et al., 2020). Traditionally, GBM treatment included surgery followed by radiotherapy, where the 3- and 5-year survival rates were 4.4 and 1.9%, respectively (Stupp et al., 2009).

Chemotherapy: Temozolomide (TMZ)

Current evidence indicates TMZ as the first-line treatment for primary and recurrent GB management, particularly for MGMT promoter-methylated tumors. During concurrent RT, the daily optimum dose of TMZ is 75 mg/m^2 for a six-week period (42 days), followed by six cycles of maintenance of 150–200 mg/m2 for five days every 28 days. In patients with poor performance status (KPS < 70), it is suggested to administer TMZ alone at 150–200 mg/m^2 for five days every 28 days after surgery. For newly diagnosed GB, there is no evidence of benefit from different TMZ doses or treatment strategies (Rodriguez Camacho et al., 2022). The phase 3 study showed that in patients with good performance status (Karnofsky performance status ≥ 60), the median overall survival (OS) was 14.6 months for radiotherapy plus temozolomide versus 12.1 months for

radiotherapy alone (hazard ratio [HR], 0.63; 95% CI, 0.52-0.75 [P < .001] (Stupp et al., 2005). Stupp and colleagues demonstrated that the benefits of adjuvant TMZ with radiotherapy lasted throughout 5 years of follow-up. The concomitant TMZ with radiotherapy in GBM produced a 2-year survival rate of 26.5% versus 10.4% survival in those who received radiotherapy alone; and 9.8% of the patients survived 5 years with temozolomide, compared with radiotherapy alone group where only 1.9% had a 5- year survival rate. An advantage of combined therapy was recorded, including patients aged 60-70 years. Methylation of the MGMT promoter was the strongest positive predictor of favourable patient outcomes (Stupp et al., 2009). There are no data suggesting the benefits of other chemotherapeutic agents such as carboplatin, procarbazine, irinotecan, and etoposide, which are mostly used for recurrent GBM (Torrisi et al., 2022). Additional alkylating chemotherapy can be Lomustine, broadly used intervention in recurrent GBM (Le Rhun et al., 2019).

2. GLIOBLASTOMA STEM CELLS

Recurrence of GBM is attributed to many factors: the heterogeneous microenvironment, which make them difficult to target with single agents; the blood–brain barrier (BBB) and abnormal intratumoral vascularization that prevent drug distribution; the highly invasive and infiltrative nature of the disease, with glioma cells penetrating the brain parenchyma at a great distance from the bulk of the tumor; the presence of glioma stem cells (GSCs), also referred to as brain tumor-initiating cells or recurrence-initiating stem cells (Osuka et al., 2017; Calinescu et al., 2021). GBM stem cells (GSCs) were first identified by Singh et al. as a subset of cells that initiate tumor growth *in vivo* (Singh et al., 2004). While rapidly proliferating cells are more likely to be found near vessels, stem-like cells are in the core region of the tumor, clustered in a cancer stem cells (CSCs) niche. The core area is more radioresistant and chemoresistant, and it is generally necrotic. These various cell distributions highlight the heterogeneity of GBM. According to the hierarchy model that explains the presence of a high degree of heterogeneity, tumors are organized hierarchically with a small number of CSCs at the apex of this hierarchy, with a low but unlimited capacity to proliferate, able to self-renew, repopulate a tumor after sublethal treatment, and generate new stem cells and daughter cells that undergo differentiation and replenish the pool of functional cells. GSCs are pluripotent cells, that can give rise to different lineages of daughter cells. For instance, a neural stem cell can generate cells that differentiate into neurons, astrocytes, and oligodendrocytes. The low mitotic activity of CSCs protects them from treatment approaches that are directed against actively dividing cells and give rise to recurrences (Biserova et al., 2021). CSCs are thought to develop stochastically in a tumor but to be mainly induced in hypoxic, low pH, and nutrient-limited environments such as the tumor niche (Mondal et al., 2018). Although extensive investigation to understand the authentic origin of CSCs has been made, some controversy remains, due to the incomplete and inconsistent experimental data. Alternatively, multiple GSC clones with distinct genetic alterations may

differentiate into specific types of gliomas. Finally, glioma cells may possess inherent plasticity that allows them to revert to GSCs under appropriate conditions. In support of the first and second models is the observation that targeted deletion of the tumor suppressor genes p53, Nf1, or Pten in NSCs generates gliomas with 100 percent penetrance, whereas deficiency of these genes in non-neurogenic brain regions does not result in tumor formation (Alcantra Llaguno S., et al., 2009). Experiments demonstrating that the reversion of neonatal cortical astrocytes in culture to NSCs can be induced by the combined loss of p16Ink4a and p19Arf provide support for the third model (Bachoo RM., et al., 2002). Nonetheless, these models still lack in vivo data. Furthermore, there is no evidence to differentiate between the first and second models (e.g., the discovery of two GSCs within the same specimen that harbour different mutations would favor the second model) (Yin CL., et al., 2014). Interestingly GSCs like GBM are known to be classified into two molecular subtypes: mesenchymal and proneural GSCs (Mao et al., 2013; Lottaz et al., 2010; Spinelli et al., 2018). In addition, primary proneural GBM can recur as mesenchymal GBM (Garnier et al., 2018). The subtype switch reflects a general feature of tumors and can be explained either by the occurrence of a molecular switch in proneural GSCs, leading to transformation into a mesenchymal profile, or by better survival of mesenchymal GSCs that were present in the primary proneural GBM (Fedele et al., 2019). The low but steady level of unlimited proliferative activity, low abundance within the tumor, treatment resistance, and association between CSCs and tumor recurrence are accepted as the general features of cancer stem cells These characteristics have been demonstrated also in glial stem cells (Eramo et al., 2006; Biserova et al., 2021).

2.1 Regulatory Mechanisms of Glioma Stem Cells

GSCs are regulated by six main mechanisms, which include intrinsic factors such as genetics, epigenetics, and metabolism as well as extrinsic qualities of niche factors, cellular microenvironment, and the host immune system (Lathia et al., 2015). Increasing evidence suggests that several crucial signal transduction pathways are involved in the maintenance of GSCs. Most significant ones are Notch, Sonic Hedgehog, Wnt/β-catenin, Akt, and STAT3 signaling pathways (Ma et al., 2018). The ligands that lead to the Notch pathway's activation, e.g., Delta-like ligand 4 (DLL4) and Jagged1 (JAG1), are expressed on the endothelium, while Notch-1 and Notch-2 themselves are expressed on GSCs. Notch activation induces the activation of the target genes Hes1 and Hey1, thereby promoting the maintenance of multipotency (Sharifzad et al., 2019). For example, Fan et al. have confirmed that Notch pathway blockade reduces the expression of stemness markers and inhibits neurosphere formation (Fan et al., 2010). The Notch cascade can also become activated by the extracellular matrix protein tenascin. Additionally, many activating receptors on GSCs, like inhibitors of differentiation 4 (ID4) and fatty-acid binding protein 7 (FABP7), facilitate Notch signaling activation. High expression of these genes promotes infiltrating potential of GBM tumors thus, the Notch signaling pathway is thought to promote migration (Sharifzad et al., 2019). Hypoxia-

inducible factor 1α (HIF-1α) promotes the Notch pathway by stabilizing its intracellular domain. NF-kB factor promotes resistance to radiation treatment in GBM. The knockdown of antiapoptotic protein A20 (TNFAIP3), which represents a regulating molecule in the NF-kB pathway, overexpressed in GSCs relative to non-stem glioblastoma cells, has shown to decrease GSCS survival and tumor growth (Hjelmeland et al., 2010). Canonical and non-canonical Wnt signaling activation develops in GSCs via genetic and epigenetic mechanisms. In GBM, mutation of FAT1 leads to abnormal activation of Wnt and promotes tumorigenesis. CSC chromatin is dependent on the ASCL1, a transcription factor that activates the Wnt pathway through the Dickkopf Wnt signaling inhibitor (DKK1). The Wnt cascade is also involved in resistance against temozolomide via the induction of MGMT (O6-methylguanine-DNA methyltransferase) expression, which preserves the genome from temozolomide-induced alkylation. Finally, the Sonic hedgehog (Shh) pathway influences the self-renewal and tumorigenicity of GSCs (Honorato et al., 2018). The Shh cascade activates the glioma-associated oncogene GLI1 and GLI2 products, which bind to the Nanog promoter and increase the expression of this stem cell marker. Nanog's transcription factor activity stimulates the production of other stemness factors. Nanog is inhibited by p53 in healthy tissues, however in GBM, TP53 activity is usually impaired, leading to Shh and Nanog activation. Besides maintaining stem cell features, Shh is involved in drug resistance via upregulation of the drug efflux P-glycoprotein and other ATP-binding cassette transporters (Biserova et al., 2021). AKT along with the WNT pathway regulates epithelial-mesenchymal transition (EMT), proliferation, and the tumorigenicity of GSCs. Recent investigations indicated that both hedgehog and TGF-β-related signaling pathways can maintain GSC phenotype to undergo proliferation, self-renew, and metastasis. Although GSCs display a minor subset of cells within the glioma microenvironment, their high cancer-initiating ability and therapy resistance highlights the tumorigenic behavior of these cells. Therefore, investigating signaling pathways correlated with GSC may discover novel approaches for selective targeting (Nasrolahi et al., 2023).

2.2 Markers of Glioma Stem Cells

GSCs are identified using cell surface markers or cell selection techniques that take advantage of phenotypes increased in this cellular subset, with sorting or validation using GSC markers that include: CD133, CD44, CD15, CD70 (CD27 L), S100A4, ALDH1A3, Nanog, OCT-4, and Nestin; the cell-surface gangliosides A2B5, CD90, and SOX2. L1CAM, KLF4, SALL4, and GFAP have also been also used for the identification of GSCs (Alves et al., 2021). Because no one marker can be considered a gold standard, a set of markers is required to establish stemness status. A better understanding of the roles of these markers as well as their involvement in many crucial cellular signaling pathways can direct potential research toward novel GBM treatments. The first accepted GSCs surface marker was CD133. This marker distinguishes between two types of stem cells: CD133-positive cells (CD133+), or cancer stem cells, and CD133-negative cells (CD133), or non-

cancer stem cells. In GBM, overexpression of CD133 has been can also be used to characterize cell self-renewal potential and resistance towards temozolomide (TMZ) by the activation of c-Jun N-terminal kinase (JNK) signaling and Notch/sonic hedgehog (SHH) pathways, respectively (Hassn Mesrati et al., 2020). Another critical feature of $CD133^+$ cells is the capacity to generate neurospheres *in vitro* and induce brain tumor formation *in vivo* models (Alves et al., 2021). $CD133^+$ cells populations were found to be highly associated with another marker named aldehyde dehydrogenase 3A1 (ALDH3A1), that promotes poor prognosis and chemoresistance (Suwala et al., 2018). Li and colleagues showed that glioblastomas expressing high levels of CD133 are extremely correlated with the overexpression of HOX gene stem cell factors. The overexpression of both HOX genes and CD133 worsen glioblastoma prognosis (Li et al., 2016; Hassn Mesrati et al., 2020). CD15 and CD133 are reported as the most useful surface markers of GSCs among others (Dirk et al., 2010). GSCs with elevated levels of CD44 in the tumor margin compared to the center correlates with the highly invasive feature, worse survival, and faster tumor progression. Fakhri et al confirmed that CD44 play a basic role in tumor invasion and migration, thus supporting several studies that described CD44 as a marker of GSCs (Hassn Mesrati et al., 2020). In addition, the presence of dual $CD133^+$/Ki-67^+ cells and associated *Nestin* or *HOX* genes is an adverse prognostic factor for GBM progression. Another marker highly expressed in GSCs is the CXCR4 chemokine receptor (CD184), which is associated with $CD133^+$ cells and increased expression of hypoxia-inducible factor (*HIF-1-α*). The same importance should be assigned to the MUSASHI-1 protein, a regulator of translation and cellular fate. (Alves et al., 2021). Many reports have shown that Nestin knockdown suppressed the proliferation, invasion, and migration of GBM cells. (Strojnik et al., 2007). Some studies suggested that Nestin expression is linked to a higher grade and a poor prognostic outcome in gliomas (Strojnik et al., 2007; Arai et al., 2012; Lv et al., 2016). GSCs proliferation is promoted by the activation of the KRAS/Notch pathway, whereas resistance to radiotherapy has been exhibited by the activation of the Akt/PI3K and p53 pathways (Hassn Mesrati et al., 2020). The role of the SOX-2 gene in the maintenance of GSC stem cell properties and chemoresistance to TMZ has been well demonstrated (Garros-Regulez et al., 2016).

SOX-2 is a gene located on chromosome 3q26.3-q27, a member of neural growth transcription factors family called SOX [sex determining region Y (SRY)-box], which controls several developmental processes and maintain stem cell activity in different tissues during embryogenesis and adult stages. Many studies have implicated SOX-2 expression with growth, tumorigenicity, metastasis, drug resistance, and prognosis of various cancers (Wuebben et al., 2017). The expression of SOX-2 is up or downregulated by various mechanisms like transcriptional factors, signaling pathways, post-transcriptional, and post-translational regulators. Transcription factors such as Oct4, Nanog, and less Stat3 co-operate with SOX-2, and this collaboration promotes the expression of genes, the production of molecules and activation of metabolic processes which guarantee the self-renewal and the maintenance of stem cell characteristics. AP-2, PROX1, PAX6, etc. are

transcriptional factors that upregulate SOX-2 and are well-expressed in the early stages of neurodevelopment, E2F3a, E2F3b, and Cyclin-dependent kinase inhibitor P21 are involved in SOX-2 expression and so control the proliferation of NPCs. Another way that regulates the expression of SOX-2 is through SOX4, which in turn is activated by the TGF-β signaling pathway and forms a complex with OCT4 in SOX-2 promoter's sites. SOX-2 expression, also participate in Shh, Wnt, and FGFR signaling pathways. SOX-2 is one of the most important transcription factors that regulate cancer stem cell properties. SOX-2 could reprogram differentiated cells into pluripotent cells in concordance with other factors and is overexpressed in various cancers; it is a marker of cancer stem-like cells (CSCs) in neurosphere cultures and is correlated with the proneural molecular subtype.

SOX-2 is expressed in all gliomas, and the proportion of SOX-2-positive cells – ranging from 6–80% of cells in the tumor – correlates with the malignancy grade (Sampetrean et al., 2013). In GBM, SOX-2 is intensely expressed in the most malignant component of the tumor and in highly proliferating cells of oligodendrogliomas. The gene encoding Sox-2 is also amplified in approximately 14.4 and 11.1% of GBM and anaplastic oligodendrogliomas, respectively, compared with EGFR amplification in 36–40% and loss of PTEN due to loss of heterozygosity in 60–80% of GBM cases. In malignant glioma samples, the intense positive staining for SOX-2 overlaps with Ki67/MIB.1-positive nuclei, which is a gold standard marker for proliferating cells. In addition, tumor regions showing intense SOX-2 staining also show frequent amplification of the *SOX-2* gene. In cultured neurospheres, the Sox-2 gene is often amplified, and therefore, the hypothesis that a genetic correlation exists between neurospheres and the most anaplastic region in glioma is validated by the expression pattern of SOX-2. The extent of SOX-2 expression is also concordant with the degree of heterogeneity observed in the cell population found in gliomas, which appear in various stages of differentiation. Gangemi et al. demonstrated that suppression of SOX-2 expression in GBM tumor-initiating cells prevented their proliferation and reduced their tumorigenicity in long-term culture conditions and no obvious short-term effects on apoptosis, cell senescence or increased differentiation were detected (Gangemi et al., 2009). They also found that the downregulation of SOX-2 using siRNA targeting the 3′ UTR of *SOX-2* mRNA in GBM cell lines results in reduced Ki67 expression in these cells. This effect was independent of defects in progression through the cell cycle and more likely due to loss of the ability of GBM cells to divide indefinitely, hence their reduced stemness. Reduced proliferation of these cells eventually leads to their premature exit from the cell cycle and eventual disappearance from the culture. Annovazzi et al. conducted a study in order to evaluate SOX-2 expression, distribution, and gene copy number status in normal nervous tissue, and in a number of neuroepithelial tumors and cell lines derived from primary GBM tumors by immunohistochemistry, western blotting, and other molecular biology techniques (Annovazzi et al., 2011). Consistent with other reports, they also observed a correlation between the expression of

SOX-2, Ki 67/MIB.1, and NESTIN. In this regard, it is possible that SOX-2 creates a permissive environment for the induction of pluripotency and tumor development.

3. MECHANISMS OF CHEMORESISTANCE IN GLIOBLASTOMA

TMZ is the major pillar of GBM treatment, but it is unfortunately also a key factor in tumor resistance and recurrence. Although subsequent radiation therapy and chemotherapy with TMZ contributed to extent survival and improve quality of life, unfortunately, more than half of GBM patients treated with TMZ do not respond to the therapy. Resistance to TMZ in GBM involves multiple molecular pathways and constitutes a multifaceted challenge. Thus, a comprehensive knowledge of the cellular populations that drive resistance, as well as the molecular processes that underpin its growth and potentiation, is of great importance (Singh et al., 2021). Various resistance mechanisms of GBM include DNA repair systems, several molecular pathways, apoptosis, metabolism, autophagic process. The unique population of undifferentiated and highly tumorigenic cancer stem cells known as glioma stem cells GSCs comprises as few as 1% of cells in any given GBM tumor. However, GSCs are thought to be responsible for TMZ resistance and most tumor recurrences after initial treatment due to their ability to regenerate tumor heterogeneity (Singh et al., 2021). TMZ chemotherapeutic treatment of glioma cells is capable of interconversion of non-GSCs into new GSCs. The drug efflux transporter (ATP-binding cassette, ABCG2) and GSCs diversity were linked to GSCs chemoresistance. GSCs exhibit diversity due to the numerous heterogenetic GSC phenotypes of tumor bulk based on distinct genomic profiles. The tumor microenvironment can also modulate GSC phenotypic change and may produce different types of GBM cell lines. In contrast, differentiated GBM cells can be reprogrammed by the tumor microenvironment and re-acquire stem cell capacity. Hypoxic condition promotes stemness and enhances MGMT upregulation through hypoxia-inducible factor 1-alpha (HIF-1α) in GSCs. Chemoresistance based on GSCs theory could be important as a hallmark of recurrent GBM. Therefore, novel therapeutic approaches that are effective and successful in eliminating both GSCs and the entire tumor bulk are urgently required. Hypotheses and studies support the idea that molecules involved in the maintenance of GSC stem-cell-like characteristics could be potential therapeutic targets for overcoming chemoresistance (Jiapaer et al., 2018).

3.1 DNA repair systems

- <u>MMR repair and temozolomide resistance - MGMT</u>

Mismatch repair (MMR) is a mechanism of DNA repair that corrects any mismatched nucleotide base pairs that occur during DNA synthesis. Under normal conditions, MMR is activated and generates breaks in the newly synthesized DNA strand. Repeated cycles of DNA breaks generated

by the MMR system in response to TMZ-induced DNA damage results in cell death (Singh et al., 2021). MLH1, MSH2, MSH6, and PMS2 genes encode MMR proteins which recognize and remove the mismatched DNA pairs occurring during the replication. In glioma cell lines, inactivation of the MMR genes MSH2 and MSH6 correlates with resistance to alkylating treatment, and in xenograft models decreased MSH2 confers temozolomide resistance. Clinically, MSH6 deficiency is associated with tumor progression during treatment with temozolomide. Defects in MMR can result in the accumulation of an abundance of mutations and a so-called hypermutated phenotype, that have been associated with responsiveness to immune checkpoint inhibitors in solid tumors including glioma (Elmore et al., 2021). The MMR mechanism and its relationship with MGMT methylation are being investigated in GBM with the goal of identifying specific MMR proteins as new prognostic markers. Interestingly, it has been shown that the combination of methylated MGMT and high MMR activity induce the best response to TMZ treatment (Singh et al., 2021). Among the several methylation lesions induced on both nitrogen and oxygen molecules within DNA nucleotides, O6-methylguanine (O6MeG) is considered the most toxic lesion induced by TMZ once metabolized from its prodrug form to MTIC [3-methyl-(triazen-1-yl)imidazole-4-car-boxamide]. It leads to methylated nucleosides and mismatch pairing of guanine with thymine rather than cytosine during the S phase and DNA replication. Although MMR recognizes these mispairs and removes the thymine residue, the enzymes are not able to remove the O6-meG. If O6MeG is not properly excised by the mismatch repair (MMR), it persists as a permanently miscoding base, triggering 'futile cycles' of MMR and resulting in the stalling of DNA replication forks or double strand breaking. As a result, MMR activity in GSCs with genetic or epigenetic abnormalities affecting MMR gene expression can impact TMZ response (Rominiyi et al., 2021). The enzyme MGMT is considered an important factor in TMZ resistance due to its direct role in maintaining genomic stability through counteracting DNA alkylation damage (Dymova et al., 2021). Under TMZ treatment, MGMT can remove the methyl group in O6-methylguanine thereby neutralizing the drug-induced DNA damage and reducing the overall efficacy of TMZ. Therefore, MGMT expression, which is determined by the CpG methylation status of the MGMT gene promoter region, could be a potential marker of the response to TMZ treatment. Hypermethylation of the MGMT gene promoter region leads to reduced MGMT expression, shifting the balance in favour of persistent O6MeG which correlates with better survival outcomes. On the contrary, unmethylated tumors (with increased MGMT activity) commonly exhibit resistance to TMZ. Thus, the epigenetic status of MGMT has been established as a surrogate marker of intrinsic resistance to TMZ (Singh et al., 2021). Moreover, there is increasing evidence from meta-analysis studies that MGMT status may alter throughout a tumor's treatment, progression, or recurrence (Feldheim et al., 2019). GBM with initial MGMT methylation had a lower methylation ratio following recurrence after TMZ therapy, indicating that MGMT promoter methylation reduction is a mechanism for acquiring therapeutic resistance to TMZ.

- Base excision repair

Aside from O6MeG, the more common (but less fatal) temozolomide-induced DNA lesions N3-methyladenine and N7-methylguanine are mostly (>90%) removed by base excision repair (BER). BER mechanism involves several enzymatic reactions carried out by AP endonuclease (APEX1), DNA polymerase (pol-b), and DNA ligases I and III, respectively (Rominiyi et al., 2021). N3-methyladenine and N7-methylguanine repair is initiated with the identification of lesions by alkylpurine-DNA-N-glycosylase (AAG), which cleaves the glycosylic bond between the damaged base and deoxyribose, leaving a basic site. After the hydrolysis reaction, AAG recruits the endonuclease, Ape1, to the site of damage, which cleaves the DNA phosphodiester backbone to produce an SSB containing a 3′-OH and 5′ deoxyribose phosphate (5'-dRP) termini. Ape1 is then replaced by DNA polymerase β, a repair DNA polymerase that possesses 5′ lyase activity that can excise the 5′-dRP to produce a single nucleotide gap. The formed gap is subsequently filled in by DNA polymerase β, and DNA ligase seals the nick to finalize the repair process (Berdis et al., 2021). Several proteins involved in the BER pathway have been associated with promoting resistance to TMZ and conferring poor prognosis in patients. (Li et al., 2022). Enzyme Poly [ADP-ribose] polymerase 1 PARP1 is considered to play a crucial role in the BER pathway by interacting with BER proteins such as DNA ligase III, DNA polymerase beta, and XRCC1. PARylation modifies these proteins through the action of PARP1 and facilitates the recruitment of DNA repair complexes by providing a docking platform. PARP1 is overexpressed in GBM but not in normal brain regions, indicating that PARP1 is involved in glioma therapy resistance. PARP inhibitors have been intensively investigated over the last three decades, with promising outcomes including improved TMZ sensitivity in GBM and perfect BBB penetrations (Lang et al., 2021) Therefore, inhibition of the BER pathway offers a very promising target to overcome TMZ resistance.

3.2 Molecular pathways in TMZ resistance

- WNT/ β-catenin signaling

Wnt signaling is essential for stem cell maintenance and differentiation. and any disruption in Wnt pathways is crucial to the biology of GSCs (Tompa et al., 2018). Wnt pathway roughly divided it into either canonical (β-catenin dependent) or non-canonical (β-catenin independent) signaling pathways which regulate the activity of gastric cancer stem cells (GCSC) via targeting CD133 (Nasrolahi et al., 2023). Irregular signaling in both pathways contribute to the progression of GBM and chemoresistance. Ligands that are highly associated with the canonical pathway include Wnt1, Wnt3a and Wnt7a (Singh et al., 2021). Based on the TCGA data, Wnt3a (an initiator of the canonical pathway) and Wnt5a (an initiator of the non-canonical pathway) are overexpressed in GBM, and also in grade II and III gliomas (Tompa et al., 2018). Xu et al., demonstrated that FoxO3a facilitates the binding of β-catenin and nuclear import and thus promotes nuclear accumulation of β-catenin which indicates the canonical pathway activation and has been shown to play a role in TMZ

resistance (Xu et al., 2017). In addition, faulty regulation of the noncanonical pathway increases the tumorigenicity of GBM (Singh et al., 2021). The upregulation of Wnt signaling alter the expression of epithelial-mesenchimal transition (EMT) activators in gliomas. Huang et al showed that endothelial cells (ECs) acquire transformation into mesenchymal stem cell (MSC)–like cells in glioblastoma (GBM), driving tumor resistance to cytotoxic treatment (Huang et al.,2020). This typical process is mediated by c-Met (also known as hepatocyte growth factor receptor, HGFR), that induces phosphorylation in β-catenin at Ser65 and subsequently augmentation in expression of multi-drug associated protein-1 leading to EC-stemness and chemoresistance (Daisy-Pricilla et al., 2022). Furthermore, β-catenin has been shown to modulate EMT through the upregulation of multiple target genes including ZEB1, Snail, Slug, Twist, and several other transcription factors, all of which play a role in promoting tumor growth (Singh et al., 2021). Activation of Wnt signaling also increases the expression DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) through Tcf/Lef binding located in the hmMGMT 5′-flanking regulatory region (Pai et al., 2017). Another protein, Fermitin family member 3 (FERMT3), also known as kindlin-3, is a cytosolic adaptor that functions as a crucial activator and modulator of integrin functioning in normal cells (Lu et al., 2017). FERMT3 regulates glioma cell activity through integrin-mediated Wnt/β-catenin signaling. Hyperactivation of this signaling pathway is mainly caused by promoter methylation of its inhibitors. (Daisy-Precilla et al., 2022). Without the protective role of its natural antagonists, the hyperactivation of Wnt pathway is hyperactivated, contributes to the maintenance of GBM cells stemness, invasiveness, and angiogenesis, as well as chemio- and radioresistance (Celińska et al., 2021). Consequently, it is probable that these effectors of Wnt/β-catenin signaling should be considered when designing targeted therapeutics for GBM.

- PI3K/Akt pathway

The dysregulation of the downstream PI3K/Akt pathway, commonly caused by the amplification of EGFR occur in ~60% of GBMs (Westphal et al., 2017). In addition, 50% of GBMs with EGFR overexpression express a mutant variation, EGFRvIII, which is a ligand-independent, constitutively active form of the receptor (Zhou et al., 2004; Oprita et al., 2021). EGFR amplification and EGFRvIII have been linked in several resistance pathways in GBM. Several downstream substrates of Akt, a serine/threonine kinase, have been linked to characteristic mechanisms of resistance to TMZ, including pyruvate dehydrogenase kinase 1 (PDK1), the hypoxia inducible factor 1 (HIF-1), the forkhead O3 (FoxO3a), nuclear factor kappa B (NF-kB), and other apoptotic regulators (Singh et al., 2021). As the gatekeeper of glucose oxidation, PDK1 inhibits the metabolism of the pyruvate in the Krebs cycle by phosphorylating the E1-a subunit of pyruvate dehydrogenase (PDH) (Velpula et al., 2017). Increased PDK1 expression in GBM cells facilitates the inactivation of PDH. Recent studies demonstrated that dichloroacetate (DCA), used clinically for the treatment of lactic acidosis inhibit the PDK1/EGFR/EGFRvIII interaction and additionally by binding the EGFRvIII over

EGFR, may become a useful alternative to treat chemotherapy resistant GBM (Franovic et al., 2007; Wang et al., 2015). The Warburg effect component mediated by EGFR can be reversed by decreasing lactate synthesis in TMZ-resistant GBMs (Velpula et al., 2017). Under hypoxic circumstances, HIF-1 is activated and plays a role in the control of glucose absorption and glycolysis in cancer cells by activating transcription of genes encoding glucose transporters and the glycolytic enzymes (Nakazawa et al., 2016; Xie et al., 2017; Infantino et al., 2021). Evidence shows that in hypoxic human glioblastoma multiforme (GBM) cells, pyruvate dehydrogenase kinase (PDK1), EGF receptor (EGFR), and HIF-1 are overexpressed. The increase of PDK1, a direct target gene of HIF-1, may boost EGFR activation. HIF-1α activation by PI3K/Akt elevates heat shock proteins 90 and 70 and stabilizes HIF-1a (Zhou et al., 2004). As a physiological response to hypoxia, the stability of HIF-1, HIF-1a, and HIF-1β activates the vascular endothelial growth factor (VEGF) gene, promoting angiogenesis (Steiner et al., 2004; Joensuu et al., 2005). It has also been demonstrated that HIF-1a stimulates the production of CXCL12 and upregulates CXCR4 CXCL12/CXCR4 activation has a role in the autocrine/paracrine process of GBM by increasing tumor cell proliferation and peripheral invasiveness (Würth et al., 2014). The CXCL12/CXCR4 axis is a viable target for reducing TMZ resistance in GBM (Wang et al., 2020). FoxO3a, a transcription factor targeted by Akt, integrates cellular signals that regulate GSC differentiation and carcinogenesis, leading in a potential tumor suppressor activity (Shi et al., 2010; Sunayama et al., 2011). FoxO3a is normally tightly controlled by PI3K/Akt; however, in GBM, when PI3K/Akt itself may be dysregulated, FoxO3a seems to act as a tumor enhancer rather than a tumor suppressor (Qian et al., 2017). Given the discordant results of studies, the precise role of FoxO3a in GBM progression and patient prognosis remains unattainable, as does its role in resistance of TMZ, warranting further investigation before it can be effectively targeted as a potential therapeutic target. In GBM, NF-κB has been identified as an oncogene, and its overactivation is correlated with a poor prognosis. RTK/PI3K/Akt pathway activation increase NF-κB transcriptional activity, and activated NF-κB translocates to the nucleus where induces transcription of pro-survival genes. Studies suggest that DNA damage induced by TMZ activates ATM kinase, which simultaneously causes MGMT repair and incorrect activation of NF-κB (Singh et al., 2021).

- JAK/STAT pathway

The JAK/STAT signaling pathway stimulates glial stemness. STAT3 affects the transition of GBM from the proneural subtype to the mesenchymal subtype and sustains cell survival, proliferation, and tumorigenesis in GBM. It has previously been reported that constitutively active STAT3 is frequently expressed in high-grade glioma. Overactivation of STAT3 is also associated with the upregulation of MGMT expression and thus promotes TMZ desensitization and chemoresistance in GBM (Singh et al., 2021). More recently, it was demonstrated that the JAK2-STAT3 signaling

pathway is disrupted in GSCs, but bone marrow and X-linked (BMX) non-receptor tyrosine kinase induces STAT3 activation to bypass the suppressor of cytokine signaling 3-mediated negative regulation of JAK2 (Kang et al., 2022).

4. INFLAMMATION IN GLIOBLASTOMA

4.1 Inflammatory Tumor Microenvironment and Inflammation in GBM

The tumor microenvironment (TME) is an intricate interconnection between the extracellular matrix, malignant cells, and associated immunological and mesenchymal cell stroma (Anderson et al., 2020; Riera-Domingo et al., 2020; Gajewski et al., 2013; Bader et al., 2020) (Figure 2). The TME is characterized by constant cell-to-cell communication via cytokines that sustain an inflammatory profile, which promotes tumor development, angiogenesis, cell invasion, and metastasis (Quail et al., 2013; Baghban et al., 2020). The connection between inflammation and cancer is a well-established concept, and now it is one of the hallmarks of cancer (Colotta et al., 2009). Inflammatory components are ubiquitous in the microenvironment of several neoplastic tissues (Balkwill et al., 2001). Although a well-regulated inflammatory response is supposed to have an anti-tumorigenic impact, chronic inflammation paradoxically has the opposite effect. It exposes cells to oncogenic transformations and molecular changes via extrinsic and intrinsic mechanisms. In the intrinsic pathway, carcinogenesis-related genetic events trigger inflammation-related programs that steer the establishment of an inflammatory milieu (Mostofa et al., 2017). Chronic inflammation is a driving force in the extrinsic pathway to facilitate cancer development (Zhao et al., 2021). The brain tumor microenvironment comprises different cells, including infiltrative inflammatory cells, cells with stem-like properties, and cells with neural, glial, and myeloid markers (Figure 2). STAT3 is the primary transcription regulator that leads to chronic inflammation-induced immunosuppression through the inhibition of pro-inflammatory cytokine and chemokine production. Several investigations have demonstrated the overexpression of STAT3 in a variety of brain tumors and its function in controlling inflammatory mediators (Yu et al., 2009). Hypoxia and aberrant vascular proliferation are caused by uncontrolled tumor development which results in the invasion of many immune cells including macrophages, eosinophils, neutrophils, and T-lymphocytes (Mostofa et al., 2017). Glioma-derived factors such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1α and -1β, IL-4, IL-6, IL-8, chemokines (e.g., CXCL-12), cyclooxygenase-2 (COX-2), prostaglandin (PG) E2, and platelet-derived growth factor (PDGF) are the key inflammatory mediators that initiate the inflammatory cycle in GBM and promote carcinogenesis by evading growth regulation, increasing angiogenesis and metastasis, avoiding apoptosis, and preserving cancer stemness (Basheer et al., 2021).

In the state of tissue homeostasis and inflammation, monocytes enter the affected tissue, and differentiate into macrophages on exposure to pro-inflammatory cytokines, chemotactic molecules, and microbial infection (Shi et al., 2011). Glioma microenvironment enriched with cytokines can recruit tumor

associated macrophages to the tumor site and polarize macrophages from one phenotype to another which are classified as classically activated (M1, anti-tumor) or alternatively activated (M2, pro-tumor) macrophages, thereby inducing tumorigenesis. In GBM, tumor-associated macrophages (TAMs) include blood-circulating monocytes and tissue resident microglia, which have shown to share both M1 and M2 phenotypes. Activated M1 macrophages elicit cytotoxicity against microbes (e.g., bacteria, viruses, etc.), anti-tumor immunity, and production of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12, IFN-γ, IL-23), chemokines (CXCL9, CXCL10, CXCL11, CXCL12, CXCL16, CCL2, CCL3, CCL5), reactive oxygen/nitrogen species, and COX-2. In contrast, activated M2 macrophages promote immunosuppression, tumor development and progression, and angiogenesis (Jetten et al., 2014; Tong et al., 2021) Macrophage M2 facilitates the recruitment of T-helper (Th) 2 cells and Tregs cells. (Th) 2 cells release cytokines such as IL-4, IL-5, and IL-10, and Tregs cells cause immune-suppression, thus eventually supporting tumor growth (Mostofa et al., 2017).

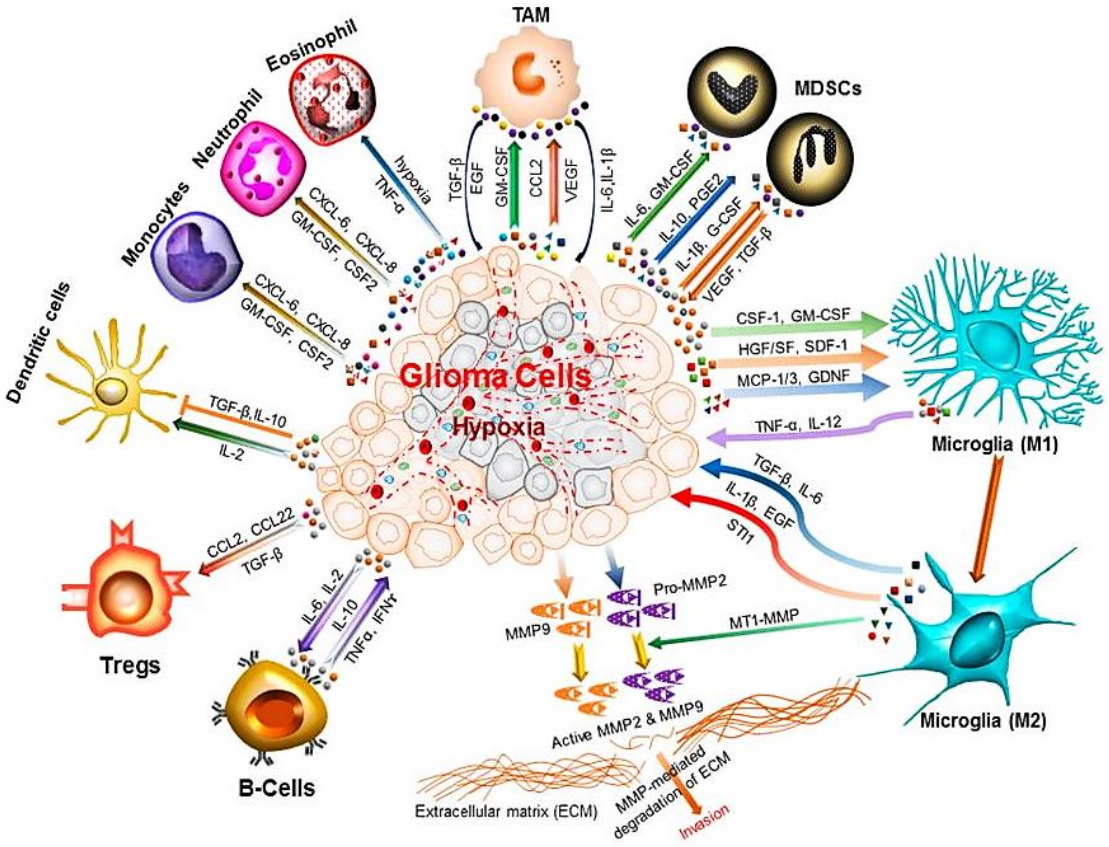


Figure 2 Brain Tumor Inflammatory Microenvironment (Mostafa et al., 2017)

4.2 Cyclooxygenase 2 (COX-2) and GBM

The overexpression of COX-2, the enzyme responsible for prostanoids synthesis, thromboxanes and prostaglandins (PGs), has been indicated as the leading promoter of the inflammatory profile of the TME (Goradel et al., 2019). The enzyme COX, officially named as prostaglandin-endoperoxide

synthase (PTGS), is a bifunctional enzyme having both cyclooxygenase and catalase activities that convert arachidonic acid into the various prostanoids, a family of lipid mediators with different biological functions. By activating a suite of G-protein-coupled receptors (GPCRs), prostanoids regulate a variety of physiological and pathological events, including inflammatory and anaphylactic reactions mediated by prostaglandins; platelet aggregation and vasoconstriction by mediated thromboxane; and platelet inhibition and vasodilation mediated by prostacyclin (Qiu et al., 2017). The two mammalian COX isozymes, COX-1 and COX-2 (or *PTGS1* and *PTGS2*), both of which were discovered during the early 1990s, are primarily localized on the luminal side of the endoplasmic reticulum and nuclear membrane in cells (Li et al., 2020). The expression of COX-2, the inducible isoform for prostanoids production is low at basal levels under normal conditions but is rapidly induced to mediate pathological events that are often associated with severe inflammatory processes in response to tissue injuries and other stimuli, such as lipopolysaccharides (LPS), excitotoxicity, cytokines, and growth factors (Qiu et al., 2017). The tumor microenvironment (TME) is an inducer of COX-2 overexpression (Ohtsuka et al., 2018). COX-2 overexpression in GBM cells and tissues appears to be regulated by several transcription factors, including nuclear factor-kB (NF-kB) and Sp1/Sp3, so could be a promising marker for identification of the tumor from normal cells (Qiu et al., 2017). It has been established that hypoxia induce the factor 1 alpha (HIF-1α), a regulator of oxygen in tumor microenvironment that increase COX-2 expression by regulating it at transcriptional level and elevate PGE2 levels (Jalota et al., 2018). Spatially, the majority of COX-2 expression is localized to the tumor's core, decreasing at the tumor's periphery and being insignificant in surrounding tissues (Dean, Hooks, 2023). Shono et al., revealed a strong correlation between COX-2 overexpression and tumor grade and high COX-2 levels are markers of poor prognosis in brain cancer patients especially those with GBM. Moreover, elevated COX-2 levels correlate with earlier recurrence and shorter survival in glioma patients (Shono et al., 2016).

4.3 Prostaglandin E2 and Prostaglandin receptors EP

Arachidonic acid (AA) is transformed into unstable intermediate PGG2, which is promptly converted into PGH2 by cyclooxygenases (COXs) and finally into five primary prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2) by cell-specific synthases (Liu et al., 2015) (Figure 3). The conversion of PGH2 to PGE2 is mediated by prostaglandin E synthase (PGES) that exists in three isoforms: membrane associated PGES-1 (mPGES-1), mPGES-2 and cytoplasmic PGES (cPGES). mPGES-1 is functionally associated with COX-2 and rapidly induced by various stimuli to generate a peak in PGH2. PGH2 is associated with a broad range of biological effects and fulfils complex (and sometimes antagonistic) roles under both normal and pathological situations (Oliver et al., 2016).

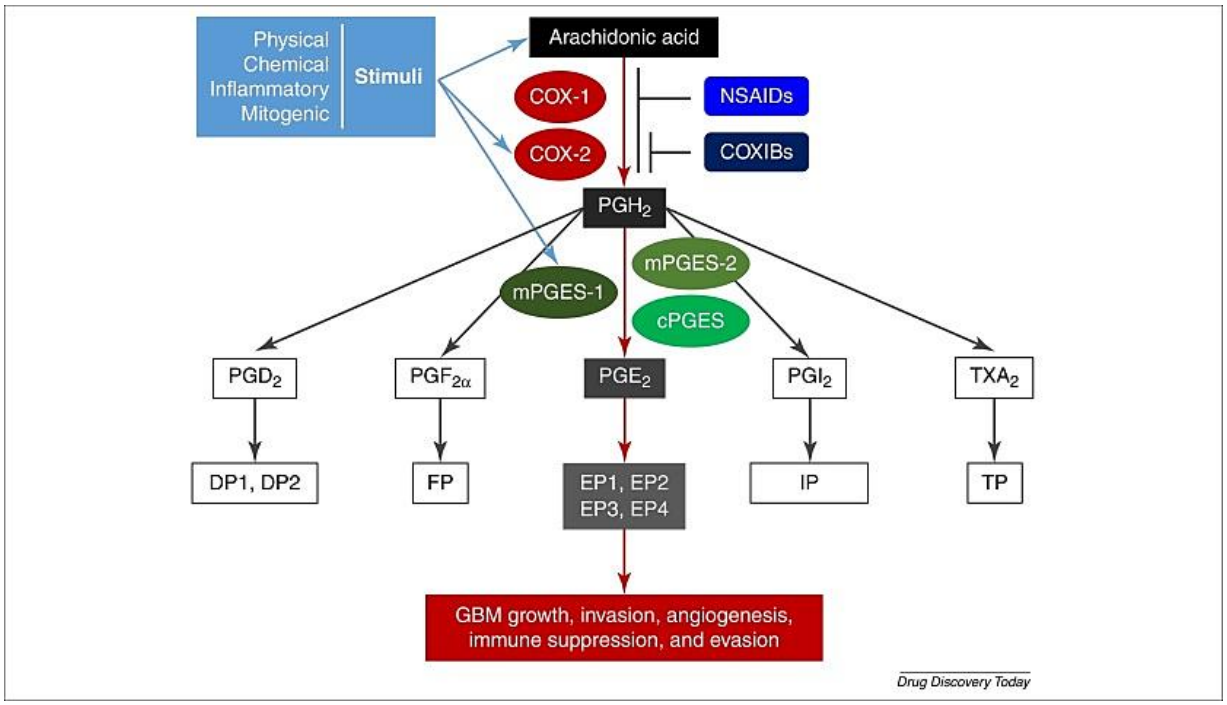


Figure 3. Proposed cyclooxygenase-2 (COX-2) signaling pathways in glioblastoma multiforme (GBM) (Qiu J., et al., 2017).

PGE2 diffuses soon after synthesis and activates its membrane receptors (EP1–4). EP1 receptors interact with the Gq-phospholipase C(PLC)-inositol trisphosphate (IP3) pathway, whose activation leads in intracellular Ca2+ release. EP2 and EP4 receptors connect to the Gs-adenyl cyclase (AC)-cyclic AMP (cAMP)-protein kinase A (PKA) pathway. EP3 interacts with a pertussis toxin-sensitive G protein to inhibit AC, leading in a cAMP reduction (Liu et al., 2015). Thereby, the interaction activates divergent downstream signaling pathways responsible for the pleiotropic actions of PGE_2 in proliferation, apoptosis, angiogenesis inflammation and immune surveillance (Figure 4). Significant crosstalk exists between the EP1, EP2, and EP4 receptors and the epidermal growth factor receptor (EGFR) signaling pathway via transactivation of the EGFR, which activates c-Src. The activation of EGFR induces cell proliferation, differentiation, or migration by activating many signal transduction cascades, including the mitogen-activated protein kinase (MAPK), PI3K/Akt, STAT, and phospholipase C (PLC) signaling pathways.

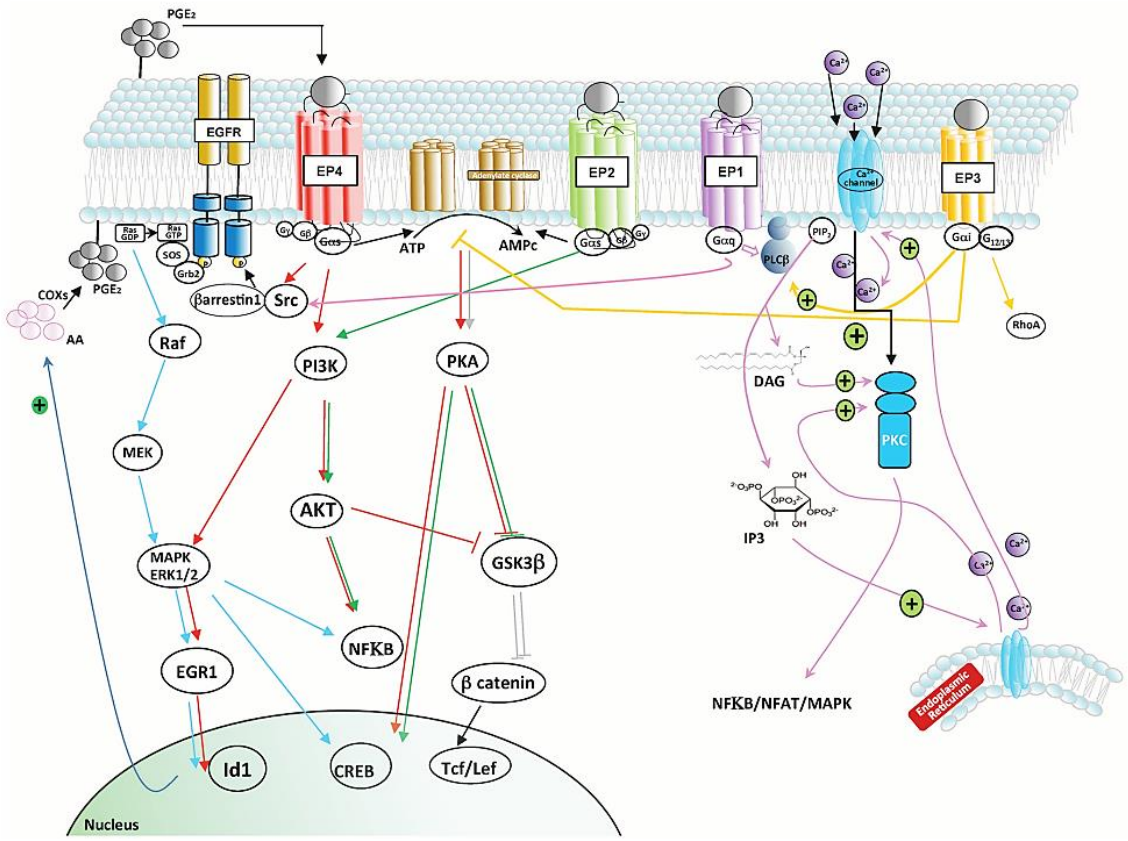


Figure 4. Signaling pathways activated by PGE_2 stimulation of the human EP receptors (Oliver et al., 2016)

Stimulation of EP2 and EP4 receptors by PGE2 induces nuclear translocation of CREB, binding to the COX-2 promoter, COX-2 expression, and synthesis of additional PGE2. Microglia produce significant levels of PGE2 in the presence of glioma-derived soluble substances, demonstrating a paracrine pathway as well. This feedback loop may provide context for the substantial connection between COX-2/PGE2 and the proportion of infiltrating glioma associated macrophages and microglia (GAMs) with high grade gliomas and poor prognosis. In addition, PGE2 stimulates VEGF via HIF-1 activation, and VEGF can boost COX-2/PGE2 synthesis, indicating that these mediators are co-regulated in a feed-forward, amplifying process This PGE2/VEGF axis may contribute to the angiogenesis and invasiveness of GBM. Macrophages, microglia, and tumor cells maintain the ability to create and respond to COX-2/PGE2 by means of autocrine/paracrine signals, therefore generating a cyclical storm of inflammatory mediators (Dean, Hooks 2023) This PGE2/VEGF axis may contribute to the prevalence of angiogenesis and invasiveness of GBM. VEGF overexpression in GBM-TME is linked with a poor prognosis. Macrophages, microglia, and tumor cells maintain the capacity to create and respond to COX-2/PGE2 via autocrine/paracrine signals, therefore sustaining a cyclical storm of inflammatory mediators. The upregulated levels of COX-2 in tumor cells and tissues are accompanied by the elevated presence of prostanoids, particularly PGE_2. The molecular mechanisms whereby induced COX-2 promotes tumorigenesis are not fully understood; however, it is becoming clear that PGE_2 is the predominant COX-2-derived prostaglandin that facilitates tumor activities, including tumor cell adhesion,

proliferation, migration, angiogenesis, immunosuppression, and metastasis. Increasing evidence also suggests that COX-2 and PGE_2 have similar roles in the development and progression of malignant CNS tumors, such as GBM (Li et al., 2020). For example, PGE2 is pro-apoptotic when intracellular and anti-apoptotic when it is released from the cells. The observation that PGE2 has potent tumor-promoting activity is centred on the considerable body of evidence obtained from rodent studies as well as research on the protective effects of non-steroidal anti-inflammatory drugs (NSAIDs) in cancer risks, thereby activating divergent downstream signaling pathways accounting for the pleiotropic actions of PGE2 in proliferation, apoptosis, angiogenesis inflammation and immune surveillance.

4.4 The role of COX-2/PGE2 pathway in Epithelial-to-Mesenchymal Transition

Overexpression of the COX-2/PGE2 axis correlates to a crucial transversal event in the inflammatory character of the TME. Thus, the stromal cells that control tumor formation stimulate the overactivation of pro-inflammatory signaling pathways in tumor cells, thereby facilitating tumor progression and EMT (Gómez-Valenzuela et al., 2021). Epithelial-to-mesenchymal transition (EMT) is a crucial biological connection in the process of tumor formation and metastasis. Some key EMT transcription factor families, including as Twist, Snail, and ZEB, have a regulatory function in tumor invasiveness and chemotherapy resistance, which is a significant factor in the poor prognosis of cancer patients. (Lu et al., 2020). It is now known that tumor cells can undergo a hybrid EMT phenotype known as partial EMT (pEMT). This pEMT phenotype combines epithelial and mesenchymal features, supporting a more invasive ability of tumor cells and promoting cancer stemness and drug resistance. Various microenvironment components, like TGF-β, vascular endothelial growth factor (VEGF), and the hypoxia-inducible factor 1 (HIF-1), are responsible for promoting pEMT (Gómez-Valenzuela et al., 2021). Lu et al., suggested that the mechanisms of plasticity and maintenance of pEMT correlate to the COX-2 inflammatory activity (Lu. et al., 2020). However, this relationship has not been elucidated. COX-2 expression is related to the intracellular pathways activated by phosphatidylinositol 3-kinase (PI3K)/Akt, Wnt/β-catenin, and NF-κB that are associated with tumor progression. The above suggested that COX-2 regulates E-cadherin expression indirectly by encouraging NF-κB nuclear translocation, which induces the downregulation of E-cadherin gene and the expression of EMT transcription factors. The overexpression of the COX-2/PGE2 axis in cells within the TME promotes EMT in tumor cells through PGE2/EP4 paracrine signaling (Figure 5). The COX-2/PGE2 axis overexpression favors the EMT phenomenon in tumor cells because of the inhibition of E-cadherin expression. The COX-2/PGE2 overexpression promotes signaling related to ROS and hypoxia, which explains the inflammatory profile of the TME in solid tumors. (Gómez-Valenzuela et al., 2021)

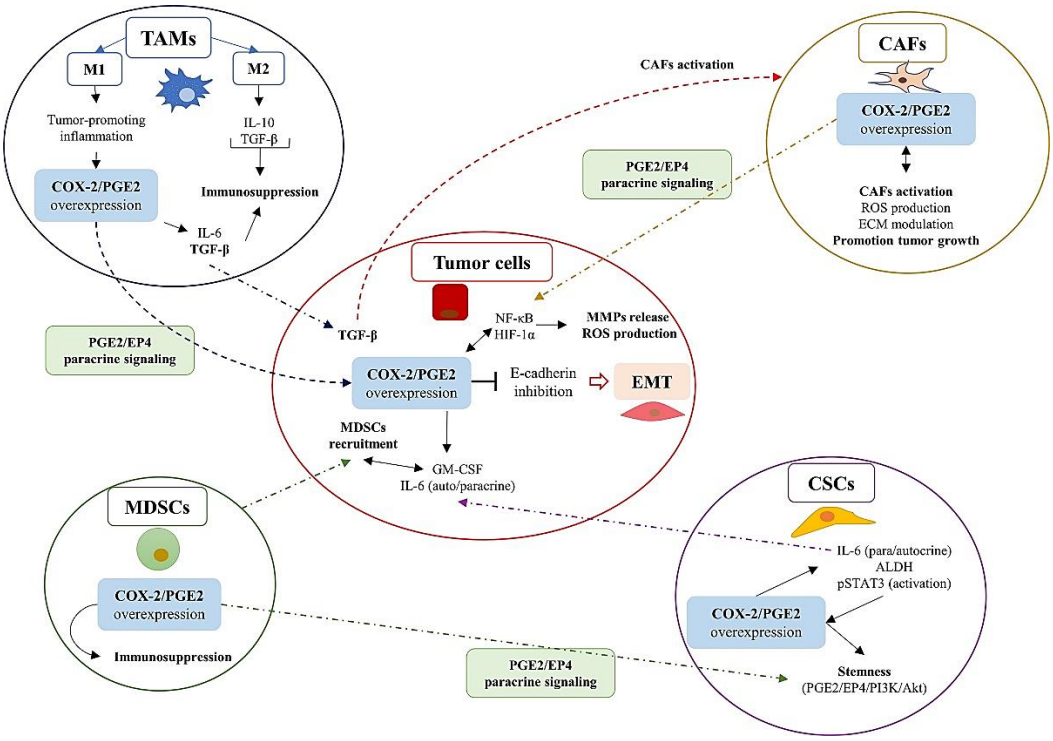


Figure 5. The overexpression of the COX-2/PGE2 axis in cells within the tumor microenvironment (TME) promotes EMT in tumor cells through PGE2/EP4 paracrine signaling (Gómez-Valenzuela et al., 2021)

4.5 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and selective COX-2 inhibitors in GBM

NSAIDs are mostly used for the treatment of patients suffering from pain and inflammatory conditions such as chronic pain, osteoarthritis, rheumatoid arthritis, postoperative surgical conditions, and even used extensively as analgesics and anti-pyretics (NSAIDs are nonselective COX inhibitors, such as aspirin, ibuprofen, and naproxen, and most commonly used selective COX-2 inhibitors (COXIBs) include celecoxib, rofecoxib, valdecoxib, and NS398. COX-2 inhibitors are emerging as therapeutic agents for glioma, including cancer stem cells, it is important to study drug combinations that could improve their efficacy under both hypoxia and normoxia (Jalota, et al., 2018). The NS398 and celecoxib, have been investigated in various cancer models, including drug resistant cancer cells, as potential cancer treatment (Lim et al., 2016; Sui et al., 2011; Cao et al., 2015). The selective COX-2 inhibitor rofecoxib, in combination with low dose of TMZ, has been proposed as an antiangiogenic strategy to treat GBM (Tuettenberg et al., 2005).

4.6 Combination of COX-2 inhibitors with Temozolomide in GBM

In recent years, the reports on using nonsteroidal anti-inflammatory drugs (NSAIDs) for cancer treatment have been on the rise. However, the mechanism of NSAIDs on preventing cancer is still not fully clear. Upregulated expression of COX-2 is associated with human tumorigenesis and tumor progression and COX-2 inhibitors show antitumor activity in different human cancers, including GBM. The use of selective COX-2 inhibitor drugs is considered by several groups as a usefulness adjunct

treatment, also owing to their ability to increase GBM sensitiveness to traditional chemotherapy and radiotherapy (Bijnsdorp et al., 2007; Kuipers et al., 2007; Qiu et al., 2017; Li et al., 2020; Suzuki et al., 2013; Wickström et al., 2015). In the study of combination therapy of COX-2 inhibitors with other antitumor drugs, the most used COX-2 inhibitor was celecoxib that, compared to other NSAIDs, presents a lower rate of gastrointestinal and renal adverse events (Li et al., 2020). Epidemiological studies and clinical trials have shown that long-term use of COX-2 inhibitors, such as celecoxib and rofecoxib, seem to reduce the incidence of several human cancers such as breast, lung, prostate, oesophageal, liver, pancreatic, gastric, and colon cancers (Harris 2009; Li et al., 2020; Nagaraju and Rayes 2019). Moreover, the drug combination therapy with alkylating agents combined with COX-2 inhibitors is able to reduce these side effects while improving antitumor activity. Wu et al. reported that COX-2 inhibition with indomethacin, celecoxib, or siRNA against COX-2, enhanced the cytotoxic effect of TMZ on glioma stem cells isolated from primary GBM and also, the COX-2 inhibition by indomethacin, combined with TMZ, significantly enhanced its cytotoxic effect in a mouse GBM orthotopic xenograft improving the overall survival displayed through the Kaplan-Meier curve (Wu et al., 2017). Tuettenberg et al., reported that the combined therapy approach based on continuous low dose TMZ in combination with the COX-2 inhibitor, rofecoxib, displayed anti-angiogenic, anti-tumor activity, and increased survival in GBM patients. No impairment of quality of life nor toxic side effects was registered during the follow up (Tuettenberg et al., 2005). Kerschbaumer et al., assessed a combined therapy with TMZ and the selective COX-2 inhibitor, celecoxib, on the functional outcome and quality of life in glioma patients who were not eligible for standard therapeutic approach for their poor health status, with a postoperative Karnofsky performance score (KPS) below 70, or old age. The patients did not present any side effects and exhibited a prolonged survival and an improvement of life quality suggesting the effectiveness of the drug combination (Kerschbaumer et al., 2015). Additional evaluation of mechanisms of action of drug combinations could also be warranted for selection of suitable drugs to combine with the COX-2 inhibitors (Li et al., 2020), Skaga et al. explored the efficacy of the combination of TMZ with CUSP9, a new treatment approach combining nine well-known drugs approved for non-oncological indications (aprepitant, auranofin, captopril, celecoxib, disulfiram, itraconazole, minocycline, ritonavir, and sertraline), in 15 patient-derived glioblastoma stem cells (GSCs). *In vitro*, the CUSP9 treatment in combination with TMZ eradicated the tumorspheres generation and abrogated the clonogenic potential of GBM primary cultures better than TMZ alone mainly acting via inhibition of the WNT pathway (Skaga et al., 2019). In Figure 6 are reported the main molecular targets of COX-2 inhibitors combined with antineoplastic drugs.

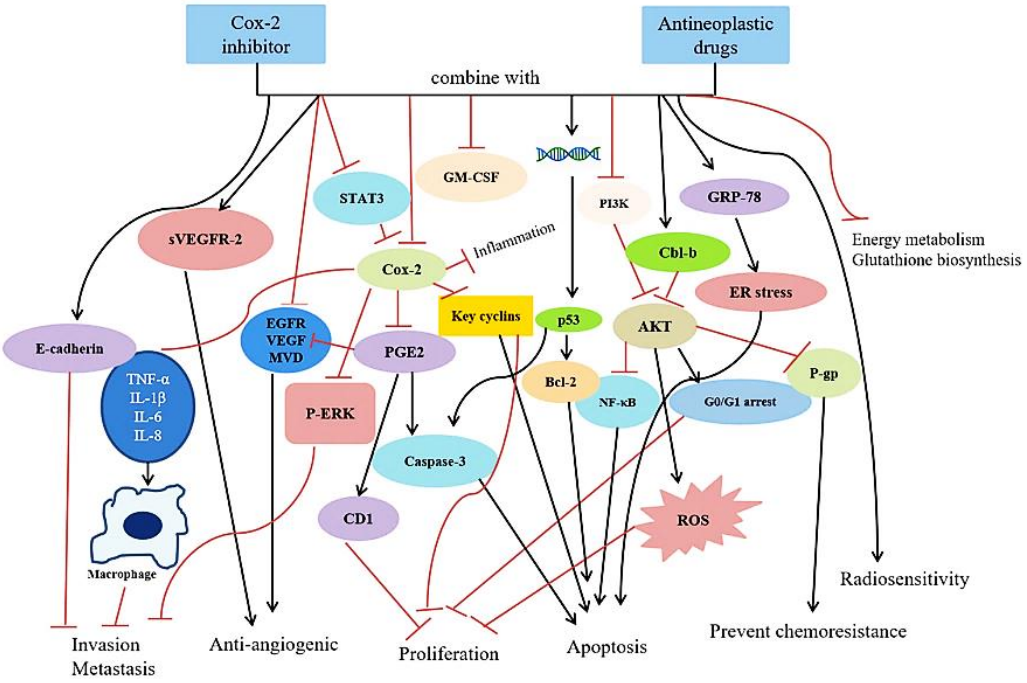


Figure 6. Illustration of molecular targets of COX-2 inhibitors and other agents (Li et al., 2020)

4.7 Molecular Targets of NS398: Mechanism of action

NS398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulphonamide (Figure 7), is a sulphonamide derivative that inhibits COX-2 specifically, with an IC_{50} of 30 nm without affecting COX-1 activity at concentrations exceeding 100 mM (> 50-fold potency for COX-2 over COX-1) (Futaki et al., 1994; Bacchi et al., 2012). The NS398 has been investigated as chemopreventive and potentially chemotherapeutic agent in several studies (Sun et al., 2006; Yao et al., 2004; Huang et al., 2005; Joki et al., 2000; Baek et al., 2007), but the precise molecular mechanism underlying these antitumor effects remain unclear. Although, the suggested mechanisms by which COX-2 inhibitors (by itself or in combination with chemo- or radiotherapy) exert their antitumor effects include inhibition of cell proliferation, inhibition of angiogenesis, apoptosis induction and inhibition of DNA repair (Kurihara et al., 2009; Bae et al., 2001; Tsujii et al., 1998; Lee et al., 2007; Kuipers et al., 2007). Yao et al., reported that NS398 (100 mM), decreased colorectal cancer cell proliferation by 35% respect to control cell, which was associated by a decreased cyclin D1 and proliferating cell nuclear antigen PCNA index, both *in vitro* and *in vivo*. Moreover, Yao and colleagues found that NS398 inhibited the capacity of cell invasion and migration through the extracellular matrix by inactivating the activities of MMP-2 and MMP-9 and suggested that metalloproteinase inhibition could account for one of the molecular mechanisms implicated in mediating the anti-invasive properties of NS398 (Yao et al., 2004). Zhang et al., in the attempt to explore the mechanism by which COX-2 promotes the metastasis

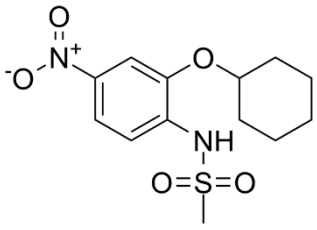


Figure 7. Chemical structure of NS398

in MG-63 osteosarcoma cells through the PI3K/AKT/NF-κB pathway, showed that NS398 increased E-cadherin levels, while the expression levels of vimentin, MMP-2, MMP-9, VEGF, p-PI3K, p-AKT, and p-IKK were significantly decreased. Along with other epidemiological and experimental studies that have demonstrated the antitumor properties of non-steroidal anti-inflammatory drugs (NSAIDS) in human cancers of the gastroenterological tract (Elder et al., 2000; Farrow et al., 1998). The work of Yu HP et al., indicated that treatment of human esophageal squamous cell carcinoma cells with NS398 significantly suppressed cell growth, promoted apoptosis, and decreased PGE2 production in a dose-dependent manner. Subsequent investigation revealed that NS-398 may significantly increase caspase-3 activity, and that the caspase-specific inhibitor AcDEVD-CHO could counteract this rise. Yu et al., suggested that NS398 could inhibit the proliferation and induce apoptosis in human ESCC cells by COX-2 dependant and COX-2 independent mechanisms. The cell cycle arrest and apoptosis appear to play a critical role in the chemopreventive and potentially chemotherapeutic effect of NS398. Joki et al., demonstrated the anti-proliferative effect of NS398 on GBM cell lines (U87MG and U251MG) and relative neurospheres (Joki et al., 2000). In a dose-dependent way, NS398 inhibited cell proliferation, spheroid formation, and tumor cell migration. There was also a moderate increase in the number of apoptotic cells in the treated spheroids but NS398 did not inhibit invasion of glioma cells (Joki et al., 2000). Jalota group investigated the effects of the combination NS398 with the anticancer drug BCNU in GBM cells under hupoxia and normoxia. In both conditions, the combination of BCNU and NS-398 decreased cell viability and accelerated caspase-dependent death in glioma cells, associated with changes in pro and anti-apoptotic markers (Jalota et al., 2018). In another study (Matsuo et al., 2004), NS398 decreased the anchorage-dependent and -independent growth of glioma cell lines independently of PHS-2 expression, indicating that certain PHS-2-independent mechanisms underlie NS398's antitumor effects. Nevertheless, the cytotoxic activity was decreased by the addition of PGE2, which is one of the primary products of PHS, indicating that PHS is the predominant mechanism.

5. BRIEF DESCRIPTION OF OUR PREVIOUS DATA

The results obtained in the three-year period of the doctorate are subsequent with those obtained in Professor Cifone's laboratory on the study of the role of COX-2 in the resistance of human GBMs.

Previous data referred in the article of Palumbo P. et al., (2020), evaluated the role of COX-2 inhibition by NS398, a highly selective COX-2 inhibitor, on human U87MG and T98G cell lines cultured as neurospheres. The neurospheres colture is commonly considered an appropriate model that recapitulate the GBM heterogeneity since is able to mimic some histological features of human gliomas, such as their multicellular organization, the hypoxic core, and gradient distributions of oxygen, pH, and metabolic wastes.

Initially, the ability of U87MG and T98G cells to generate neurospheres when cultured in a specific medium was verified. Both cell lines were able to generate *in vitro* neurospheres at different time points.

In particular, the U87MG cell line started to organize in neurospheres after 8-10 days, T98G after 12-15 days. Neurospheres appeared as cellular aggregates with a spherical shape floating in the culture medium (Figure 15).

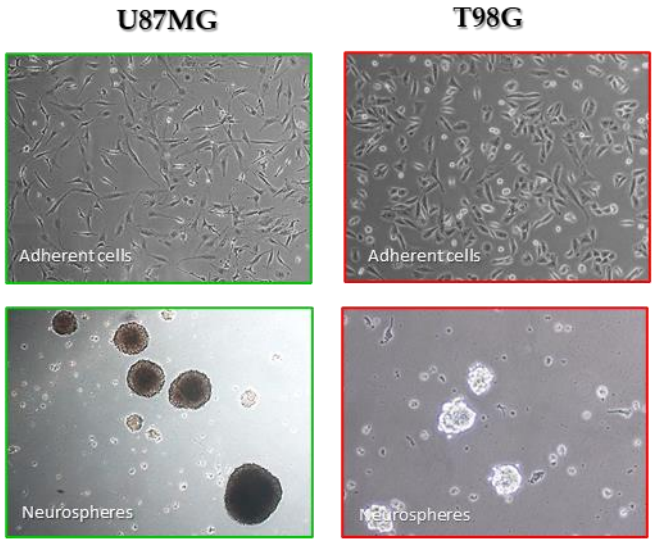


Figure 15. GBM cell lines U87MG and T98G, cultured in standard culture medium (adherent cells) and in DMEM/F12 medium serum free with EGF, b-FGF and B27 supplement for neurosphere growth. Representative images from the analysed glioma cell lines are shown (Original magnification 10×).

In addition, the phenotype of neurospheres derived from U87MG and T98G cells was evaluated by flow cytometric analysis of the main stemness markers, β-tubulin III, nestin, and SOX-2, and of the glial cell marker, GFAP. All these markers were also analyzed in relative adherent cells to highlight the differences with respect to neurosphere-forming cells (Table 3). In accordance with previous reports (Palumbo et al., 2017; Ludwig and Kornblum 2017), the percentages of positive cells as well as the Median Fluorescence Intensity (MFI) for β-tubulin III, SOX-2, and nestin were significantly higher (>80%) in U87MG neurospheres. The percentage of positive cells in U87MG adherent cells never exceeded 55% for β-tubulin III and SOX-2, and the MFI values were overall lower than those of the respective neurospheres. Instead, the percentage of positive cells for nestin was comparable between adherent cells and neurospheres and was between 92% and 96%, but the MFI value of adherent cells was about half that detected in relative neurospheres (Table 3). The percentages of T98G positive cells for β-tubulin III, SOX-2, and nestin were not different between neurosphere-forming and adherent cells; however, the MFI values in neurospheres were significantly higher when compared to adherent cells (Table 3). As expected, the MFI value of GFAP, marker of glial differentiation seemed significantly down-modulated in neurospheres, although a comparable percentage of positive cells among adherent and neurosphere was shown only in U87MG, but not in T98G cells.

		U87MG adherent cell	U87MG neurospheres	P value
β-tubulin	% positive cells	51.30 ± 4.21	89.47 ± 7.23	*
	Mean value	45.21 ± 3.78	210.60 ± 10.30	**
Sox-2	% positive cells	50.57 ± 3.02	95.41 ± 9.21	*
	Mean value	36.57 ± 2.47	129.77± 20.82	*
Nestin	% positive cells	92.41 ± 6.84	96.31 ± 4.39	ns
	Mean value	249.38 ± 15.86	511.61 ± 34.90	*
GFAP	% positive cells	69.91 ± 2.38	59.71 ± 3.40	ns
	Mean value	139.14 ± 11.70	60.69 ± 5.11	*

		T98G adherent cell	T98G neurospheres	P value
β-tubulin	% positive cells	80.52 ± 4.34	89.88 ± 5.73	ns
	Mean value	117.51 ± 9.87	189.4 ± 13.85	*
Sox-2	% positive cells	86.84 ± 6.4	97.63 ± 7.7	ns
	Mean value	44.58 ± 36.84	90.03 ± 83.02	*
Nestin	% positive cells	89.4 ± 6.96	96.64 ± 4.8	ns
	Mean value	99.58 ± 7.43	411.38 ± 36.11	***
GFAP	% positive cells	52.96 ± 4.01	4.11 ± 0.98	*
	Mean value	58.88 ± 1.99	28 ± 1.45	*

*Table 3: Percentage (%) and values of Median Fluorescence Intensity (MFI) of β-tubulin III, SOX-2, nestin and GFAP in U87MG and T98G respectively, maintained in standard culture conditions (adherent cells) and in DMEM/F12 medium serum free with EGF, FGF–β and B27 supplement for neurosphere generation. Data are from one representative out of three independent experiments in duplicates ± SD. For comparison between two means, Student's unpaired t-test was used (*p < 0.05, **p < 0.01, ***p < 0.001).*

The choose of these GBM cell lines (U87MG and T98G) was made on their different genetic profiles and COX-2 expression level. Considering the crucial role of COX-2 in the GBM stemness, aggressiveness, and chemoresistance, the basal levels of COX-2 were evaluated in GBM cell line-derived neurospheres by western blot. Results in Figure 16 A showed a different basal COX-2 expression in U87MG and T98G neurospheres, as already demonstrated (Ponten et al., 2008).

To evaluate the role of COX-2 in GBM neurospheres, the specific COX-2 activity inhibitor, NS398, was used in neurosphere cultures and the measurement of PGE2 levels was evaluated following NS398 treatment at several concentrations (10, 100, and 200 μM) for 48 h. The treatment with NS398 at 100 and 200 μM significantly reduced the PGE2 levels in the supernatants of the U87MG-derived neurospheres (Figure 16 B). In particular, the effect was dose-dependent, being much more intense at higher concentrations of NS398 ≥ 100 μM ($p < 0.0001$ vs CNTR) respect compared to 10 μM ($p < 0.05$ vs CNTR) (Figure 17). In T98G-neurospheres, which basically released considerably lower amounts of PGE2, the addition of COX-2 inhibitor markedly lowered PGE2 at 100 μM ($p < 0.01$ vs CNTR) and at 200 μM ($p < 0.05$ vs CNTR) (Figure 16 B). The different COX-2 expression detected in the U87MG-

and T98G-neurospheres could justify the different PGE2 levels as well as the different effectiveness of the NS398 inhibitor. The control cells were treated with drug vehicle DMSO (0.6% v/v) and indicated for all experiments as CNTR.

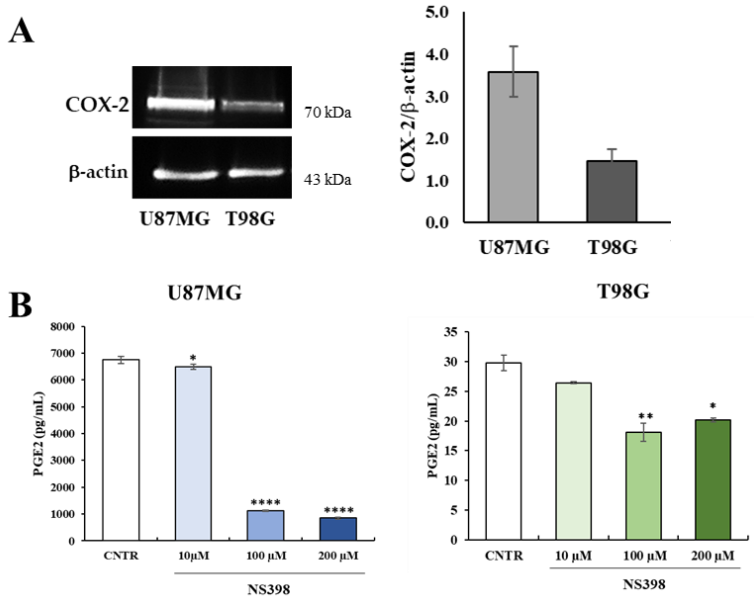


*Figure 16. Basal expression of COX-2 detected in GBM neurospheres derived from U87MG, T98G and U251MG. A representative western blotting image of COX-2 and proteins in untreated (control, CNTR) neurospheres. Densitometric analysis was performed by normalizing vs. β-actin and presented as COX-2/β-actin. Data from three independent experiments are expressed as mean ± SEM. (B) PGE2 levels were detected in supernatants of U87MG- and T98G-derived neurospheres after 48 h culture in the presence of NS398 at indicated concentrations. CNTR: control, drug vehicle alone. The results are relative to one representative out of two experiments performed in triplicate, and are expressed as mean ± SD. For comparative analysis of groups of data, one-way ANOVA followed by Dunnett post hoc test was used (*p < 0.05, **p < 0.01, ****p < 0.0001 vs CNTR).*

The NS398 inhibitor at the concentrations of 10, 100, and 200 μM did not significantly modify the number of viable neither ofor dead cells derived of from U87MG- and T98G-derived neurospheres (Figure 17 A and B, respectively). Neurosphere-derived cell viability was also evaluated by flow cytometric analysis with PI staining, and the percentage of viable and dead cells is reported in the histograms in Figure 17 C and D with results similar to Trypan blue test. DMSO used to dissolve NS398 at 0.6% (v/v) final concentration had no effect has not any effect on its own.

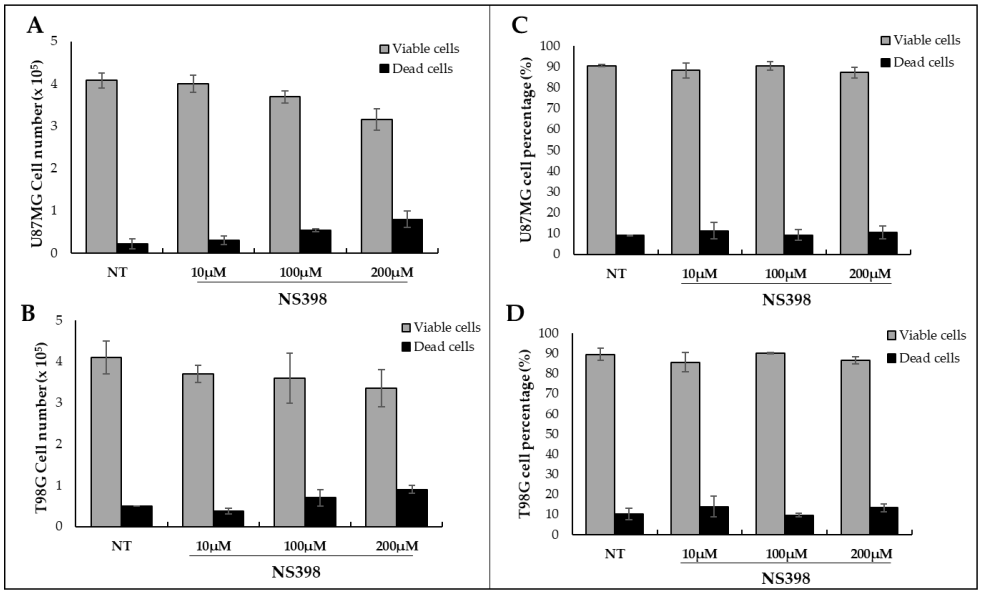


Figure 17. Concentration-dependent response of NS398, COX-2 selective inhibitor, on viability of human glioblastoma (GBM) cells. U87MG and T98G neurospheres were treated with NS398 at 10 μM, 100 μM, 200 μM, or DMSO (NT) for 48 h, and number of viable and dead cells (A and B, respectively) was assessed by Trypan blue exclusion test and by propidium iodide (PI) staining (C and D, respectively). Results are presented as the mean ± SEM of three independent experiments in duplicate. For comparative analysis of groups of data, two-way ANOVA followed by Bonferroni post hoc test was used ($p<0.05$ vs NT).*

It has been verified that neurospheres generated from both GBM cell lines were highly influenced by NS398 treatment, a reduced area (Figure 18 A, B), morphological changes (Figure 18 C, D) and a high level of autophagy (Figure 19). In particular, NS398-treated neurospheres from both GBM cell lines resulted smaller than control ones. This result was confirmed also with Scanning Electon Microscopy images at low magnification clearly underlined a reduction of spheres' size in NS398-treated cells when compared to untreated ones. Moreover, all NS398-treated neurospheres had an altered morphology, were smaller than control spheres, and were not well structured, with a clear cell disaggregation and with an extremely irregular outline. The membranes of treated neurospheres appeared much rougher compared to untreated spheres representing an abundant membrane activity due to the presence of rich vesicle-like membrane protrusions (Figure 18 C, D).

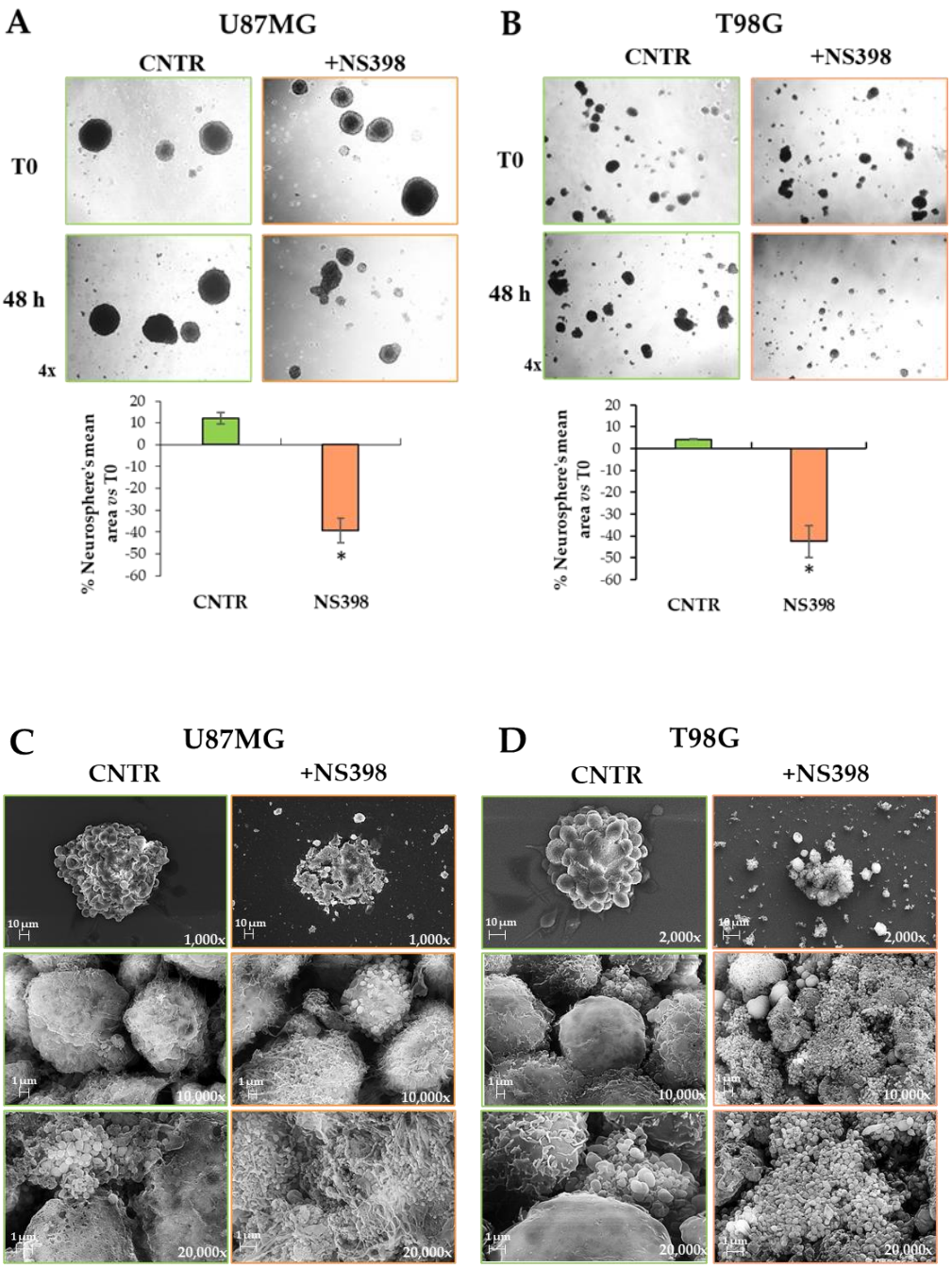


*Figure 18. Representative phase-contrast images (4× magnification) of (A) U87MG and (B) T98G neurospheres in the absence (CNTR) or presence of COX-2 activity inhibitor NS398 (100 μM for 48 h). The quantitative analysis of neurospheres' mean area was expressed as percentage vs relative T0. The results are representative of two independent experiments and are expressed as mean values of duplicates ± SEM. For comparison between two means, Student's unpaired t-test was used (*p < 0.05). NS398 affects the neurospheres' morphology. Representative SEM images of (C) U87MG- and (D) T98G-derived neurospheres (A and B, respectively) after 48 h treatment with vehicle alone (CNTR) or NS398 at a concentration of 100 μM.*

It has been also verified that COX-2 inhibition induced the autophagy pathway in GBM stem cells detected by fluorescent staining of acidic vesicular organelles (AVOs) and by analysis of main autophagic proteins. Autophagy is a catabolic process that facilitates the recycling of cellular constituents in response to stressing conditions, such as nutrient deprivation or infection. Autophagy occurs at basal levels to perform homeostatic functions and is rapidly induced in metabolic stress (Guo

and White 2016; Yang, et al., 2019). Autophagy plays a double-sided role in cancer, working as both a guardian and a cancer cell killer, based on the different stages of the cancer and the surrounding environment (Figure 9). This paradoxical behaviour has been proven by the fact that autophagy blockade can greatly increase the susceptibility of glioma cells to cytostatic treatments, at the same time a genetically or pharmacologically induced amplification of autophagy is associated with higher tumor removal *in vivo* and *in vitro*. Even with the awareness of its "double face", autophagy induction has been proposed as another potential anti-tumoral mechanism to counteract several cancers, including GBM (Trejo-Solís et al., 2018).

The exposure to NS398 (100 μM for 48 h) induced an evident amount of AVOs in the cytoplasm, as clearly shown by the bright orange/red acidic compartments, suggesting the ability of COX-2 inhibitor to induce autophagy (Figure 19 A and C). To further prove the ability of NS398 to trigger the autophagic process, the fluorescent signal of AVOs was measured by FACS analysis (Figure 19 A and 19 C), and the main autophagy-related proteins (LC3B and Beclin-1) were evaluated by western blotting (Figure 19 B and D).

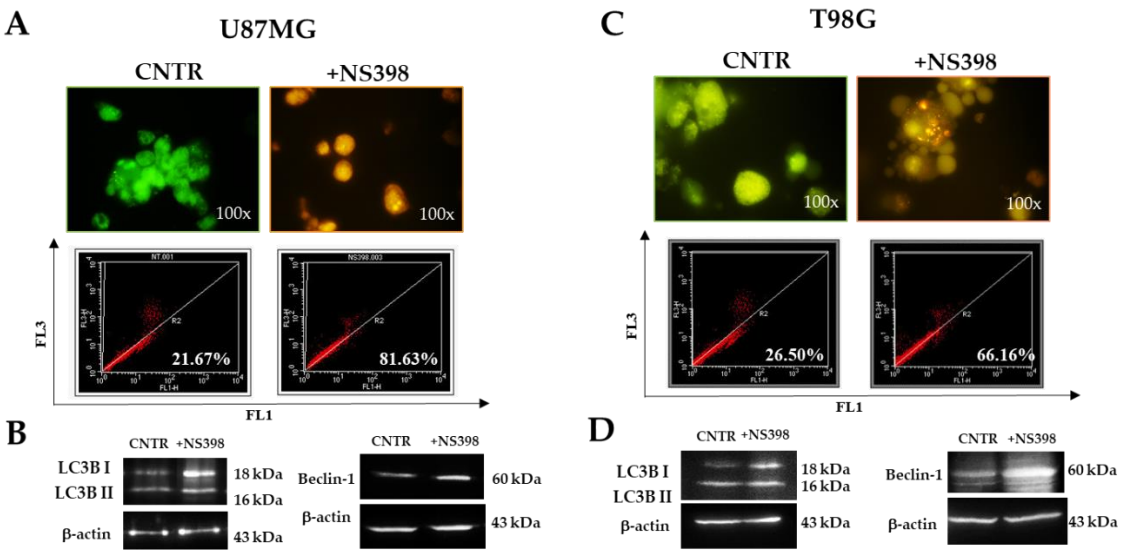


Figure 19. NS398 induces autophagy in GBM neurospheres. Fluorescence representative images of Acridine Orange-stained (A) U87MG- and (C) T98G-derived neurospheres treated in presence or absence (CNTR) of NS398 at the concentration of 100 μM for 48 h. Under AO staining, the cytoplasm and nucleolus emit green fluorescence, whereas the acidic compartments fluoresce bright orange/red. The orange/red fluorescence indicates acidic vesicular organelles (AVOs), hallmark of autophagic process. The control cells were exposed to DMSO alone (CNTR). Flow cytometric analysis of red and green fluorescence of AVOs in U87MG- and T98G-derived neurospheres treated or not (CNTR) with NS398 is shown. Representative western blots of autophagy markers, LC3B I/II and Beclin-1, in U87MG- and T98G-neurospherse (B and D, respectively). The results are representative of three independent experiments.

Another crucial data presented in the work of Palumbo et al. (2020), was that COX-2 inhibitor modulated extracellular Vesicles (EV) shedding from U87MG and T98G neurospheres affecting adherent cells behaviour. EVs are nanometer particles released from all cells, that play a role in regulating cell-cell

communication. In cancer biology, the role of EV has been the subject of great study, it has been shown to directly influence tumor development in several tumors including gliomas. In recent years, accumulating evidence highlights the EVs role as mediators in stress response of cancer cells, and therefore at the base of the mechanisms of progression, metastasis, and resistance to therapy. Furthermore EVs, released into the circulation, may provide a circulating biomarker predictive of response to therapy and indicative of microenvironmental conditions related to disease progression, such as hypoxia (O'Neill et al., 2019). EVs can be responsible for molecules transfer, from one cell to another, such as enzymes, ligands, receptors, DNA, complementary DNA (cDNA), mRNA, microRNA (miR), among others (Balaj et al., 2011). A number of these molecules are active when they reach the receiving cell, therefore, EVs regulate various processes, many of which are correlated with cancer progression and resistance to therapy (Quezada et al., 2018). The involvement of EVs in GBM progression involves several aspects: cell viability and resistance to treatment, immune response, angiogenesis, migration and invasion (Yekula et al., 2020).

After exposure of neurospheres to NS398 at 100 µM for 48 h, the EV fraction secreted in supernatants was collected and characterized by transmission electron microscopy (TEM), showing a continuous bilayer membrane with a typical rounded morphology similar to EV released by DMSO-treated neurospheres (CNTR) (Figure 20 A and 22 B). To further characterize the EV from GBM neurospheres, the expression of tetraspanins specific EV markers, CD63 and CD81 (Théry et al., 2018), was assessed. The levels of CD63 and CD81 from CNTR and NS398-treated samples were similar to each other,'s further confirming the nature of isolated EV and that the NS398 did not modify the EV structure (Figure 20 C and D).

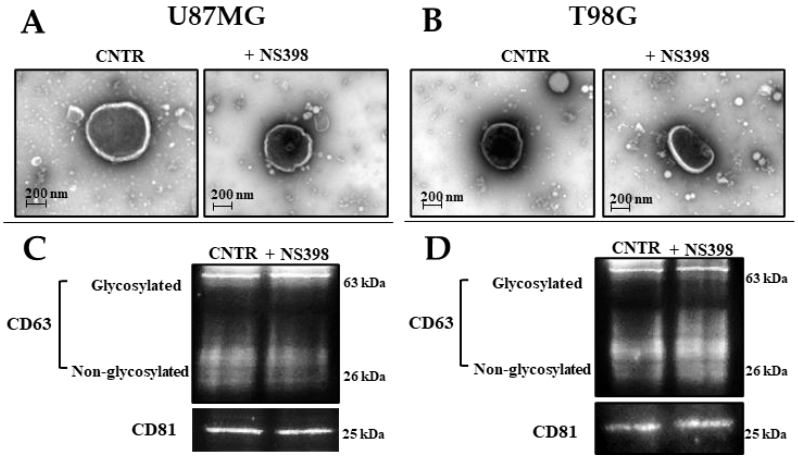


Figure 20. Characterization of EV released from U87MG- and T98G-neurospheres' cultures. Representative TEM images of EV from (A) U87MG- and (B) T98G-derived neurospheres cultured in the absence (CNTR) or presence of NS398 (100 µM). Images were acquired at magnification of 50,000× (scale bar 200 nm). Expression of EV specific markers, CD63 and CD81, in EV recovered from (C) U87MG- and (D) T98G-derived neurospheres exposed to NS398 (100 µM, 48 h) or not (CNTR) was detected by Western blotting technique.

Before verifying the ability of EV derived from GBM neurospheres to affect the GBM adherent cells biology, the PKH26 fluorescent staining was performed to detect the intracellular uptake of EV into

GBM cells (Figure 21). Adherent GBM cells were exposed to EV released by neurospheres previously cultured with NS398 at 100 µM for 48 h or with a DMSO-drug vehicle (CNTR). Images from the microscopic analysis showed that PKH26-labeled EV were effectively internalized by adherent GBM cell lines. The NS398 treatment did not influence the EV uptake in adherent recipient U87MG and T98G cells, resulting comparable in all samples (Figure 21).

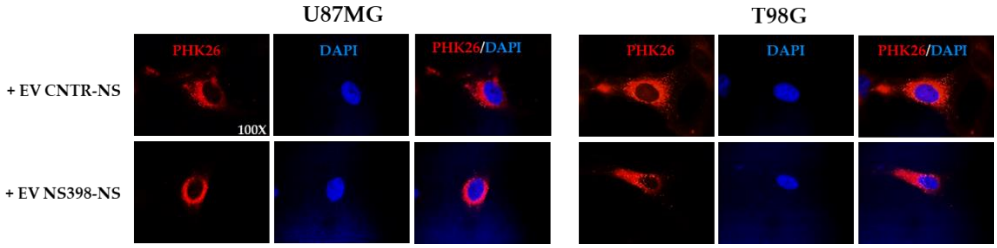


Figure 21. Internalization of EV derived from control (CNTR) and NS398-treated neurospheres on adherent U87MG and T98G. The EV uptake by recipient cells was evaluated by PKH67 dye (red signal). Adherent U87MG and T98G cells were cultured with EV obtained from relative neurosphere cultures untreated (+EV CNTR-NS) or treated with NS398 (+EV NS398-NS). DAPI staining (blue signal) was used to counterstain cell nucleus. Merged images for PHK67 and DAPI are also shown. Representative images from two independent experiments are shown.

The EV secreted by NS398-treated neurospheres induced high levels of AVO's accumulation detected by a diffuse orange/red fluorescence in both recipient adherent cell lines (Figure 22 A and B). On the other hand, the addition of EV released by DMSO-treated neurospheres had no effect in terms of AVOs formation. In Figure 22 C and D, the results of experiments aimed at evaluating the effects of NS398 as a single agent on autophagy induction are shown. The direct addition of COX-2 inhibitor led to an increase of AVOs amount in both GBM cell lines, which was similar to what observed after the addition of EV released by NS398-treated neurospheres.

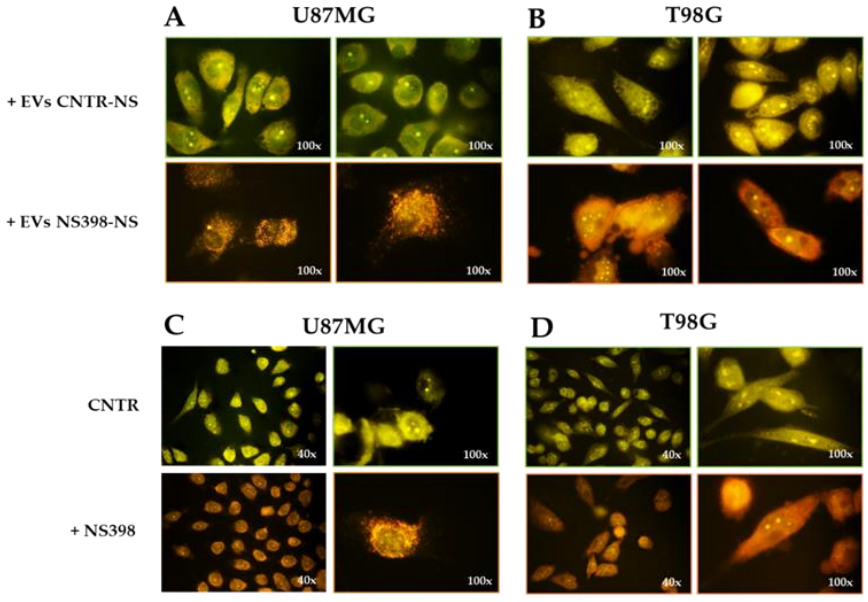
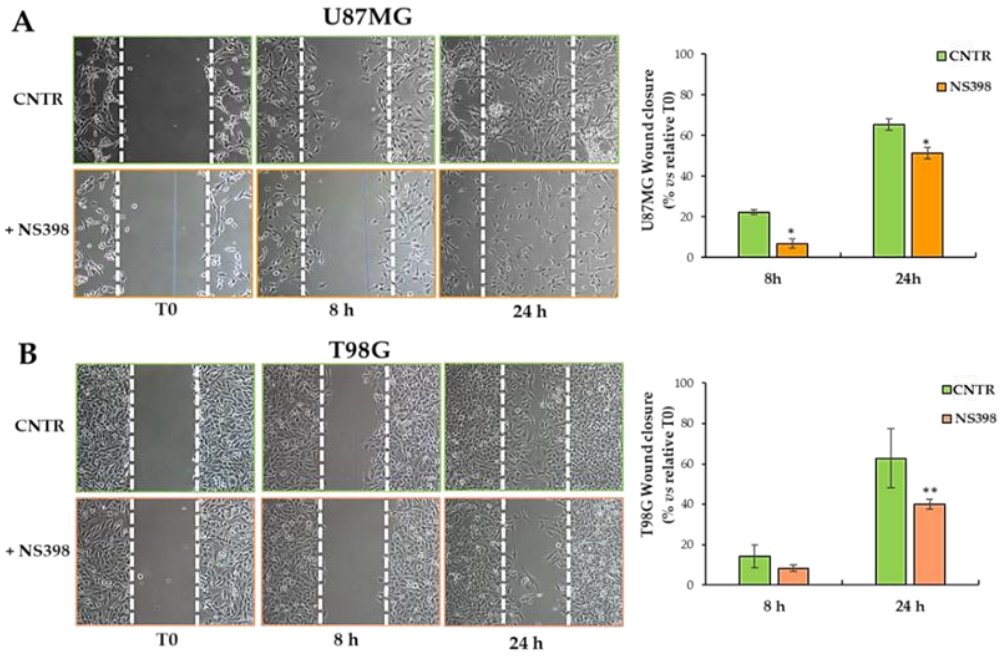


Figure 22. Evaluation of indirect and direct induction of autophagy process by NS398. (A) U87MG- and (B) T98G-treated with EV obtained from control (+EV CNTR-NS) and NS398-treated neurospheres (+EV NS398-NS). (C) U87MG- and (D)

T98G-directly exposed to NS398. Two images for each condition are shown and are representative of two independent experiments. CNTR = DMSO-treated cells

Moreover, NS398 inhibitor and EV released by NS398-treated neurospheres hampered the migration ability of adherent GBM cell lines (Figure 23). The scratched monolayers of CNTR cells were completely repaired after about 25–28 h. On the contrary, NS398 treatment drastically hindered the U87MG monolayer repair both at 8 h and 24 h (Figure 23 A). Similarly, NS398 treatment negatively influenced the wound closure rate of T98G cells being the effects statistically relevant just at 24 h (Figure 23 B). Under these experimental conditions, while cell viability was not affected by NS398 treatment, the proliferation rate of both cell lines decreased compared to control cells, although to a different trend. The U87MG cell growth was significantly influenced by NS398, with a reduced rate of 10–12% and 25–30% at 8 and 24 h, respectively, while the number of T98G cells treated with NS398 was 5–7% and 10–15% lower than control cultures at 8 and 24 h, respectively.

The ability of the EV secreted from CNTR and NS398-treated neurospheres to influence GBM adherent cell migration was also evaluated. The addition of EV from NS398-treated neurospheres significantly delayed the re-epithelialization of both U87MG and T98G scratched monolayers with respect to the relative T0 (Figure 23 C and D). These results suggested that GBM cell lines exposed to EV purified from NS398-treated neurospheres media for 48 h showed a delay in re-epithelizationhad a reduced mobility with respect to cells exposed to EV from CNTR neurospheres.



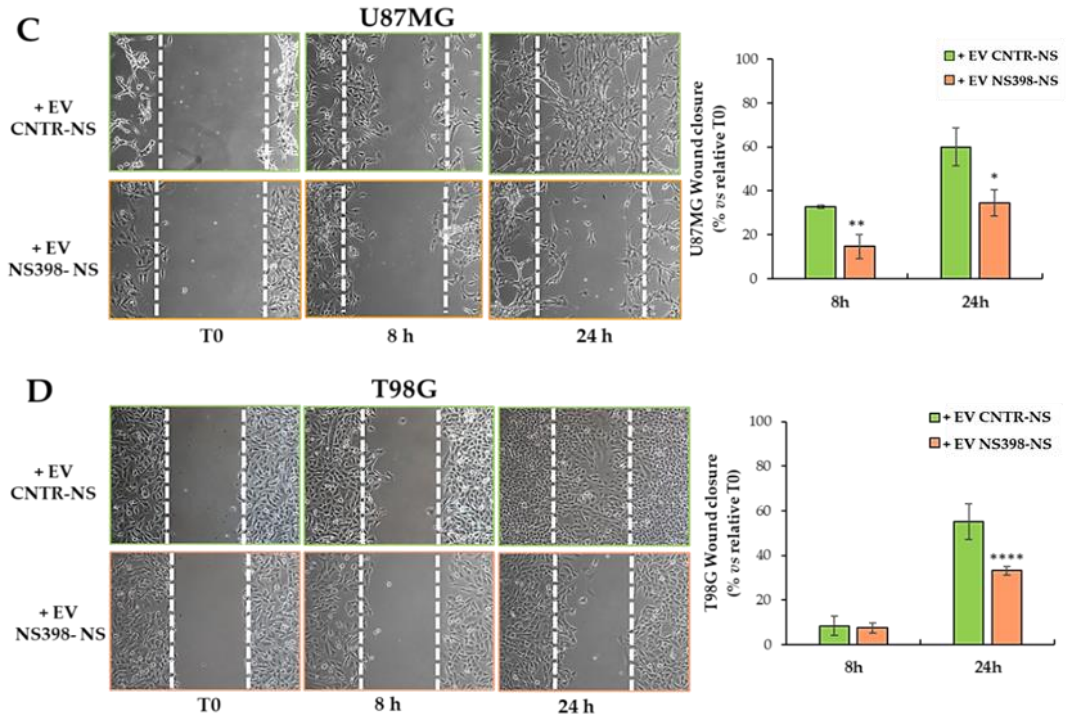


*Figure 23. NS398 inhibited migratory ability of GBM cell lines evaluated by wound healing assay. Representative phase-contrast images and relative quantification of scratch assays performed in (A) U87MG and (B) T98G cell lines exposed to NS398 or with drug vehicle DMSO (CNTR), immediately after the scratch at the initial time (T0) and after 8 h and 24 h. Representative phase-contrast micrographs and quantification of scratch assays performed in (C) U87MG and (D) T98G GBM cell lines after incubation with EV derived from control (CNTR) neurospheres (+EV CNTR-NS) or NS398-treated neurospheres (+EV NS398-NS). Original magnification 10x. The results are expressed as percentage of wound closure vs relative T0 (mean ± SD) and are representative of two independent experiments performed in duplicate. For comparative analysis of groups of data, two-way ANOVA followed by Bonferroni post hoc test was used (*p < 0.05, **p < 0.01, ****p < 0.0001).*

6. AIMS OF THE EXPERIMENTAL WORK

Although the complex biomolecular context that defines GBM malignancy is not yet fully understood, several studies are focused at identifying the mechanisms responsible for GBM progression, therapy resistance, and high recurrence rate to identify new potential targets for anti-GBM therapy.

The connection between inflammation and cancer is a well-established concept, and now it is considered to be one of the hallmarks of cancer. Gliomas, like others malignant tumors, are characterized by a moderate inflammatory microenvironment and the inflammatory process seems to be involved in all the steps of tumorigenesis, promoting the genomic instability, proliferation, and survival of malignant cells, as well as angiogenesis, resistance to therapy, local or systemic immunosuppression, and also raising the metastatic process. Aberrant cyclooxygenase 2 (COX-2) expression and its enzymatic product Prostaglandin 2 (PGE2), which play a crucial role in the pathophysiology of several inflammatory disorders, have been implicated in the development, growth, and progression of several human malignant tumors, including GBM.

Based on our previous experimental data and having in mind to continue to explore the involvement of COX-2 in the chemoresistance of human GBM, we wanted to investigate if TMZ, the main drug used for GBM treatment, was able to modulate the expression and activity of COX-2.

To this purpose, we evaluated:

- The ability of TMZ to influence COX-2 expression and activity, and MGMT status in two GBM cell lines, T98G and U251MG, showing different sensitivity to TMZ and different COX-2 expression.
- The involvement of the Wnt/β-catenin pathway in GBM chemoresistance and stemness potential by dectection of β-catenin, MGMT, and SOX-2 expression levels.
- The ability of NS398 to overcome the TMZ resistance of T98G, investigating the relationships between COX-2/PGE2 system, β-catenin, MGMT, and stemness potential.

The drug combination effect of NS398 and TMZ was also evaluated on the COX-2 negative U251MG cell line at the same experimental conditions to understand whether the mechanism behind the NS398 effects could be directly dependent by COX-2.

7. MATERIALS AND METHODS

7.1 GBM cell lines and culture conditions

Human GBM grade IV cell lines T98G were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and U251MG were acquired from Cell Lines Service (CLS), respectively. All cell lines were manipulated according to the instructions given in the product sheet to maintain a stable genetic profile. These cell lines were chosen as established GBM *in vitro* model for their different sensitivity to TMZ due to the different TP53 mutational status as well as the expression of the MGMT repair enzyme. The T98G cells, expressing high levels of MGMT, p53 and PTEN mutated, and p14ARF/p16 deleted are known to be TMZ-resistant cells, as extensively reported (Lee 2016; Montaldi et al., 2015). Also, T98G cells are defined "TMZ-resistant", showing a LC50 ranged in >250 mM to 1585 mM, and U251MG cells are considered "TMZ-sensitive", showing a LC50 around 50 µM (Lee 2016). U251MG cell line, being COX-2 negative was used to evaluate whether the mechanism behind the effects of NS398 could be directly dependent by COX-2, and being MGMT-negative was used as TMZ-sensitive cellular model.

GBM cells were grown in adherent condition in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) of fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (standard medium, St-M) (Euro Clone, West York, UK). The cells were maintained at 37 °C in an incubator with 5% CO_2. After reaching 70/80% confluence cells were detached using trypsin-EDTA solution (Euro Clone, West York, UK). The culture medium was totally replaced by centrifugation for 10 min at 400*xg* every three days.

7.2 Drugs preparation and treatments

GBM cells were treated in the absence or presence of the selective COX-2 inhibitor, NS398 (N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide) acquired from Sigma Aldrich (Sigma Aldrich, Saint Louis, MO, USA). Initially, cells were treated with increasing concentrations of the selective COX-2 inhibitor, NS398 (50, 100, 200 and 400 µM) until 72 h to select the optimal concentration (100 µM). According to manufacturer instruction, the COX-2 inhibitor was stored as stock solutions in dimethyl sulfoxide (DMSO) solvent at -20 °C and diluted in cell culture medium just before use. The effect of NS398 was also assessed onto adherent GBM cell lines. For the choice of NS398 concentration range (50–400 µM) and treatment times (up to 72 h) we relied on previous reports and in vitro studies on GBM cell lines (Kuipers et al., 2007; Jalota et al., 2018). Moreover, the NS398 concentration of 200 µM inhibit COX-2 activity and proliferation rate of T98G, TMZ-resistant cells, therefore, it was chosen for the subsequent experiments designed to analyze the effects of combined treatment (NS398+TMZ). As control, an equal volume of DMSO was added in samples (not treated - CNTR). In culture media the final concentration of DMSO was 0.60% (v/v), and no cytotoxic effect was detected after exposure with DMSO.

The temozolomide (TMZ), the main chemotherapeutic agent clinically used to treat GBM, was acquired by Sigma-Aldrich (Saint Louis, MO, USA) and was prepared as stock solutions in sterile DMSO at a concentration of 51.5 mM. To select the drug concentration of TMZ for subsequent experiments, the TMZ at several final concentrations (10, 100, 200, and 400 μM) was tested by dose–response curve for 24, 48, and 72 h on T98G and U251MG cells. In particular, the specific concentration of chemotherapeutic agent able to reduce proliferation of each cell line by 50% at 72 h was used for the subsequent experiments designed to analyze the effects of drug combination treatment (NS398+TMZ).

7.3 Analysis of cell viability and proliferation

The viability and the number of dead cells of GBM cells, treated with NS398 in combination or not with TMZ, was measured Trypan blue staining. Moreover, number of viable adherent GBM cells were evaluated after TMZ, NS398, and drug combination treatment using the IncuCyte ® Live Cell Imager system (Essen BioSciences, Inc., Ann Arbor, MI, USA). Adherent cells were plated in a 96-multiwell culture plate at 1000 cells/cm^2 and allowed to attach overnight. Media were replaced with media containing TMZ (10, 100, 200, and 400 μM) or NS398 (50, 100, 200, and 400 μM), and NucLight Rapid Red live-cell nuclear dye (1:4000 dilution), a lentivirus-based reagent that stably integrates into the host cell line without affecting cell growth or morphology, was added in each well. The IncuCyte® Live Cell instrument captured images every 4 h using phase contrast and red (400 msec exposure) channels. Four image sets were acquired from several points of the well, using a 10× objective lens, and all the treatment conditions were run in triplicates. At the end of the experiment, the proliferation rate, based on NucLight dye uptake, was analyzed by IncuCyte ® S3 2018C software (Essen BioSciences, Inc.). The treatment with the compound vehicle alone, DMSO, was referred to as “control” (CNTR).

7.4 Western Blot analysis

Cell pellets of treated or not neurosphere and adherent cells were homogenized in ice-cold RIPA buffer (Merck KGaA, Darmstadt, Germany) supplemented with 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 5 mM of EDTA (ethylenediaminetetraacetic acid) and 100 mM of protease inhibitor cocktail (Sigma Aldrich, Saint Louis, MO, USA). The total protein amount was evaluated by DC Protein Assay (Bio-Rad, Hercules, CA, USA) using BSA as standard. Protein lysates (25 μg/lane) were mixed with sample buffer; samples were denatured (at 100°C for 4 minutes) and run on 10% SDS polyacrylamide gel under reducing conditions with β-mercaptoethanol 5% and electroblotted on to nitrocellulose membranes. Proteins were transferred onto 0.45 μm nitrocellulose membrane sheets for 1 hour at 4°C at 70 V using a Mini Trans-Blot Cell apparatus (Bio-Rad). Non-specific binding sites were blocked with 5% non-fat dry milk for 1 hour (room temperature) and then overnight incubated at 4°C with primary antibodies. The list of primary antibodies is here reported:

- rabbit monoclonal anti-COX-2 (Cell Signaling Technology, USA; dilution 1:1,000),

- mouse monoclonal antibody anti-MGMT (BD Biosciences, San José, CA, USA; dilution 1:500),
- mouse monoclonal antibody anti-SOX2 (Origene, Rockville, MD, USA; dilution 1:1,000),
- rabbit polyclonal antibody anti-β-catenin (Cell Signaling Technology, USA; dilution 1:1,000)
- mouse monoclonal antibody for anti-β-actin antibody (Bio-Rad, Hercules, CA, USA; dilution 1:1,000).

The horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Sigma–Aldrich (Saint Louis, MO, USA). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech), according to the manufacturer's instructions. Band relative densities were determined using a chemiluminescence documentation system ALLIANCE (UVITEC, Cambridge UK), and values were given as relative units. All data were normalized to β-actin protein levels, used as internal control, and results were expressed as arbitrary units.

7.5 Distribution of cell cycle phases and apoptotic cells detection

The cell cycle and apoptosis were examined in adherent T98G and U251MG cell lines exposed to COX-2 inhibitor, NS398, TMZ as single agents and their combination for 3 days. At the end of incubation time, adherent cells were detached using trypsin–EDTA solution (EuroClone, West York, UK), counted, and fixed by using a cooled 70% ethanol solution (Sigma-Aldrich, Saint Louis, MO, USA), in PBS, with gentle mixing at 4°C for 30 minutes. Fixed cells (10^5 cells/ml) were transferred to plastic BD tubes (BD Biosciences, San José, CA, USA), washed with ice-cold PBS, and resuspended in a solution containing 50 μg/ml Propidium Iodide (PI), 0.1% Nonidet-P40, and RNase A (6 μg/10^6 cell) (all reagents acquired from Sigma-Aldrich, Saint Louis, MO, USA) for 30 min in the dark at 4°C. The percentages of cells in the G1, G2/M, and S phases were calculated by software Modfit LT for Mac V3.0 using FACSCalibur instrument (BD Biosciences). The assay was carried out in duplicate. Apoptotic cells were determined by their hypochromic subdiploid nuclei staining. Data from 10,000 events per sample have been collected and analyzed using FACS Calibur instrument (BD Biosciences) equipped with cell cycle analysis software (Modfit LT for Mac V3.0).

7.6 Prostaglandin 2 (PGE2) level assay

The levels of secreted PGE2 in NS398-, TMZ- or drug combination-treated cells were assayed by prostaglandin E2 enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA) as described in the manufacturer's instructions. Supernatants of adherent GBM cells and neurospheres were collected and assayed at a final dilution of 1:10 for U87MG and not diluited for T98G cultures. Data are presented as pg/mL. The lowest detectable PGE2 concentration was 7.8 pg/mL.

7.7 Colony formation assay

The colony formation assay, or clonogenic assay, is an *in vitro* cell survival test used for study of chemical or anti-cancer agents evaluating the cells' ability to generate clones following treatment, allowing the evaluation of cytotoxic and/or antiproliferative effects (Balla et al., 2019). Briefly, adherent T98G and U251MG were seeded in 6-well plates at a concentration of 1,000 cells/well and were incubated at 37°C overnight for attachment. The next day cell medium was exchanged, cells were treated with a single dose of NS398 (200 µM), TMZ (200 µM for T98G and 10 µM for U251MG), and their combination and placed in the CO_2 incubator for 3 days. After this treatment, cells were maintained in a fresh medium until colony formation attested on 20 and 18 days for T98G and U251MG cultures, respectively. Formed colonies were fixed with cold methanol for 20 min and stained with a crystal violet solution (0.1% in PBS) at room temperature for 10 min and air-dried. Colonies were visualized and images were captured by optical microscopy (Eclipse 50i, Nikon, Tokyo, Japan). Colonies formed were counted using the ColonyCountJ, a semi-automated colony counting program of ImageJ, which provides the number of colonies (Maurya et al., 2017). The experiment set was carried out twice in duplicate, and histogram plots are the average number from three analyses.

7.8 Immunofluorescence staining of MGMT and SOX-2

The T98G adherent cells were grown on coverslips in a 12-well plate (seeded at 3,000 cells/cm^2), let to adhere overnight, and then treated with NS398, TMZ, and combination for 3 days. The coverslips were washed and fixed with 4% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA) for 5 min, and blocked with 3% BSA (Sigma-Aldrich) for 20 min at room temperature. Cells were incubated overnight at 4°C with mouse monoclonal antibody anti-human MGMT (BD Biosciences, San José, CA, USA; dilution 1:500). Next, the coverslips were stained using a FITC conjugated goat anti-mouse polyclonal IgG secondary antibody (Bethyl Laboratories, Inc, Montgomery, TX, USA) for 1 hour at room temperature and washed. For SOX-2 immunostaining, cells were incubated overnight at 4°C with FITC-conjugated mouse monoclonal antibody anti-SOX2 (Origene, 9620 Medical Center Drive Suite 200 Rockville, MD, USA; dilution 1:100). All samples were then incubated for 45 minutes at room temperature with TRITC labeled phalloidin (Sigma-Aldrich), a highly selective bicyclic peptide used for staining actin filaments (also known as F-actin). The coverslips were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and then examined at 100× magnifications with the fluorescent microscope (Eclipse 50i, Nikon, Tokyo, Japan).

7.9 Statistical Analysis

Statistical analysis was performed by the GraphPad Prism 6.0 (GraphPad Software, San Diego, Ca). All data shown are from at least two independent experiments conducted in duplicate or triplicate and are expressed as the mean ± SD or mean ± SEM, where specified. For comparison between two means, Student's unpaired t-test was used. For comparisons of the mean values among groups, a one-way or two-way ANOVA followed by Tukey *post hoc* or Dunnett test, as indicated, were used. Results were considered significant if $P<0.05$.

8. RESULTS

8.1 TMZ influence the COX-2 expression and activity of GBM cell lines

In view to assess the role of COX-2 in chemoresistance, the effect of TMZ on COX-2 levels was investigated in two GBM cell lines, T98G, and U251MG, expressing different sensitivity to TMZ and different COX-2 levels. First of all, the TMZ influence on cell proliferation was estimated on the two adherent GBM cell lines treated with increasing concentrations of drug (10, 100, 200, 400 µM) or drug vehicle DMSO (CNTR) by the IncuCyte® Live Cell Imager instrument. Images were acquired every 4 h for a total timeline of 72 h. The cell proliferation of the T98G, TMZ-resistant cell line, as expected, was significantly affected only at the highest concentration of 400 µM, thus confirming its elevated chemoresistance (Figure 24 A). On the contrary, the proliferation of the U251MG cell line was significantly reduced at all TMZ concentrations respect to CNTR cells (Figure 24 B).

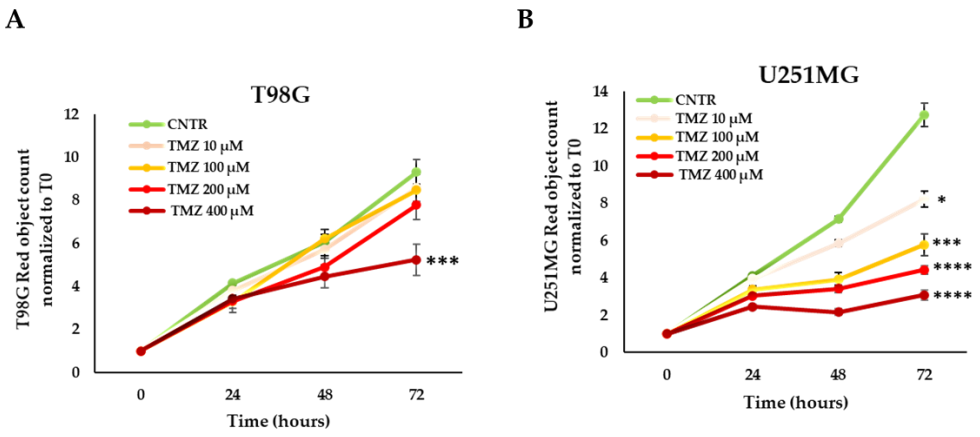


Figure 24. Effect of TMZ on proliferation rate of GBM cell lines, T98G and U251MG. Proliferation was calculated through IncuCyte® Live Cell Imager. Cell cultures were exposed to TMZ (10, 100, 200, 400 µM) for a total of 72 h and stained with the NucLight Rapid Red reagent, a cell permeable DNA stain that specifically stains nuclei in live cells. The IncuCyte NucLight Rapid Red Reagent addition to does not affect cell growth and morphology and provides homogenous staining of nuclei. The results are relative to one representative out of three experiments performed in triplicate and are expressed as mean ± SD. For comparative analysis of groups of data, two-way repeated-measures ANOVA followed by Dunnett's post hoc test was used (p< 0.05, *** p< 0.001, **** p< 0.0001 vs. CNTR).*

With the aim of analysing the ability of TMZ to impact the COX-2 expression in GBM cells, the T98G and U251MG were treated with the different concentrations of TMZ for 72 h, and the COX-2 levels were measured. In T98G cells, the COX-2 mRNA levels were significantly increased after exposure to TMZ at 200 µM ($p<0.05$ vs CNTR) and considerably improved at 400 µM when compared with CNTR cells ($p<0.0001$ vs CNTR) (Figure 25 A). Additionally, western blot analyses demonstrated the COX-2 protein levels were upregulated at increasing TMZ concentrations and appeared significant at

concentrations of TMZ ≥ 200 µM with respect to CNTR (Figure 25 B). In the COX-2 negative U251MG cells, TMZ did not stimulate COX-2 expression at all tested concentrations (Figure 25 C).

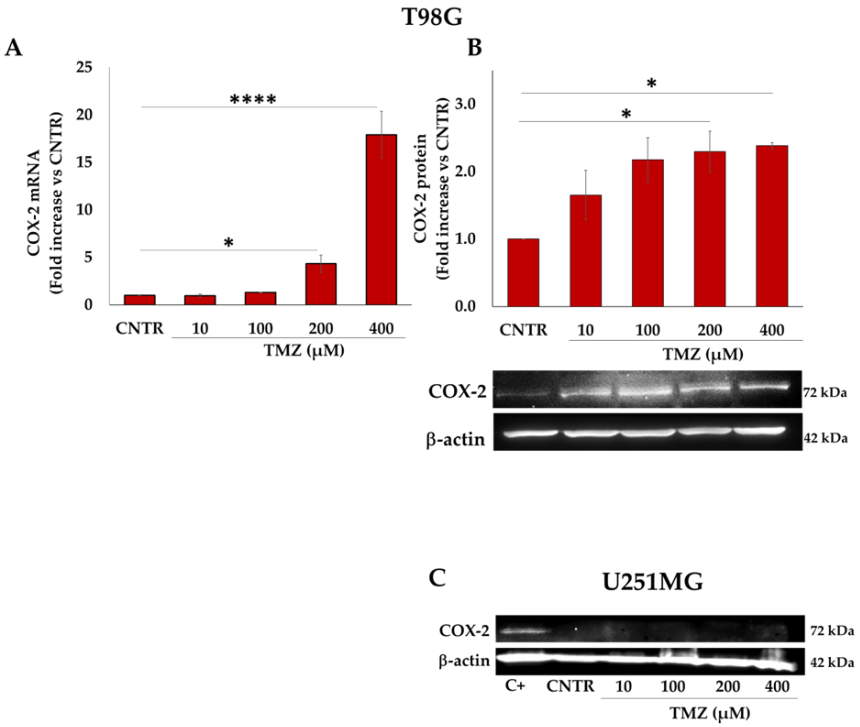


*Figure 25. Effect of TMZ chemotherapy drug on COX-2 expression in GBM cell lines. T98G and U251MG were exposed to increasing concentrations of TMZ (10, 100, 200, 400 µM) for 72 h, and COX-2 levels were evaluated. (**A**) Results from Real-time PCR of COX-2 in T98G, COX-2 positive cells, were presented and expressed as mean ± SEM of two independent experiments in triplicate (fold increase vs. CNTR). (**B**, **C**) Western blotting assay for COX-2 protein was performed on both GBM cell lines in the presence of vehicle (CNTR) or TMZ at 10, 100, 200, 400 µM as indicated. Densitometric analysis is presented normalizing vs. β-actin. Data are relative to three independent experiments (mean ± SEM). C+ = positive control (not treated T98G cells). For comparative analysis of groups of data, one-way ANOVA followed by Dunnett's post hoc test was used (* p< 0.05, **** p< 0.0001 vs. CNTR).*

8.2 Influence of TMZ treatment on β-Catenin, MGMT, and SOX-2 levels in GBM cells

Based on these results about the up-regulation of COX-2 by TMZ on TMZ-resistant cells and given the contribution of the Wnt/β-catenin pathway in GBM chemoresistance and stemness ability, we wanted to verify whether TMZ was able to modulate the key proteins closely associated with the activity of COX-2 and strongly implicated in the progression and resistance of GBM: the β-catenin, MGMT, and SOX-2.

In Figure 26, the effects of increasing doses of the alkylating agent (10–400 µM) for 72 h on the β-catenin, MGMT, and SOX-2 expression were reported. The results showed in T98G an overexpression of β-catenin proportional to the increasing TMZ concentrations, which was significant at TMZ ≥ 200 µM (Figure 26 A). Equally, the MGMT expression was upregulated accordingly to TMZ concentrations, resulting significant at 200 and 400 µM (Figure 26 B). A significant and dose-dependent increase was also verified in the protein expression of SOX-2 at 10 µM, 100 µM, 200 µM, and 400 µM TMZ with respect to CNTR (Figure 26 C). In contrast, in the U251MG cells, TMZ did not affect the β-catenin

expression (Figure 26 D) nor induce the SOX-2, or MGMT expression, since the cell line is MGMT negative (MGMT-) (data not shown).

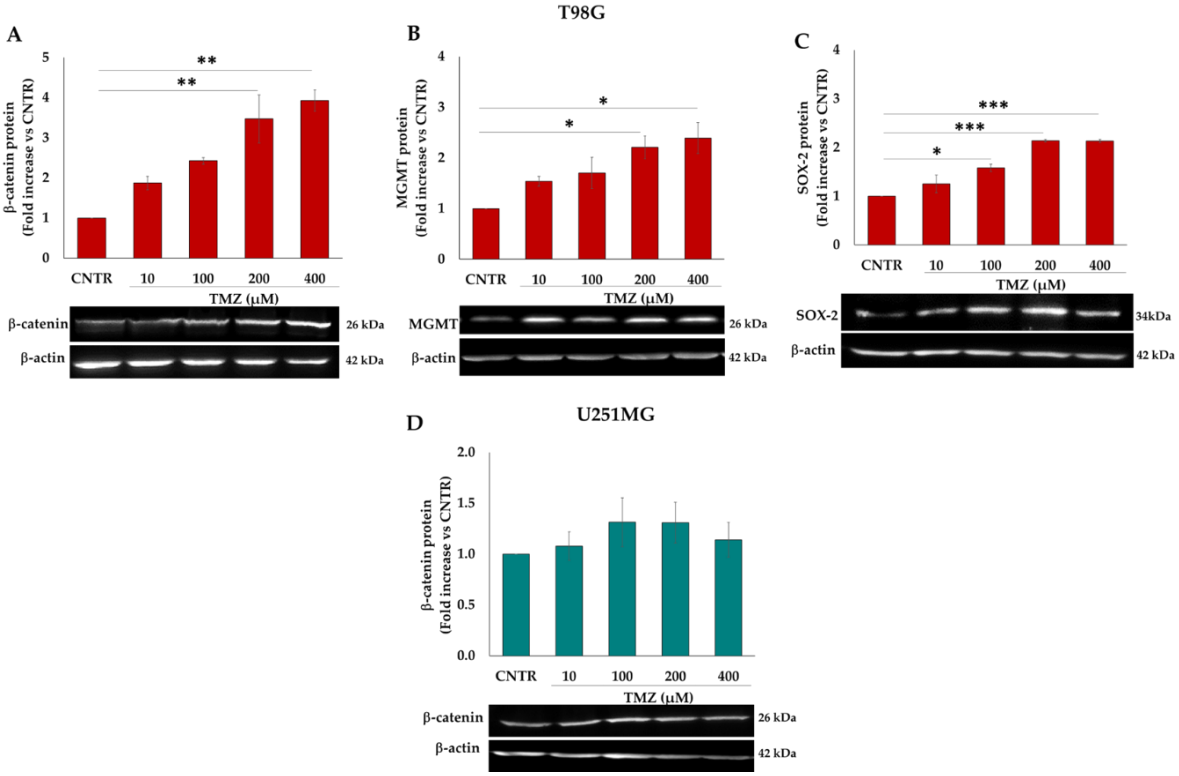


Figure 26. TMZ induces an upregulation of β-catenin, MGMT, and SOX-2 levels in TMZ-resistant and COX-2 positive cell line. GBM cell lines, T98G and U251MG, were treated with increasing concentrations of TMZ (10, 100, 200, 400 μM) for 72 h. (A, D) Western blotting images for β-catenin are shown. (B) MGMT and (C) SOX-2 expression levels were detected by western blotting in T98G cells. Densitometric analysis was done by normalizing vs. β-actin. Data from three independent experiments are shown (mean ± SEM) and expressed as fold increase vs. CNTR. Images from one representative out of three independent experiments are shown. For comparative analysis of groups of data, one-way ANOVA followed by Dunnett's post hoc test was used ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CNTR).*

8.3 PGE2 release and cell proliferation of GBM cell lines affected by NS398

The effect of NS398 on the COX-2 activity was evaluated by PGE2 levels detection in the supernatant of adherent cell cultures after treatment with different concentrations of NS398 (50–400 μM) for 3 days. In T98G cells, the NS398 strongly reduced the COX-2 activity at higher concentrations (200 and 400 μM) (Figure 29 A). As expected, PGE2 levels were undetectable in the culture medium of U251MG cells (data not shown).

The NS398 concentration range of 50–400 μM was used to evaluate cell growth for 72 h by IncuCyte® Live Cell Imager instrument. The NS398 exposure influenced the proliferation rate of the T98G COX-2-positive cell line and showed significant differences vs CNTR at 200 μM and 400 μM (Figure 27 B). Of note, NS398 treatment at all concentrations had no effect on U251MG proliferation (Figure 27 C). Considering these results, the concentration of 200 μM NS398, being able to effectively lower COX-2 activity and T98G proliferation rate was chosen for the next experiments aimed to investigate the effects

of combined treatment NS398+TMZ. In addition, the results obtained on U251MG (COX-2 negative cells) confirmed the specificity of the NS398 inhibitor.

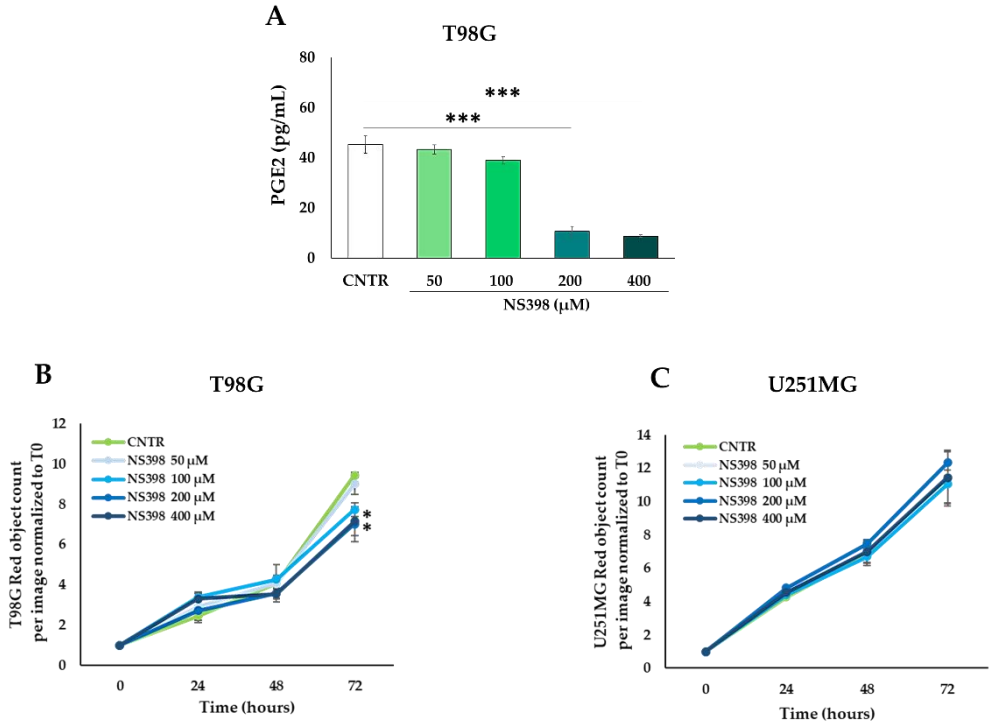


*Figure 27. Effects of NS398 on COX-2 activity and cell proliferation rate of GBM cell lines. (A) Detection of PGE2 levels in supernatants of NS398-treated adherent T98G cells were assayed by ELISA (mean ± SEM of two experiments in duplicate). The one-way ANOVA for repeated measures followed by Dunnett's post hoc test (*** $p < 0.001$ vs. CNTR) was used. (B) Effect of NS398 on the cell proliferation rate of T98G and U251MG exposed or not (CNTR) for 72 h with increasing concentrations of NS398 (50–400 µM) was evaluated. Cells were stained with NucLight Rapid Red reagent to detect live and healthy cells, and cell growth curves were analysed through IncuCyte® Live Cell Imager. The results are relative to one representative out of three experiments performed in triplicate (mean ± SD). For comparative analysis of groups of data, two-way repeated-measures ANOVA followed by Dunnett's post hoc test was used (* $p < 0.05$ vs. CNTR).*

8.4 COX-2 inhibitor in a drug combination approach enhances TMZ effect on GBM cell viability, cell cycle distribution, and apoptosis.

To verify the effect of the COX-2 inhibitor, NS398, on TMZ resistance, both the GBM cell lines, T98G and U251MG, were treated with single agents and their combination for 72 h, and the viability, cell cycle, and apoptosis levels were evaluated. Based on the results described above, the concentrations of TMZ 200 µM for T98G cells and 10 µM for U251MG cells were chosen since their inhibitory effect on proliferation was about 50%.

In the T98G cell line, NS398 and TMZ, as single drugs, did not significantly increase the percentage of dead cells when compared to CNTR (Figure 28 A); TMZ was able to considerably enhance the dead cell percentage only in U251MG, to confirm the high chemoresistance of T98G. The co-treatment (NS398+TMZ) induced a significant increase in cell mortality in T98G, showing a synergistic effect (Figure 28 A). Conversely, drug combination induced an effect similar to TMZ alone in COX-2 negative

U251MG (Figure 28 B). Microscopic images of the cell cultures treated with NS398, TMZ, and their combination revealed the same trend.

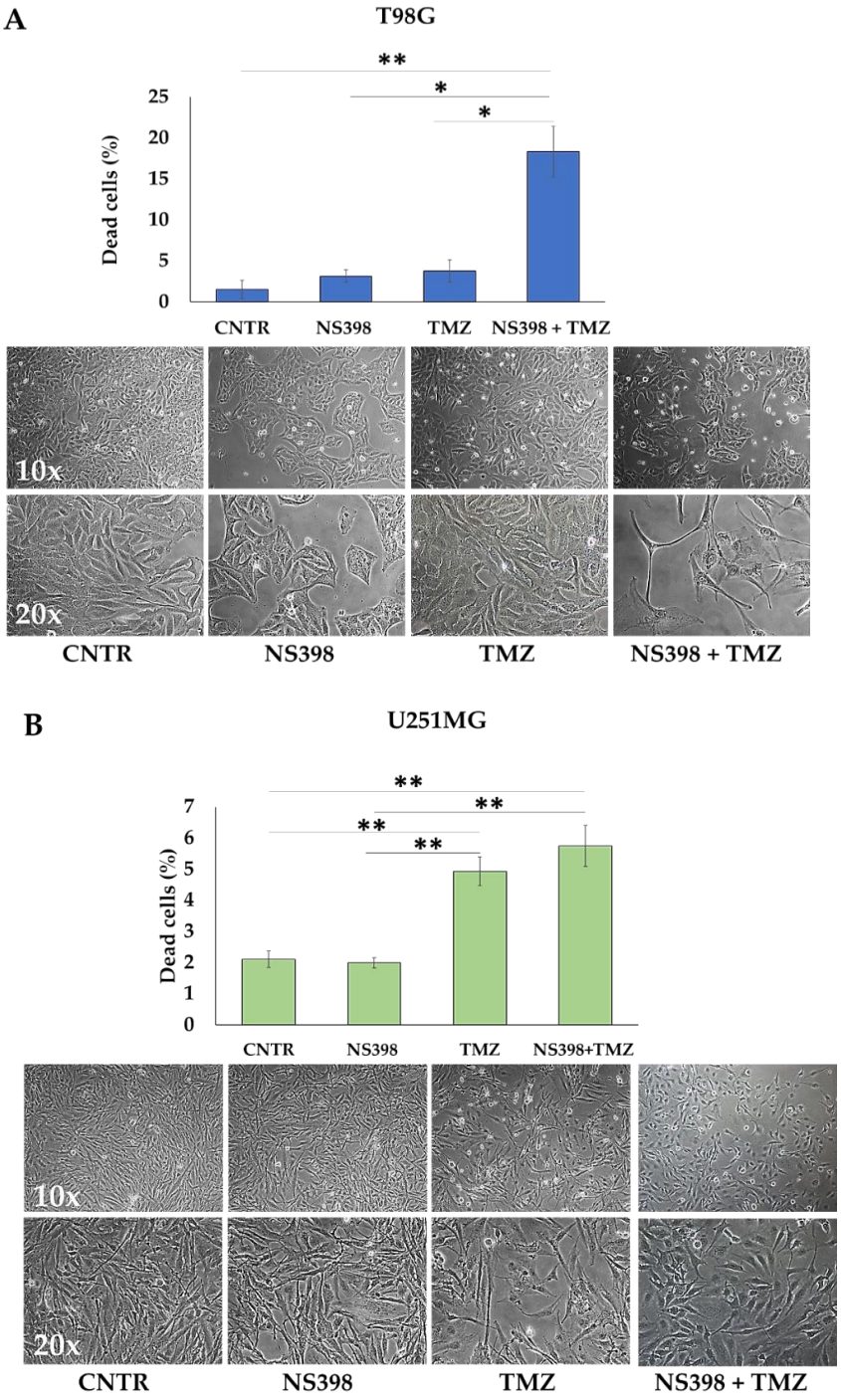
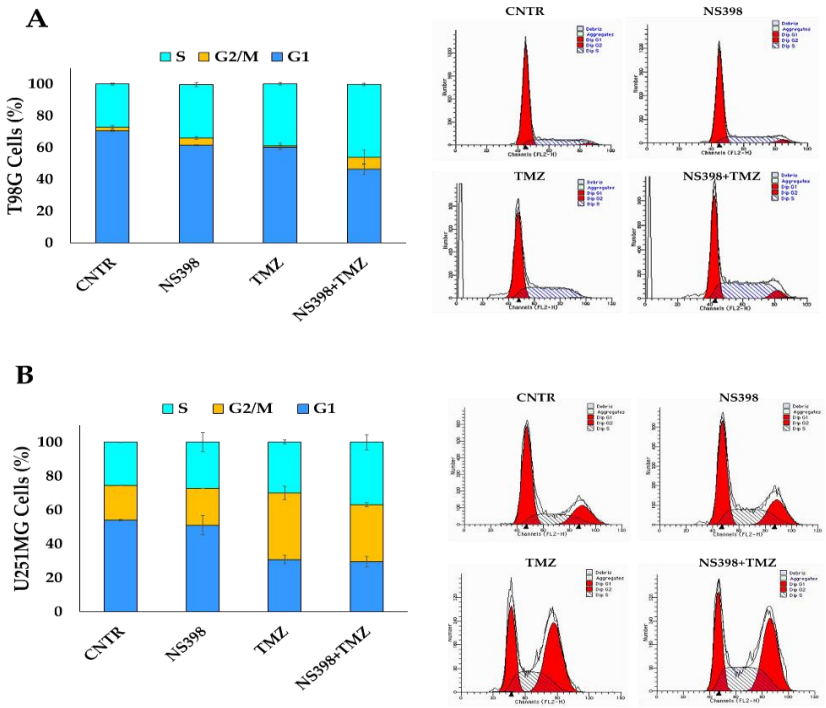


Figure 28. Effects of COX-2 inhibitor, NS398, alone or in combination with TMZ was evaluated on GBM cell viability. (A) T98G and (B) U251MG cells were incubated for 72 h with or without (CNTR) of NS398 (200 µM), TMZ (200 µM for T98G, and 10 µM for U251MG) and their combination. The percentage of dead cells was evaluated by Trypan blue test (mean ± SEM). The experiments were from independent experiments repeated three times in triplicate. For comparative analysis of groups of data, one-way ANOVA followed by Tukey post hoc test was used ($p < 0.05$, ** $p < 0.01$). Representative phase-contrast microscopic images from one out of three independent experiments are shown (10× and 20× magnification).*

The effect of NS398, TMZ, and NS398+TMZ on cell cycle phase distribution was measured by cytofluorimetric analysis on T98G and U251MG cells. The results demonstrated that NS398 treatment significantly lowered the percentage of T98G cells in the G1-phase. Similarly, the TMZ determined an evident decrease of cells in the G1-phase, but also an increase in the S-phase. Of interest, the NS398+TMZ combination modified the cell cycle phase distribution, causing a significant reduction in the G1-phase cell population respect to control, TMZ, and NS398 as single agents, accompanied by an increase in the fraction of G2/M-phase when compared with CNTR and TMZ-treated cells (Figure 29 B, C) and a significant cell accumulation in S-phase respect to CNTR and NS398 (Figure 29 A, C). The TMZ treatment in U251MG cells caused effects similar to the combination: a reduction in the G1 phase cell population, accompanied by an increase in the fraction of G2/M-phase when compared with CNTR and NS398-treated cells at 72h (Figure 29 B, C)



C

		3 days treatment		
		Phase G1 (%)	Phase G2/M (%)	Phase S (%)
T98G	CNTR	70.46 ± 0.44	2.36 ± 0.58	27.17 ± 0.97
	NS398	61.51 ± 0.29*	4.57 ± 1.35	33.59 ± 0.93
	TMZ	60.06 ± 1.35*	1.06 ± 0.71	39.88 ± 1.66*
	NS398+TMZ	46.50 ± 3.40**°° §	7.55 ± 0.87* §§	46.50 ± 4.51*°
U251MG	CNTR	54.10 ± 0.40	20.42 ± 0.21	25.47 ± 0.19
	NS398	51.10 ± 5.67	21.74 ± 0.08	27.15 ± 5.59
	TMZ	30.74 ± 2.58*°	39.36 ± 3.83*°	29.89 ± 1.24
	NS398+TMZ	29.57 ± 3.09*°	33.55 ± 1.28*°	36.87 ± 4.38

Figure 29. Analysis of cell cycle distribution phases of GBM cell lines after exposure to NS398 alone or combined with TMZ. (A, B) T98G and U251MG cells were exposed to NS398, TMZ, and NS398+TMZ for 72 h, and the analysis of phases of cell cycle was performed by flow cytometry. (C) Values of flow cytometric histograms showing the percentage distribution of cycle phases referring to two independent experiments in duplicate (mean ± SEM). The flow cytometric profiles from a representative experiment are also shown. For comparative analysis of groups of data, a one-way analysis of variance (ANOVA) with post hoc Tukey test was used ($p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs. CNTR; ° $p < 0.05$, °° $p < 0.01$ vs. NS398; § $p < 0.05$, §§ $p < 0.01$ vs. TMZ).*

We then further investigated the level of apoptotic cells following treatment with single drugs and their combination. In Figure 30, the NS398 alone provoked a slight increase of apoptosis of T98G treated for 72 h, as detectable by the sub-G1 peak. Alternatively, the exposure for the same time to TMZ alone failed to induce a significant level of apoptosis. Of note, the addition of NS398+TMZ led to a much higher and statistically significant level of apoptosis than all other culture conditions (Figure 30 A). In U251MG, NS398, which alone did not increase the sub-G1 population, in combination with TMZ, did not significantly influence the percentage of the apoptotic cell compared to TMZ alone (Figure 30 B). Overall, in the U251MG cell line, COX-2 negative, the COX-2 inhibition by NS398 did not strengthen the antiproliferative, apoptotic, and cytostatic effect of TMZ. Otherwise, in COX-2 positive, TMZ-resistant T98G cells, the reduction in COX-2 expression and activity led to the appearance of susceptibility to TMZ, resulting in a significant cell proliferation reduction, cell cycle arrest, and increased apoptosis level, suggesting the involvement of COX-2 activity underlying the chemoresistance mechanisms.

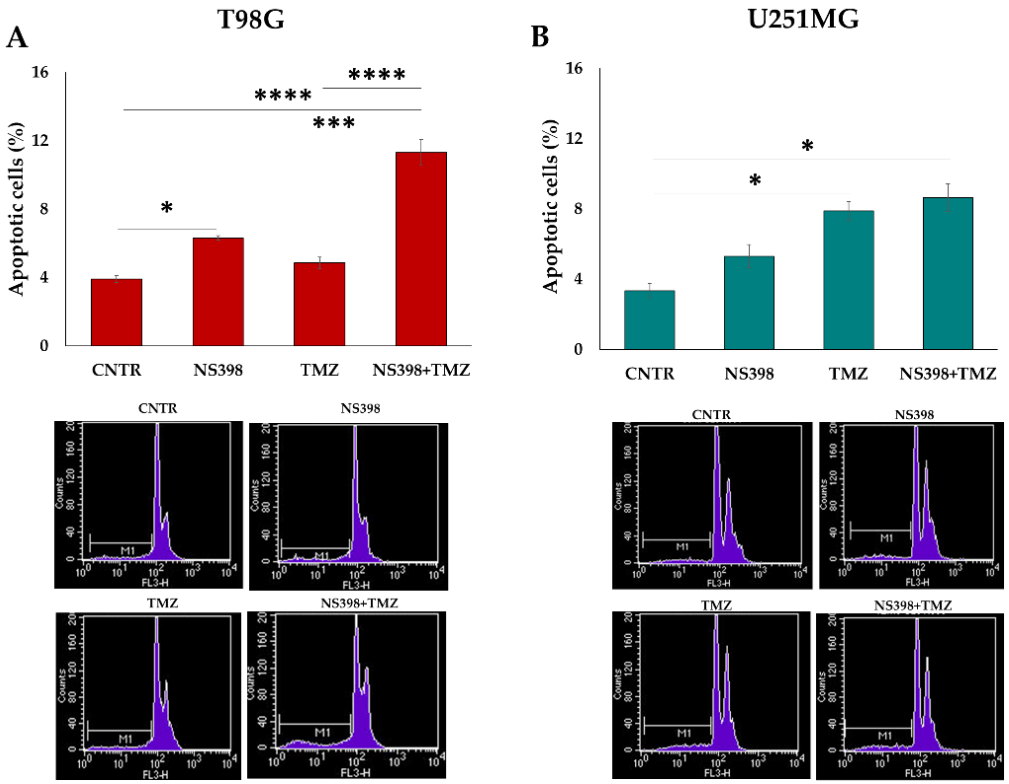


Figure 30. Analysis of apoptotic cells following the exposure of GBM cells to NS398, alone or combined with TMZ. GBM cell lines were exposed to the NS398 and TMZ, alone or in combination for 72 h, and flow cytometry analysis was performed to detect the apoptotic cells in (A) T98G and (B) U251MG. Histograms show the results from two independent experiments in duplicate (mean ± SEM). Flow cytometric profiles from one representative experiment are shown. The ANOVA one-way test followed by Tukey's test for multiple comparisons was performed ($p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).*

8.5 COX-2 pharmacological inhibition, in combination with TMZ, abrogates the clonogenic potential of GBM cell lines.

The biological response of T98G and U251MG cells to drug combination and/or to single agent treatment has been further assessed, evaluating their clonogenic ability. The clonogenic assay is a gold standard technique used for *in vitro* evaluation of anti-cancer agents (Balla et al., 2019). Results in Figure 33 highlighted that NS398 alone significantly affected the colony generation in the COX-2 positive cell line, T98G; as expected, the amount of colonies generated after TMZ treatment of T98G cells was not significantly different by CNTR (Figure 31 A). Surprisingly, the combination NS398+TMZ considerably hindered the T98G clonogenic potential (Figure 31 A). No effect was observed in the COX-2 negative cell line, U251MG, exposed to NS398 compared with CNTR; instead, after treatment with TMZ, very few colonies were generated (Figure 31 B). Additionally, in the clonogenic ability, no significant difference was detected between the NS398+TMZ combination and TMZ alone treatment in U251MG (Figure 31 B).

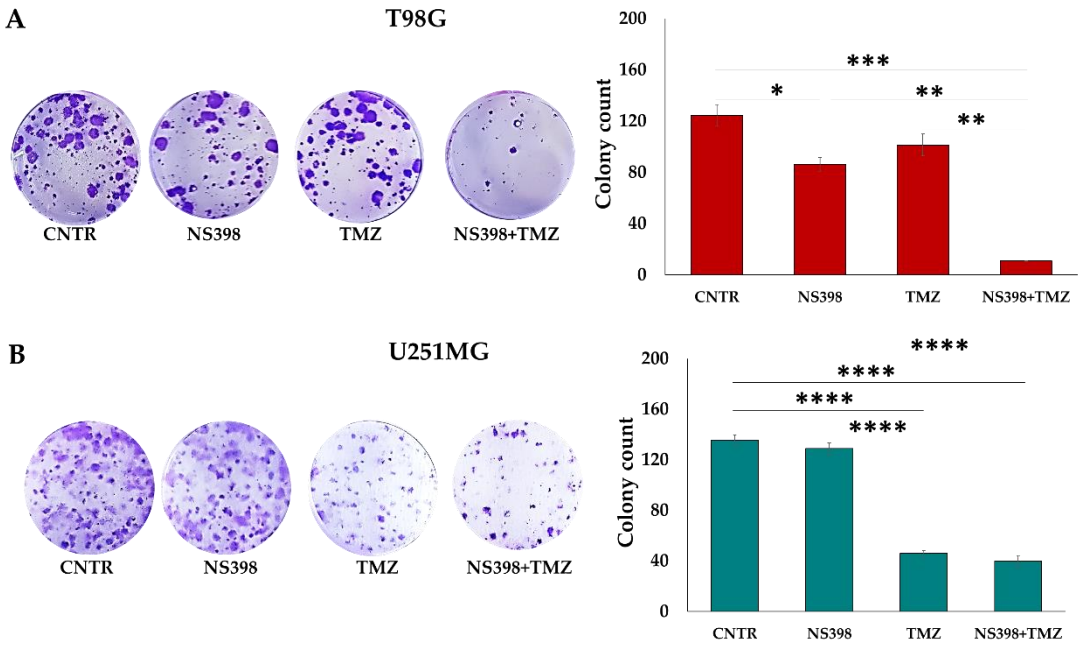


Figure 31. Effects of NS398 alone or combined with TMZ on colony formation ability of GBM cell lines. (A) T98G and (B) U251MG, untreated (CNTR) and treated for 72 h with NS398 and TMZ, alone or in combination, were cultured until the colony formation (~20 days for T98G and ~18 days for U251MG cells). Representative phase contrast images of colonies are shown. The colonies count was made through the ColonyCountJ program, and quantitative results are expressed as total colony counts from three independent experiments in duplicate (mean values ± SEM). For comparative analysis of groups of data, one-way ANOVA followed by Tukey post hoc test was used ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).*

8.6 NS398 reverses the TMZ-induced COX-2 upregulation in T98G cells

Consistent with all these results, we wanted to evaluate whether the drug combination was able to modulate the COX-2 levels in T98G cells. To this aim, the COX-2 gene expression was assessed through real-time PCR in T98G after 72 h of treatment with single agents or their combination, and results are

presented in Figure 32 A. According to previous data on the T98G cell line, the TMZ exposure caused a relevant increase in COX-2 mRNA and protein levels. Of note, treatment with NS398 single agent downmodulated the basal COX-2 levels and, when combined with TMZ, could counteract the TMZ-induced COX-2 upregulation in T98G (Figure 32 A, B). PGE2 levels were assayed in T98G cell supernatants, and the results further confirmed the previous ones, being the PGE2 production affected by treatments in an overlapping way (Figure 32 C). In the U251MG, being a COX-2 negative cell line, the COX-2 expression was not influenced by any treatments (Figure 32 D).

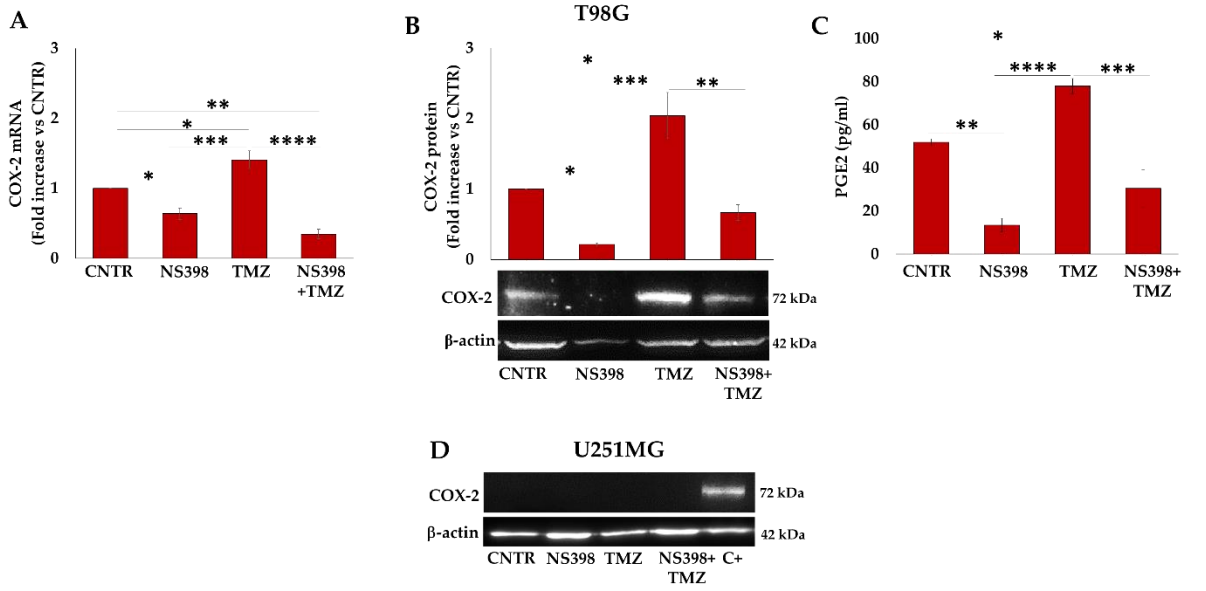


Figure 32. NS398 counteracted TMZ-induced COX-2 overexpression in T98G cells. (A) The Real-Time PCR of the COX-2 gene was achieved in T98G cells incubated for 72 h with NS398 and TMZ, alone or in combination. GAPDH was used as internal control. Data from three experiments performed in duplicate (mean ± SEM) are shown as fold increase vs. untreated cells (CNTR). (B and D) Western blotting assay for COX-2 on T98G and U251MG is shown. C+ = positive control (not treated T98G). Densitometric analysis was made by normalization with β-actin. Data from three independent experiments are expressed as fold increase vs. CNTR (mean ± SEM). Representative images from one experiment are shown. (C) PGE2 levels were quantified in T98G supernatants by ELISA. Results are expressed as mean ± SEM and are relative to two experiments performed in duplicate. For comparative analysis of data, the one-way ANOVA followed by Tukey post hoc test was performed ($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. CNTR).*

8.7 COX-2 inhibition in T98G cells reduces the overexpression of chemoresistance-related pathways: MGMT, β-catenin and SOX-2

To further investigate the ability of NS398 to counteract the TMZ resistance, the MGMT mRNA and protein levels were evaluated in the TMZ-resistant cell line, T98G, exposed for 72 h to NS398 and TMZ, alone or in combination. The COX-2 inhibitor significantly lowered the basal and TMZ-induced MGMT expression either at the transcriptional and post-transcriptional level (Figure 33 A, B). According to these results, immunofluorescence images of T98G reported the ability of combined treatment to markedly suppress the MGMT protein (Figure 33 C). Although it is known that the U251MG does not express MGMT (Chahal M., et al., 2012), we have verified the effect of the NS398, TMZ, and

combination also on this cell line. No treatments were able to induce the MGMT protein expression in these cells (Figure 33 D).

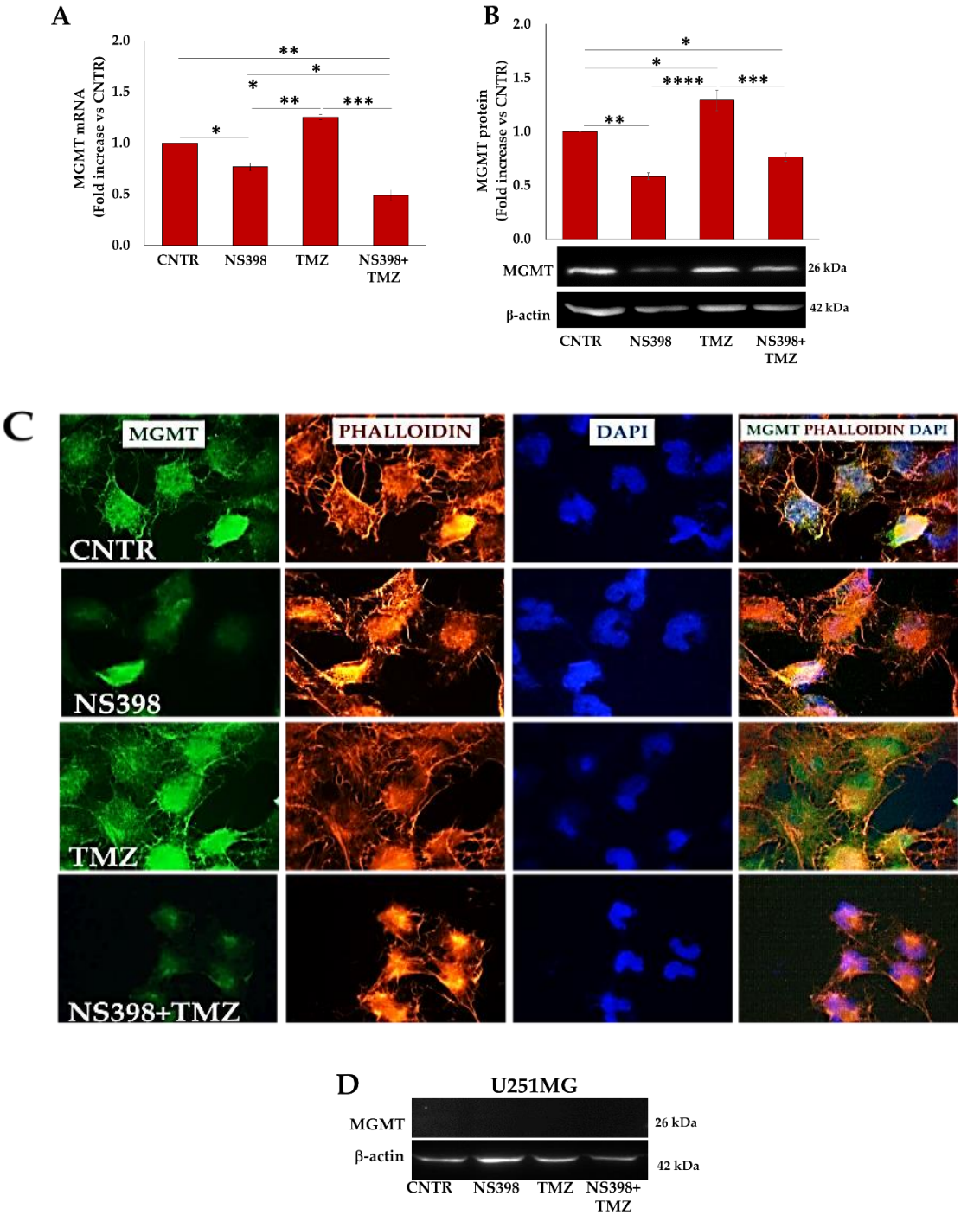


Figure 33. COX-2 inhibitor reduced TMZ-induced MGMT upregulation in T98G cells. Cells were exposed to NS398 and TMZ, alone or in combination for 72 h, and MGMT levels were assessed. (A) Relative mRNA expression levels of MGMT in TMZ-resistant T98G cell line are shown as fold increase vs. untreated cells (CNTR) (mean ± SEM). (B, D) Western blotting assay for MGMT was performed on T98G and U251MG. Densitometric analysis is presented normalizing vs. β-actin (loading control). Data from three independent experiments are shown as the mean ± SEM and expressed as fold increase vs. CNTR. For comparative analysis of data, a one-way analysis of variance (ANOVA) with post hoc Tukey test was used ($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). (C) Representative immunofluorescence images of T98G cells stained with anti-MGMT antibody (green) and with TRITC-phalloidin (red) to reveal F-actin from one out of three independent experiments are shown. Nuclei were counterstained with DAPI (blue). (100× magnification).*

To better elucidate the mechanism underlying the NS398 ability in modulating the TMZ-resistance, we analysed the β-catenin expression in the T98G cell line exposed for 3 days to NS398 and TMZ, alone or in combination. In GBM, β-catenin is engaged in the stem-like state maintaining as well as in the progression and recurrence of the tumor (Latour et al., 2021). Results in Figure 34 show that the COX-2 inhibitor slightly downregulated the β-catenin expression compared with CNTR, while TMZ alone significantly upregulated its expression (Figure 34). Of interest, the concurrent treatment (NS398+TMZ) counteracted the β-catenin expression induced by TMZ, considerably reducing it even below the baseline level.

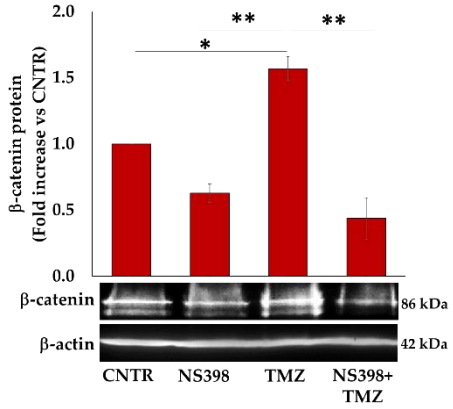


Figure 34. NS398 counteracted TMZ-induced β-catenin overexpression in T98G cells. The expression level of β-catenin was determined by western blotting assay in the T98G cells incubated for 72 h in the presence or absence (CNTR) of NS398 and TMZ, alone or in combination. The densitometric analysis was performed normalized bands intensity to those of β-actin (used as a loading control). Data shown are means ± SEM of three independent experiments and are expressed as fold increase vs. CNTR. For comparative analysis of data, a one-way analysis of variance (ANOVA) with Tukey post hoc test was used ($p < 0.05$; ** $p < 0.01$).*

In conclusion, with the Wnt/β-catenin pathway being involved in GBM stem-like maintenance and considering the close link between GBM resistance and cancer stem cell presence, we evaluated the SOX-2 expression following exposure of T98G cells to NS398 alone or in combination with TMZ. As reported above (Table 3), we observed that T98G cells expressed high basal levels of stemness marker SOX-2. The treatment with COX-2 inhibitor for 72 h slightly affected the SOX-2 expression, but not at a significant level (Figure 35 A). A high increase in SOX-2 expression of T98G after TMZ treatment was detected, thus confirming previous data (Valtorta et al., 2017). Notably, NS398 significantly counteracted the TMZ-induced SOX-2 expression (~3-fold decrease vs. TMZ alone) (Figure 35 A). Representative images of immunofluorescence staining of T98G cells, showing the SOX-2 protein, localized mainly in nuclei, supported the above results (Figure 35 B).

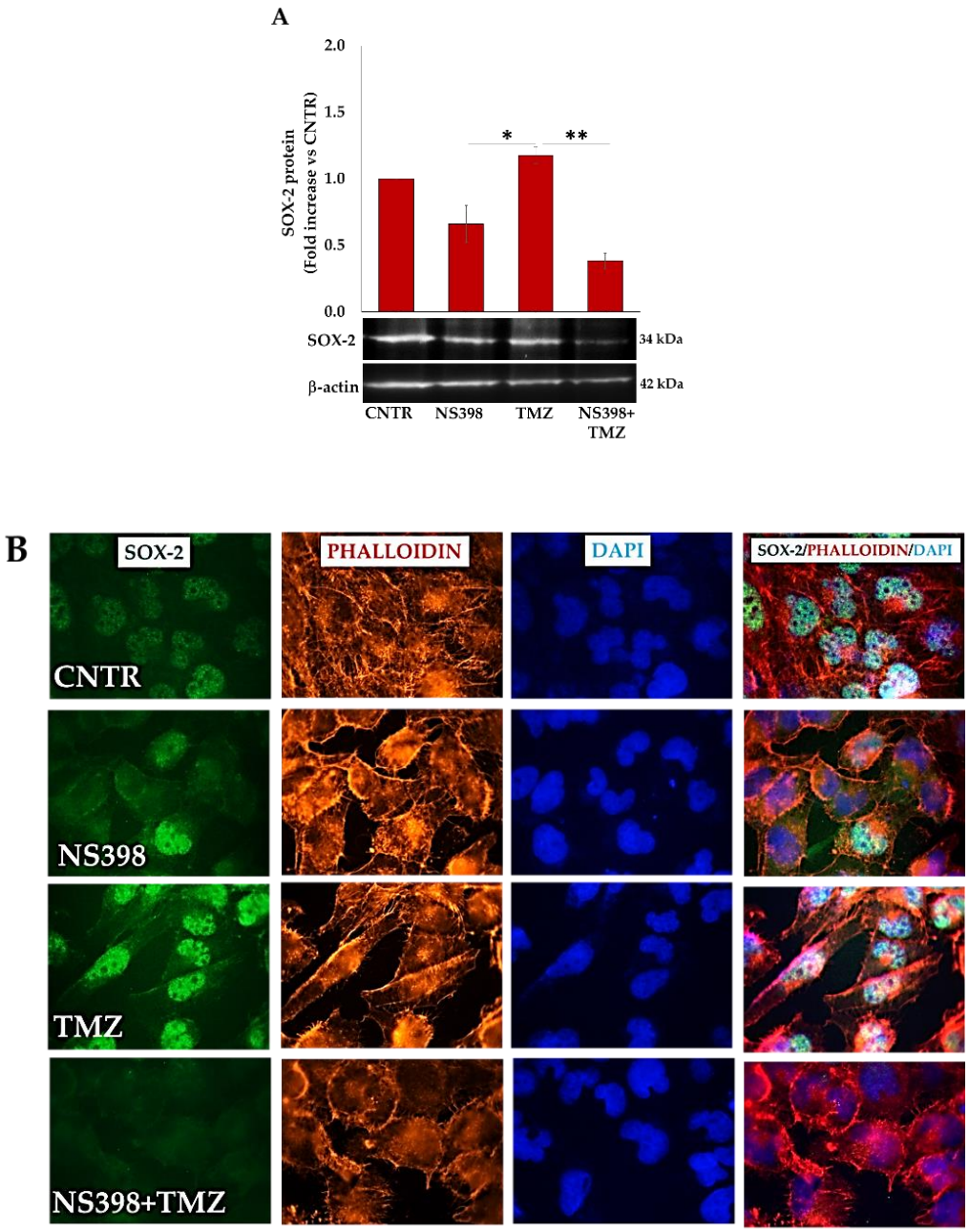


Figure 33. NS398 counteracted TMZ-induced SOX-2 overexpression in T98G cells. (A) Western blotting for SOX-2 was performed on T98G cells treated for 72 h with NS398 and TMZ, alone or in combination. Obtained values from densitometric analysis, were normalized vs. β-actin. Data are from three independent experiments, and values are expressed as fold increase vs. CNTR (mean ± SEM). Images from one representative out of three independent experiments are shown. For comparative analysis of data, a one-way ANOVA with post hoc Tukey test was used ($p < 0.05$; ** $p < 0.01$). (B) Representative immunofluorescence images of T98G cells treated as above described and stained with anti-SOX-2 antibody (green) and with TRITC-phalloidin (red) to reveal F-actin, from one out of three independent experiments are shown. Nuclei were counterstained with DAPI (blue). All images were acquired at 100× magnification.*

9. DISCUSSION

Current therapeutic approaches for Glioblastoma (GBM) cure point to reducing or destroying the tumor cells by surgical methods and radio- and chemotherapy. Despite advances in therapy, outcomes for patients with GBM remain dismal. Disease recurrence after surgery, radiation, and chemotherapy, typically occurs within six months, and patients inevitably succumb to disease progression at just over one year (Stupp et al., 2005).

GBM, like many other malignant tumors, is characterized by a moderately inflammatory microenvironment that promotes all stages of tumorigenesis, progression, and invasiveness, as well as resistance to therapy (D'Alessio et al., 2019).

Therapeutic resistance represents the main challenge in the development of successful treatments, so understanding the mechanisms underlying the development of therapeutic resistance is essential. However, the mechanisms of resistance are multiple: cancer stem cell repopulation, epithelial-mesenchymal transition, inflammatory infiltration, and immunosuppression play an important role in this process (Tong et al., 2018). The latest evidence demonstrates the influence of the COX2/PGE2/EP axis not only in mechanisms of tumor development but also in progression and treatment resistance. COX-2 expression was found to be highly associated with increased cancer stem cell population in multiple types of cancer, including glioma (Tafani et al., 2011). COX-2 induction in tumor cells contributes to tumor formation, growth, and metastasis, predominantly via synthesizing PGE2 that acts on its EP receptors to regulate cell proliferation, migration, apoptosis, and angiogenesis (Qiu et al., 2017; Jiang et al., 2017). As shown by previous studies, COX-2 is highly expressed in many human malignant glioma cell lines, cultured either in adherent conditions or in a specific medium, promoting stem cell growth.

Since PGE2 mainly functions through binding and activating prostaglandin receptors (EPs), together with COX-2 and EP, PGE2 forms a functional COX2/PGE2/EP axis with important biological and pathological functions (Tong et al., 2018).

Several studies discussing the link between inflammation and tumorigenesis have recommended COX-2 inhibitors for cancer therapy. In addition, clinical trials demonstrated that long-term use of COX-2 inhibitors, such as celecoxib and rofecoxib, could reduce the incidence of various human cancers and could significantly improve the efficacy of chemotherapy by preventing multidrug resistance (Nagaraju and El-Rayes, 2019). Elevated COX-2 levels have been associated with an increase of cancer stem cells number, not only in GBM but also in other cancers (Pang et al., 2016).

To counteract the inflammatory process in GBM and to improve sensitivity to treatment, the use of COX-2 inhibitor drugs is being evaluated in addition to standard therapy. NS398, a selective COX-2 inhibitor not yet approved by the Food and Drug Administration (FDA) for clinical use, has been used *in vivo* and *in vitro* in many types of cancer (Bergqvist et al., 2019). The NS398 inhibitory effects on COX-2 in GBM cells were investigated in human U87MG and T98G cell lines (Joki et al., 2000).

In our previous experimental work it has been demonstrated that neurospheres, arising from U87MG and T98G, both GBM cell lines, are strongly influenced by NS398 treatment, showing morphological changes, reduced growth rate, and increased autophagy. NS398 also was able to affect the biology of extracellular vesicles (EVs) released from neurospheres; in fact, EVs from GBM cells pretreated with the COX-2 inhibitor impaired the migratory capacity and induced autophagy in U87MG and T98G adherent cells, confirming the effects observed after the direct addition of the inhibitor. Such evidence can be explained since the NS398 was able to influence the cargo of the EVs released from neurospheres, and it can be hypothesized that NS398, when added to neurospheres, could be trapped in the EVs, which would then serve as a vehicle for the drug administration (Kalimuthu et al., 2018). However, further research is needed to definitely assess the influence of NS398 on EVs loading.

Of note, these results suggest that the two COX-2 positive GBM cell lines were equally influenced by NS398, despite the intrinsic diversity and different genetic characteristics, such as the TP53 gene status, MGMT activity, and base excision repair (BER) or BRCA1 pathways, which in turn are associated to a different sensitivity or resistance to TMZ (Lee 2016). Although both expression and enzymatic activity of COX-2 were considerably higher in U87MG than in T98G, the NS398 inhibitor exhibited similar effects on cell growth and viability, autophagy, and cell migration. Kuipers et al. demonstrated that COX-2 inhibitors, including NS398, caused a reduction in cell growth and radiation resistance in both COX-2-positive (U87MG) and negative (D384, U251MG) GBM lines. These results led the authors to conclude that the effects of non-steroidal anti-inflammatory drugs (NSAIDs) could be independent of enzyme expression and probably caused by interaction with other targets than COX-2 (Kuipers et al., 2007).

In 2013, Gurpinar et al. published a detailed description of the major COX independent mechanisms of NSAIDs and COX-2 inhibitors, including inhibition of cyclic guanosine monophosphate phosphodiesterases (cGMP PDEs), Wingless-related integration site (Wnt) signaling, peroxisome proliferator activated receptor δ (PPARδ) activity, phosphatidylinositol-3-kinase (PI3K)/3-phosphoinositide-dependent kinase-1 (PDK-1)/Protein Kinase B (Akt) pathway (Gurpinar et al. 2013). In line with these findings, our results show that both GBM cell lines are similarly sensitive to NS398 treatment, notwithstanding the relevant difference in COX-2 expression and activity. Additional experiments with negative COX-2-GBM cell lines will be necessary to verify whether the mechanism(s) behind the observed effects of NS398 could be COX-2 independent. Wu et al. recently described that the use of COX-2 inhibitors such as indomethacin, celecoxib, or siRNA increased the cytotoxic effect of TMZ on glioma stem cells isolated from primary GBM (Wu et al., 2017). Although the two COX-2 positive cell lines used in our experiments had different sensitivity to TMZ, a similar response was obtained after treatment with NS398. Although additional studies are necessary to provide a complete view of the mechanisms involved, these data help to expand the knowledge of biological effects induced by NS398.

Despite the substantial evidence from the literature supporting the implication of COX-2 in TMZ resistance of GBM (Qiu et al., 2017; Wu et al., 2017), as far as we know, there is no data that the gold standard drug for the GBM can influence COX-2 expression and/or activity in GBM cells. Thus, the effect of NS398 and TMZ in combination therapy has been evaluated in two GBM cell lines, T98G and U251MG, showing different COX-2 expression level, different MGMT status, and sensitivity to TMZ. In particular, the two cell lines were used to evaluate whether the alkylating agent TMZ might influence the COX-2 expression and activity and if the mechanism behind the observed effects of NS398 might be COX-2 independent.

To maintain a cell proliferation rate greater than 50%, TMZ was administered at doses of 200 µM to T98G cells and 10 µM to U251MG cells, with the latter dose being within the clinically relevant TMZ dose range (35 µM). The obtained results showed that TMZ treatment in resistant T98G cells, but not sensitive and COX-2 null U251MG cells, led to a dose-dependent and considerable upregulation of COX-2 and to an increase in PGE2 release. It is known that COX-2 expression is modulated by growth factors, proinflammatory cytokines, such as TNF, IL-1, and IL-6, and Toll-like receptors (TLRs). Thus, the COX-2 promoter has an NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) response element in addition to other cytokine and growth factor response elements (Gupta et al., 2018; Shi et al., 2015). Aberrant constitutive activation of NF-κB is a frequent occurrence in GBM, and its activation induces the malignant phenotype, negatively impacting the prognosis of GBM patients (Soubannier et al., 2017). The NF-κB pathway interplays with epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), which are often aberrantly activated in GBM, through a number of mechanisms, involving both protein kinase B/AKT (AKT)-dependent and -independent pathways (Khabibov et al., 2022). Notably, TMZ treatment of T98G cells enhanced the release of EGF and its receptor EGFR, resulting in the stimulation of the multidrug resistance gene (MDR1) (Munoz et al., 2014). Based on this evidence, the ability of TMZ to induce the COX-2 overexpression may be attributed to the drug's activity at the EGF/EGFR pair level, which leads to NF-kB upmodulation and, consequently, an upregulation of COX-2 expression. According to earlier studies, TMZ can upregulate Wnt/-β-catenin signaling, MGMT expression, and stemness potential, all of which are involved in GBM-chemoresistance (Tong et al., 2018; Wickstrom et al., 2015; Buchanan and DuBois 2006). The results shown confirmed that TMZ enhanced the β-catenin, MGMT, and SOX-2 expression only in T98G cells but had no influence on U251MG cells. These findings imply that the effects on T98G cells are due to the TMZ-induced up-regulation of COX-2, which is responsible for the overexpression of the Wnt/β-catenin pathway that, in turn, positively influences the expression of MGMT and SOX-2.

In an effort to investigate the role of the COX-2/PGE2 system in the TMZ resistance mechanisms, the effect of the specific COX-2 inhibitor, NS398, in T98G and U251MG cell lines has been explored. The first data indicate that NS398, alone or in combination with TMZ, influenced T98G, COX-2 positive cells, while no effect was observed in U251MG, confirming that the NS398 activity was strongly

correlated with COX-2 inhibition. Remarkably, in addition to suppressing the enzymatic activity of COX-2 as anticipated, the NS398 was also capable of significantly reducing the gene and protein expression of COX-2 in T98G when combined with TMZ. Hence, here we demonstrated that the treatment with COX-2 inhibitor NS398 enhanced the chemosensitivity to TMZ of GBM cells due to a downregulation of the TMZ-induced COX-2 overexpression. This result could be explained through the role of PGE2, which exerts positive feedback on the expression of COX-2, and NS398 could inhibit this mechanism by suppressing its generation. Moreover, NS398 was also able to counteract the TMZ-induced upregulation of β-catenin, MGMT, and SOX-2 in T98G, indicating the pivotal role of the COX-2/PGE2 system in the cascade of pathways triggered by TMZ and implicated in chemoresistance reduction.

Although NS398 is one of the early found COX-2 selective inhibitors, and it has often been used as a prototype COX-2 inhibitor for in vitro and in vivo experiments on various types of cancers, including GBM, it has not yet been approved by the FDA for clinical use (Rouzer and Marnett 2020; Jalota A., et al., 2018; Ferreira et al., 2021; Bergqvist et al., 2019; Majchrzak-Celinska et al., 2019). All the obtained results, both from NS398 treatment and drug combination treatment (NS398+TMZ), confirm the ability of NS398 to affect the biology of GBM cell lines as well as the relative derived-neurospheres. Moreover, the effect of other COXIBs, such as celecoxib, is being evaluated to determine the reproducibility of the effects obtained with NS398 on chemoresistance and to better understand the mechanisms involved. At present, pharmacokinetic studies and clinical trials with NS398 are still pending. Altogether, these data lead us to hypothesize that the pharmacological inhibition of COX-2 could be considered a valid therapeutic approach to complement standard therapies in patients affected by GBM.

Future perspectives will be to use longer culture times to gain insights on whether the restoration of chemosensitivity represents a long-lasting mechanism or if the cells could activate further resistance mechanisms. Also, more GBM cell lines are differently sensitive o TMZ, and more approaches, such as the COX-2-gene silencing and the experiments of COX-2 transfection in COX-2 null GBM cells, are still in progress to better investigate the mechanism of TMZ resistance. On the other hand, the study of inhibition of the COX-2/PGE2/EP signal pathway in preclinical models could be of particular interest, given the prominent role played by this pathway not only in GBM chemoresistance but also in immunotherapy resistance (Authier et al., 2015; Pu et al., 2021). GBM patient-derived xenografts (PDX) implanted in a humanized mouse model, immunodeficient mice with peripheral blood mononuclear cells derived from the same patient, recreating the patient's immune-GBM interface, could represent an innovative preclinical model particularly appealing to solve this aim (Buqué and Galluzzi 2018; Haddad et al., 2021).

All these findings strongly support the role of the COX-2/PGE2 system in GBM biology and, in particular, in resistance to TMZ. The COX-2 inhibition influenced in a similar way GBM cell lines even though they show individual genetic features. In addition, COX-2 inhibition abrogated TMZ-induced COX-2 up-regulation and all COX-2-dependent pathways involved in TMZ-resistance. Although further

studies are needed to gain a complete picture of the actors involved in the observed effects, overall, our data help to broaden the complex interplay of TMZ-resistance.

10. REFERENCES

- Agnihotri S, Wolf A, Munoz DM, Smith CJ, Gajadhar A, Restrepo A, et al. A GATA4-regulated tumor suppressor network represses formation of malignant human astrocytomas. J Exp Med. 2011;208(4):689-702.
- Agnihotri S, Wolf A, Picard D, Hawkins C, Guha A. GATA4 is a regulator of astrocyte cell proliferation and apoptosis in the human and murine central nervous system. Oncogene. 2009;28(34):3033-46.
- Alcantara Llaguno S, Chen J, Kwon CH, Jackson EL, Li Y, Burns DK, Alvarez-Buylla A, Parada LF. Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. Cancer Cell. 2009 Jan 6;15(1):45-56. doi: 10.1016/j.ccr.2008.12.006. Erratum in: Cancer Cell. 2009 Mar 3;15(3):240.
- Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol. 2008;10(5):619-24.
- Al-Samadi A, Awad SA, Tuomainen K, Zhao Y, Salem A, Parikka M, et al. Crosstalk between tongue carcinoma cells, extracellular vesicles, and immune cells in in vitro and in vivo models. Oncotarget. 2017;8(36):60123-34.
- Alvarado-Ortiz E, de la Cruz-López KG, Becerril-Rico J, Sarabia-Sánchez MA, Ortiz-Sánchez E, García-Carrancá A. Mutant p53 Gain-of-Function: Role in Cancer Development, Progression, and Therapeutic Approaches. Front Cell Dev Biol. 2020; 8:607670.
- Alves ALV, Gomes INF, Carloni AC, Rosa MN, da Silva LS, Evangelista AF, et al. Role of glioblastoma stem cells in cancer therapeutic resistance: a perspective on antineoplastic agents from natural sources and chemical derivatives. Stem Cell Res Ther. 2021;12(1):206.
- Anderson NM, Simon MC. The tumor microenvironment. Curr Biol. 2020;30(16): R921-r5.
- Annovazzi L, Mellai M, Caldera V, Valente G, Schiffer D. SOX2 expression and amplification in gliomas and glioma cell lines. Cancer Genomics Proteomics. 2011 May-Jun;8(3):139-47.
- Arai H, Ikota H, Sugawara K, Nobusawa S, Hirato J, Nakazato Y. Nestin expression in brain tumors: its utility for pathological diagnosis and correlation with the prognosis of high-grade gliomas. Brain Tumor Pathol. 2012;29(3):160-7.
- Arita H, Narita Y, Fukushima S, Tateishi K, Matsushita Y, Yoshida A, et al. Upregulating mutations in the TERT promoter commonly occur in adult malignant gliomas and are strongly associated with total 1p19q loss. Acta Neuropathol. 2013;126(2):267-76.
- Atkin-Smith GK, Tixeira R, Paone S, Mathivanan S, Collins C, Liem M, et al. A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure. Nat Commun. 2015; 6:7439.
- Authier A, Farrand KJ, Broadley KW, Ancelet LR, Hunn MK, Stone S, et al. Enhanced immunosuppression by therapy-exposed glioblastoma multiforme tumor cells. Int J Cancer. 2015;136(11):2566-78.
- Avril T, Vauleon E, Tanguy-Royer S, Mosser J, Quillien V. Mechanisms of immunomodulation in human glioblastoma. Immunotherapy. 2011;3(4 Suppl):42-4.
- Bacchi S, Palumbo P, Sponta A, Coppolino MF. Clinical pharmacology of non-steroidal anti-inflammatory drugs: a review. Antiinflamm Antiallergy Agents Med Chem. 2012;11(1):52-64.
- Bachoo RM, Maher EA, Ligon KL, Sharpless NE, Chan SS, You MJ, Tang Y, DeFrances J, Stover E, Weissleder R, Rowitch DH, Louis DN, DePinho RA. Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. Cancer Cell. 2002 Apr;1(3):269-77.
- Bader JE, Voss K, Rathmell JC. Targeting Metabolism to Improve the Tumor Microenvironment for Cancer Immunotherapy. Mol Cell. 2020;78(6):1019-33.
- Bae SH, Jung ES, Park YM, Kim BS, Kim BK, Kim DG, et al. Expression of cyclooxygenase-2 (COX-2) in hepatocellular carcinoma and growth inhibition of hepatoma cell lines by a COX-2 inhibitor, NS-398. Clin Cancer Res. 2001;7(5):1410-8.

- Baek JY, Hur W, Wang JS, Bae SH, Yoon SK. Selective COX-2 inhibitor, NS-398, suppresses cellular proliferation in human hepatocellular carcinoma cell lines via cell cycle arrest. World J Gastroenterol. 2007;13(8):1175-81.

- Baghban R, Roshangar L, Jahanban-Esfahlan R, Seidi K, Ebrahimi-Kalan A, Jaymand M, et al. Tumor microenvironment complexity and therapeutic implications at a glance. Cell Commun Signal. 2020;18(1):59.

- Balaj L, Lessard R, Dai L, Cho YJ, Pomeroy SL, Breakefield XO, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. Nat Commun. 2011; 2:180.

- Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357(9255):539-45.

- Balla MMS, Yadav HD, Pandey BN. Tumorsphere assay provides a better in vitro method for cancer stem-like cells enrichment in A549 lung adenocarcinoma cells. Tissue Cell. 2019; 60:21-4.

- Basheer AS, Abas F, Othman I, Naidu R. Role of Inflammatory Mediators, Macrophages, and Neutrophils in Glioma Maintenance and Progression: Mechanistic Understanding and Potential Therapeutic Applications. Cancers (Basel). 2021;13(16).

- Bautista F, Paci A, Minard-Colin V, Dufour C, Grill J, Lacroix L, et al. Vemurafenib in pediatric patients with BRAFV600E mutated high-grade gliomas. Pediatr Blood Cancer. 2014;61(6):1101-3.

- Behling F, Barrantes-Freer A, Skardelly M, Nieser M, Christians A, Stockhammer F, et al. Frequency of BRAF V600E mutations in 969 central nervous system neoplasms. Diagn Pathol. 2016;11(1):55.

- Berdis AJ. Examining the Role of Specialized DNA Polymerases in the Development of Temozolomide Resistance in Glioblastoma Multiforme. OBM Neurobiology 2021; 5(2): 096.

- Bergqvist F, Ossipova E, Idborg H, Raouf J, Checa A, Englund K, et al. Inhibition of mPGES-1 or COX-2 Results in Different Proteomic and Lipidomic Profiles in A549 Lung Cancer Cells. Front Pharmacol. 2019; 10:636.

- Bijnsdorp IV, van den Berg J, Kuipers GK, Wedekind LE, Slotman BJ, van Rijn J, et al. Radiosensitizing potential of the selective cyclooygenase-2 (COX-2) inhibitor meloxicam on human glioma cells. J Neurooncol. 2007;85(1):25-31.

- Binabaj MM, Bahrami A, ShahidSales S, Joodi M, Joudi Mashhad M, Hassanian SM, et al. The prognostic value of MGMT promoter methylation in glioblastoma: A meta-analysis of clinical trials. J Cell Physiol. 2018;233(1):378-86.

- Birzu C, French P, Caccese M, Cerretti G, Idbaih A, Zagonel V, et al. Recurrent Glioblastoma: From Molecular Landscape to New Treatment Perspectives. Cancers (Basel). 2020;13(1).

- Bischof J, Westhoff M-A, Wagner JE, Halatsch M-E, Trentmann S, Knippschild U, et al. Cancer stem cells: The potential role of autophagy, proteolysis, and cathepsins in glioblastoma stem cells. Tumor Biology. 2017;39(3):1010428317692227.

- Biserova K, Jakovlevs A, Uljanovs R, Strumfa I. Cancer Stem Cells: Significance in Origin, Pathogenesis and Treatment of Glioblastoma. Cells. 2021;10(3).

- Brandenburg S, Müller A, Turkowski K, Radev YT, Rot S, Schmidt C, et al. Resident microglia rather than peripheral macrophages promote vascularization in brain tumors and are source of alternative pro-angiogenic factors. Acta Neuropathol. 2016;131(3):365-78.

- Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. Cell. 2013;155(2):462-77.

- Brito C, Azevedo A, Esteves S, Marques AR, Martins C, Costa I, et al. Clinical insights gained by refining the 2016 WHO classification of diffuse gliomas with: EGFR amplification, TERT mutations, PTEN deletion and MGMT methylation. BMC Cancer. 2019;19(1):968.

- Broekman ML, Maas SLN, Abels ER, Mempel TR, Krichevsky AM, Breakefield XO. Multidimensional communication in the microenvirons of glioblastoma. Nat Rev Neurol. 2018;14(8):482-95.

- Buchanan FG, DuBois RN. Connecting COX-2 and Wnt in cancer. Cancer Cell. 2006;9(1):6-8.

- Buqué A, Galluzzi L. Modeling Tumor Immunology and Immunotherapy in Mice. Trends Cancer. 2018;4(9):599-601.
- Calinescu AA, Kauss MC, Sultan Z, Al-Holou WN, O'Shea SK. Stem cells for the treatment of glioblastoma: a 20-year perspective. CNS Oncol. 2021;10(2):Cns73.
- Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell. 1990;60(3):509-20.
- Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 2008 Oct 23;455(7216):1061-8.
- Cao Y, Qu J, Li C, Yang D, Hou K, Zheng H, et al. Celecoxib sensitizes gastric cancer to rapamycin via inhibition of the Cbl-b-regulated PI3K/Akt pathway. Tumour Biol. 2015;36(7):5607-15.
- Cartron PF, Loussouarn D, Campone M, Martin SA, Vallette FM. Prognostic impact of the expression/phosphorylation of the BH3-only proteins of the BCL-2 family in glioblastoma multiforme. Cell Death Dis. 2012;3(11):e421.
- Ceccarelli M, Barthel FP, Malta TM, Sabedot TS, Salama SR, Murray BA, et al. Molecular Profiling Reveals Biologically Discrete Subsets and Pathways of Progression in Diffuse Glioma. Cell. 2016;164(3):550-63.
- Chaichana KL, Jusue-Torres I, Navarro-Ramirez R, Raza SM, Pascual-Gallego M, Ibrahim A, et al. Establishing percent resection and residual volume thresholds affecting survival and recurrence for patients with newly diagnosed intracranial glioblastoma. Neuro Oncol. 2014;16(1):113-22.
- Chahal M, Abdulkarim B, Xu Y, Guiot MC, Easaw JC, Stifani N, Sabri S. O6-Methylguanine-DNA methyltransferase is a novel negative effector of invasion in glioblastoma multiforme. Mol Cancer Ther. 2012 Nov;11(11):2440-50. doi: 10.1158/1535-7163.MCT-11-0977
- Charles NA, Holland EC, Gilbertson R, Glass R, Kettenmann H. The brain tumor microenvironment. Glia. 2011;59(8):1169-80.
- Clark AJ, Ware JL, Chen MY, Graf MR, Van Meter TE, Dos Santos WG, et al. Effect of WT1 gene silencing on the tumorigenicity of human glioblastoma multiforme cells. J Neurosurg. 2010;112(1):18-25.
- Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 2009;30(7):1073-81.
- Coniglio SJ, Eugenin E, Dobrenis K, Stanley ER, West BL, Symons MH, et al. Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. Mol Med. 2012;18(1):519-27.
- Cowppli-Bony A, Bouvier G, Rué M, Loiseau H, Vital A, Lebailly P, et al. Brain tumors and hormonal factors: review of the epidemiological literature. Cancer Causes Control. 2011;22(5):697-714.
- Cruz JVR, Batista C, Afonso BH, Alexandre-Moreira MS, Dubois LG, Pontes B, et al. Obstacles to Glioblastoma Treatment Two Decades after Temozolomide. Cancers (Basel). 2022;14(13).
- Cuddapah VA, Robel S, Watkins S, Sontheimer H. A neurocentric perspective on glioma invasion. Nat Rev Neurosci. 2014;15(7):455-65.
- D'Alessio A, Proietti G, Sica G, Scicchitano BM. Pathological and Molecular Features of Glioblastoma and Its Peritumoral Tissue. Cancers (Basel). 2019;11(4).
- da Fonseca AC, Badie B. Microglia and macrophages in malignant gliomas: recent discoveries and implications for promising therapies. Clin Dev Immunol. 2013; 264124.
- Daisy Precilla S, Biswas I, Kuduvalli SS, Anitha TS. Crosstalk between PI3K/AKT/mTOR and WNT/β-Catenin signaling in GBM - Could combination therapy checkmate the collusion? Cell Signal. 2022; 95:110350.
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417(6892):949-54.
- de Vrij J, Maas SL, Kwappenberg KM, Schnoor R, Kleijn A, Dekker L, et al. Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells. Int J Cancer. 2015;137(7):1630-42.

- Dean PT, Hooks SB. Pleiotropic effects of the COX-2/PGE2 axis in the glioblastoma tumor microenvironment. Front Oncol. 2022; 12:1116014.
- Dirks PB. Brain tumor stem cells: the cancer stem cell hypothesis writ large. Mol Oncol. 2010;4(5):420-30.
- Djuzenova CS, Fiedler V, Memmel S, Katzer A, Hartmann S, Krohne G, et al. Actin cytoskeleton organization, cell surface modification and invasion rate of 5 glioblastoma cell lines differing in PTEN and p53 status. Exp Cell Res. 2015;330(2):346-57.
- Dymova MA, Kuligina EV, Richter VA. Molecular Mechanisms of Drug Resistance in Glioblastoma. Int J Mol Sci. 2021;22(12).
- Elmore KB, Schaff LR. DNA Repair Mechanisms and Therapeutic Targets in Glioma. Curr Oncol Rep. 2021;23(8):87.
- England B, Huang T, Karsy M. Current understanding of the role and targeting of tumor suppressor p53 in glioblastoma multiforme. Tumour Biol. 2013;34(4):2063-74.
- Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, et al. Chemotherapy resistance of glioblastoma stem cells. Cell Death Differ. 2006;13(7):1238-41.
- Esemen Y, Awan M, Parwez R, Baig A, Rahman S, Masala I, et al. Molecular Pathogenesis of Glioblastoma in Adults and Future Perspectives: A Systematic Review. Int J Mol Sci. 2022;23(5).
- Eskilsson E, Røsland GV, Solecki G, Wang Q, Harter PN, Graziani G, et al. EGFR heterogeneity and implications for therapeutic intervention in glioblastoma. Neuro Oncol. 2018;20(6):743-52.
- Fabian D, Guillermo Prieto Eibl MDP, Alnahhas I, Sebastian N, Giglio P, Puduvalli V, et al. Treatment of Glioblastoma (GBM) with the Addition of Tumor-Treating Fields (TTF): A Review. Cancers (Basel). 2019;11(2).
- Fan X, Khaki L, Zhu TS, Soules ME, Talsma CE, Gul N, et al. NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells. 2010;28(1):5-16.
- Fedele M, Cerchia L, Pegoraro S, Sgarra R, Manfioletti G. Proneural-Mesenchymal Transition: Phenotypic Plasticity to Acquire Multitherapy Resistance in Glioblastoma. Int J Mol Sci. 2019;20(11).
- huang J, Kessler AF, Monoranu CM, Ernestus RI, Löhr M, Hagemann C. Changes of O(6)-Methylguanine DNA Methyltransferase (MGMT) Promoter Methylation in Glioblastoma Relapse-A Meta-Analysis Type Literature Review. Cancers (Basel). 2019;11(12).
- Ferreira MT, Gomes RN, Panagopoulos AT, de Almeida FG, Veiga JCE, Colquhoun A. Opposing roles of PGD (2) in GBM. Prostaglandins Other Lipid Mediat. 2018; 134:66-76.
- Ferreira MT, Miyake JA, Gomes RN, Feitoza F, Stevannato PB, da Cunha AS, et al. Cyclooxygenase Inhibition Alters Proliferative, Migratory, and Invasive Properties of Human Glioblastoma Cells In Vitro. Int J Mol Sci. 2021;22(9).
- Frandsen S, Broholm H, Larsen VA, Grunnet K, Møller S, Poulsen HS, et al. Clinical Characteristics of Gliosarcoma and Outcomes from Standardized Treatment Relative to Conventional Glioblastoma. Front Oncol. 2019; 9:1425.
- Franovic A, Gunaratnam L, Smith K, Robert I, Patten D, Lee S. Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. Proc Natl Acad Sci U S A. 2007;104(32):13092-7.
- Friedl P, Vischer P, Freyberg MA. The role of thrombospondin-1 in apoptosis. Cell Mol Life Sci.
- Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, Otomo S. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins. 1994;47(1):55-9.
- Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol. 2013;14(10):1014-22.

- Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res. 2004;64(19):7011-21.
- Gangemi RM, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, et al. SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem Cells. 2009;27(1):40-8.
- Garnier D, Meehan B, Kislinger T, Daniel P, Sinha A, Abdulkarim B, et al. Divergent evolution of temozolomide resistance in glioblastoma stem cells is reflected in extracellular vesicles and coupled with radiosensitization. Neuro Oncol. 2018;20(2):236-48.
- Garros-Regulez L, Garcia I, Carrasco-Garcia E, Lantero A, Aldaz P, Moreno-Cugnon L, et al. Targeting SOX2 as a Therapeutic Strategy in Glioblastoma. Front Oncol. 2016; 6:222.
- Gittleman H, Ostrom QT, Stetson LC, Waite K, Hodges TR, Wright CH, et al. Sex is an important prognostic factor for glioblastoma but not for nonglioblastoma. Neurooncol Pract. 2019;6(6):451-62.
- Giusti I, Delle Monache S, Di Francesco M, Sanità P, D'Ascenzo S, Gravina GL, et al. From glioblastoma to endothelial cells through extracellular vesicles: messages for angiogenesis. Tumour Biol. 2016;37(9):12743-53.
- Giusti I, Di Francesco M, Cantone L, D'Ascenzo S, Bollati V, Carta G, et al. Time-dependent release of extracellular vesicle subpopulations in tumor CABA I cells. Oncol Rep. 2015;34(5):2752-9.
- Godlewski J, Ferrer-Luna R, Rooj AK, Mineo M, Ricklefs F, Takeda YS, et al. MicroRNA Signatures and Molecular Subtypes of Glioblastoma: The Role of Extracellular Transfer. Stem Cell Reports. 2017;8(6):1497-505.
- Gómez-Valenzuela F, Escobar E, Pérez-Tomás R, Montecinos VP. The Inflammatory Profile of the Tumor Microenvironment, Orchestrated by Cyclooxygenase-2, Promotes Epithelial-Mesenchymal Transition. Front Oncol. 2021; 11:686792.
- Gouazé-Andersson V, Delmas C, Taurand M, Martinez-Gala J, Evrard S, Mazoyer S, et al. FGFR1 Induces Glioblastoma Radioresistance through the PLCγ/Hif1α Pathway. Cancer Res. 2016;76(10):3036-44.
- Gritsch S, Batchelor TT, Gonzalez Castro LN. Diagnostic, therapeutic, and prognostic implications of the 2021 World Health Organization classification of tumors of the central nervous system. Cancer. 2022;128(1):47-58.
- Grochans S, Cybulska AM, Simińska D, Korbecki J, Kojder K, Chlubek D, et al. Epidemiology of Glioblastoma Multiforme-Literature Review. Cancers (Basel). 2022;14(10).
- Guo JY, White E. Autophagy, Metabolism, and Cancer. Cold Spring Harb Symp Quant Biol. 2016; 81:73-8.
- Gupta SC, Kunnumakkara AB, Aggarwal S, Aggarwal BB. Inflammation, a Double-Edge Sword for Cancer and Other Age-Related Diseases. Front Immunol. 2018; 9:2160.
- Gurpinar E, Grizzle WE, Piazza GA. COX-Independent Mechanisms of Cancer Chemoprevention by Anti-Inflammatory Drugs. Front Oncol. 2013; 3:181.
- Gustafson MP, Lin Y, New KC, Bulur PA, O'Neill BP, Gastineau DA, et al. Systemic immune suppression in glioblastoma: the interplay between CD14+HLA-DRlo/neg monocytes, tumor factors, and dexamethasone. Neuro Oncol. 2010;12(7):631-44.
- Haddad AF, Young JS, Amara D, Berger MS, Raleigh DR, Aghi MK, et al. Mouse models of glioblastoma for the evaluation of novel therapeutic strategies. Neurooncol Adv. 2021;3(1): vdab100.
- Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. Nat Neurosci. 2016;19(1):20-7.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
- Hanif F, Muzaffar K, Perveen K, Malhi SM, Simjee Sh U. Glioblastoma Multiforme: A Review of its Epidemiology and Pathogenesis through Clinical Presentation and Treatment. Asian Pac J Cancer Prev. 2017;18(1):3-9.
- Harris RE. Cyclooxygenase-2 (cox-2) blockade in the chemoprevention of cancers of the colon, breast, prostate, and lung. Inflammopharmacology. 2009;17(2):55-67.

- Hashemi Goradel N, Najafi M, Salehi E, Farhood B, Mortezaee K. Cyclooxygenase-2 in cancer: A review. J Cell Physiol. 2019;234(5):5683-99.
- Hassn Mesrati M, Behrooz AB, A YA, Syahir A. Understanding Glioblastoma Biomarkers: Knocking a Mountain with a Hammer. Cells. 2020;9(5).
- He J, Mansouri A, Das S. Alpha Thalassemia/Mental Retardation Syndrome X-Linked, the Alternative Lengthening of Telomere Phenotype, and Gliomagenesis: Current Understandings and Future Potential. Front Oncol. 2017; 7:322.
- Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005;352(10):997-1003.
- Heimberger AB, Suki D, Yang D, Shi W, Aldape K. The natural history of EGFR and EGFRvIII in glioblastoma patients. J Transl Med. 2005; 3:38.
- Hellwinkel JE, Redzic JS, Harland TA, Gunaydin D, Anchordoquy TJ, Graner MW. Glioma-derived extracellular vesicles selectively suppress immune responses. Neuro Oncol. 2016;18(4):497-506.
- Herbener VJ, Burster T, Goreth A, Pruss M, von Bandemer H, Baisch T, et al. Considering the Experimental use of Temozolomide in Glioblastoma Research. Biomedicines. 2020;8(6).
- Hjelmeland AB, Wu Q, Wickman S, Eyler C, Heddleston J, Shi Q, et al. Targeting A20 decreases glioma stem cell survival and tumor growth. PLoS Biol. 2010;8(2): e1000319.
- Honorato JR, Hauser-Davis RA, Saggioro EM, Correia FV, Sales-Junior SF, Soares LOS, et al. Role of Sonic hedgehog signaling in cell cycle, oxidative stress, and autophagy of temozolomide resistant glioblastoma. J Cell Physiol. 2020;235(4):3798-814.
- Hopkins BD, Hodakoski C, Barrows D, Mense SM, Parsons RE. PTEN function: the long and the short of it. Trends Biochem Sci. 2014;39(4):183-90.
- Huang DS, Shen KZ, Wei JF, Liang TB, Zheng SS, Xie HY. Specific COX-2 inhibitor NS398 induces apoptosis in human liver cancer cell line HepG2 through BCL-2. World J Gastroenterol. 2005;11(2):204-7.
- Huang M, Zhang D, Wu JY, Xing K, Yeo E, Li C, et al. Wnt-mediated endothelial transformation into mesenchymal stem cell-like cells induces chemoresistance in glioblastoma. Sci Transl Med. 2020;12(532).
- Infantino V, Santarsiero A, Convertini P, Todisco S, Iacobazzi V. Cancer Cell Metabolism in Hypoxia: Role of HIF-1 as Key Regulator and Therapeutic Target. Int J Mol Sci. 2021;22(11).
- Inoue K, Ogawa H, Yamagami T, Soma T, Tani Y, Tatekawa T, et al. Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels. Blood. 1996;88(6):2267-78.
- Izumoto S, Tsuboi A, Oka Y, Suzuki T, Hashiba T, Kagawa N, et al. Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme. J Neurosurg. 2008;108(5):963-71.
- Jadoon SS, Ilyas U, Zafar H, Paiva-Santos AC, Khan S, Khan SA, et al. Genomic and Epigenomic Features of Glioblastoma Multiforme and its Biomarkers. J Oncol. 2022; 4022960.
- Jalota A, Kumar M, Das BC, Yadav AK, Chosdol K, Sinha S. A drug combination targeting hypoxia induced chemoresistance and stemness in glioma cells. Oncotarget. 2018;9(26):18351-66.
- Janjua TI, Rewatkar P, Ahmed-Cox A, Saeed I, Mansfeld FM, Kulshreshtha R, et al. Frontiers in the treatment of glioblastoma: Past, present and emerging. Adv Drug Deliv Rev. 2021; 171:108-38.
- Jetten N, Verbruggen S, Gijbels MJ, Post MJ, De Winther MP, Donners MM. Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. Angiogenesis. 2014;17(1):109-18.
- Jiang J, Qiu J, Li Q, Shi Z. Prostaglandin E2 Signaling: Alternative Target for Glioblastoma? Trends Cancer. 2017;3(2):75-8.
- Jiang T, Nam DH, Ram Z, Poon WS, Wang J, Boldbaatar D, et al. Clinical practice guidelines for the management of adult diffuse gliomas. Cancer Lett. 2021; 499:60-72.

- Jiapaer S, Furuta T, Tanaka S, Kitabayashi T, Nakada M. Potential Strategies Overcoming the Temozolomide Resistance for Glioblastoma. Neurol Med Chir (Tokyo). 2018;58(10):405-21.

- Jimenez-Pascual A, Hale JS, Kordowski A, Pugh J, Silver DJ, Bayik D, et al. ADAMDEC1 Maintains a Growth Factor Signaling Loop in Cancer Stem Cells. Cancer Discov. 2019;9(11):1574-89.

- Jimenez-Pascual A, Siebzehnrubl FA. Fibroblast Growth Factor Receptor Functions in Glioblastoma. Cells. 2019;8(7).

- Joensuu H, Puputti M, Sihto H, Tynninen O, Nupponen NN. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol. 2005;207(2):224-31.

- Joensuu H, Puputti M, Sihto H, Tynninen O, Nupponen NN. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol. 2005;207(2):224-31.

- Joki T, Heese O, Nikas DC, Bello L, Zhang J, Kraeft SK, et al. Expression of cyclooxygenase 2 (COX-2) in human glioma and in vitro inhibition by a specific COX-2 inhibitor, NS-398. Cancer Res. 2000;60(17):4926-31.

- Kalimuthu S, Gangadaran P, Rajendran RL, Zhu L, Oh JM, Lee HW, et al. A New Approach for Loading Anticancer Drugs Into Mesenchymal Stem Cell-Derived Exosome Mimetics for Cancer Therapy. Front Pharmacol. 2018; 9:1116.

- Kang H, Lee H, Kim D, Kim B, Kang J, Kim HY, et al. Targeting Glioblastoma Stem Cells to Overcome Chemoresistance: An Overview of Current Therapeutic Strategies. Biomedicines. 2022;10(6):1308.

- Kanzawa T, Bedwell J, Kondo Y, Kondo S, Germano IM. Inhibition of DNA repair for sensitizing resistant glioma cells to temozolomide. J Neurosurg. 2003;99(6):1047-52.

- Karayan-Tapon L, Quillien V, Guilhot J, Wager M, Fromont G, Saikali S, et al. Prognostic value of O6-methylguanine-DNA methyltransferase status in glioblastoma patients, assessed by five different methods. J Neurooncol. 2010;97(3):311-22.

- Kerschbaumer J, Schmidt FA, Grams AE, Nowosielski M, Pinggera D, Brawanski KR, et al. Dual Anti-angiogenic Chemotherapy with Temozolomide and Celecoxib in Selected Patients with Malignant Glioma Not Eligible for Standard Treatment. Anticancer Res. 2015;35(9):4955-60.

- Khabibov M, Garifullin A, Boumber Y, Khaddour K, Fernandez M, Khamitov F, et al. Signaling pathways and therapeutic approaches in glioblastoma multiforme (Review). Int J Oncol. 2022;60(6).

- Kijima N, Hosen N, Kagawa N, Hashimoto N, Kinoshita M, Oji Y, et al. Wilms' tumor 1 is involved in tumorigenicity of glioblastoma by regulating cell proliferation and apoptosis. Anticancer Res. 2014;34(1):61-7.

- Knobbe CB, Merlo A, Reifenberger G. Pten signaling in gliomas. Neuro Oncol. 2002;4(3):196-211.

- Knutson KL, Disis ML. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer Immunol Immunother. 2005;54(8):721-8.

- Komori T. Grading of adult diffuse gliomas according to the 2021 WHO Classification of Tumors of the Central Nervous System. Lab Invest. 2022 Feb;102(2):126-133

- Koshy M, Villano JL, Dolecek TA, Howard A, Mahmood U, Chmura SJ, et al. Improved survival time trends for glioblastoma using the SEER 17 population-based registries. J Neurooncol. 2012;107(1):207-12.

- Koul D. PTEN signaling pathways in glioblastoma. Cancer Biol Ther. 2008;7(9):1321-5.

- Kowalski-Chauvel A, Gouaze-Andersson V, Baricault L, Martin E, Delmas C, Toulas C, et al. Alpha6-Integrin Regulates FGFR1 Expression through the ZEB1/YAP1 Transcription Complex in Glioblastoma Stem Cells Resulting in Enhanced Proliferation and Stemness. Cancers (Basel). 2019;11(3).

- Kraus JA, Glesmann N, Beck M, Krex D, Klockgether T, Schackert G, et al. Molecular analysis of the PTEN, TP53 and CDKN2A tumor suppressor genes in long-term survivors of glioblastoma multiforme. J Neurooncol. 2000;48(2):89-94.

- Kucharzewska P, Christianson HC, Welch JE, Svensson KJ, Fredlund E, Ringnér M, et al. Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. Proc Natl Acad Sci U S A. 2013;110(18):7312-7.

- Kuipers GK, Slotman BJ, Wedekind LE, Stoter TR, Berg J, Sminia P, et al. Radiosensitization of human glioma cells by cyclooxygenase-2 (COX-2) inhibition: independent on COX-2 expression and dependent on the COX-2 inhibitor and sequence of administration. Int J Radiat Biol. 2007;83(10):677-85.

- Kurdi M, Butt NS, Baeesa S, Kuerban A, Maghrabi Y, Bardeesi A, et al. Sensitivity Assessment of Wilms Tumor Gene (WT1) Expression in Glioblastoma using qPCR and Immunohistochemistry and its Association with IDH1 Mutation and Recurrence Interval. Biologics. 2021; 15:289-97.

- Kurihara Y, Hatori M, Ando Y, Ito D, Toyoshima T, Tanaka M, et al. Inhibition of cyclooxygenase-2 suppresses the invasiveness of oral squamous cell carcinoma cell lines via down-regulation of matrix metalloproteinase-2 production and activation. Clin Exp Metastasis. 2009;26(5):425-32.

- Lang F, Liu Y, Chou FJ, Yang C. Genotoxic therapy and resistance mechanism in gliomas. Pharmacol Ther. 2021; 228:107922.

- Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CL, Rich JN. Cancer stem cells in glioblastoma. Genes Dev. 2015;29(12):1203-17.

- Latour M, Her NG, Kesari S, Nurmemmedov E. WNT Signaling as a Therapeutic Target for Glioblastoma. Int J Mol Sci. 2021;22(16).

- Le Rhun E, von Achenbach C, Lohmann B, Silginer M, Schneider H, Meetze K, et al. Profound, durable and MGMT-independent sensitivity of glioblastoma cells to cyclin-dependent kinase inhibition. Int J Cancer. 2019;145(1):242-53.

- Lee EJ, Choi EM, Kim SR, Park JH, Kim H, Ha KS, et al. Cyclooxygenase-2 promotes cell proliferation, migration and invasion in U2OS human osteosarcoma cells. Exp Mol Med. 2007;39(4):469-76.

- Lee SY. Temozolomide resistance in glioblastoma multiforme. Genes Dis. 2016;3(3):198-210.

- Li B, Severson E, Pignon JC, Zhao H, Li T, Novak J, et al. Comprehensive analyses of tumor immunity: implications for cancer immunotherapy. Genome Biol. 2016;17(1):174.

- Li J, Koczor CA, Saville KM, Hayat F, Beiser A, McClellan S, et al. Overcoming Temozolomide Resistance in Glioblastoma via Enhanced NAD (+) Bioavailability and Inhibition of Poly-ADP-Ribose Glycohydrolase. Cancers (Basel). 2022;14(15).

- Li S, Jiang M, Wang L, Yu S. Combined chemotherapy with cyclooxygenase-2 (COX-2) inhibitors in treating human cancers: Recent advancement. Biomed Pharmacother. 2020; 129:110389.

- Lim JS, Park Y, Lee BM, Kim HS, Yoon S. Co-treatment with Celecoxib or NS398 Strongly Sensitizes Resistant Cancer Cells to Antimitotic Drugs Independent of P-gp Inhibition. Anticancer Res. 2016;36(10):5063-70.

- Liu B, Qu L, Yan S. Cyclooxygenase-2 promotes tumor growth and suppresses tumor immunity. Cancer Cell Int. 2015; 15:106.

- Lopes Abath Neto O, Aldape K. Morphologic and Molecular Aspects of Glioblastomas. Neurosurg Clin N Am. 2021;32(2):149-58.

- Lottaz C, Beier D, Meyer K, Kumar P, Hermann A, Schwarz J, et al. Transcriptional profiles of CD133+ and CD133- glioblastoma-derived cancer stem cell lines suggest different cells of origin. Cancer Res. 2010;70(5):2030-40.

- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. 2007;114(2):97-109.

- Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol. 2016;131(6):803-20.

- Louis DN, Perry A, Wesseling P, Brat DJ, Cree IA, Figarella-Branger D, et al. The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. Neuro Oncol. 2021;23(8):1231-51.

- Lu C, Cui C, Liu B, Zou S, Song H, Tian H, et al. FERMT3 contributes to glioblastoma cell proliferation and chemoresistance to temozolomide through integrin mediated Wnt signaling. Neurosci Lett. 2017;657:77-83.
- Lu Y, Sun T-J, Chen Y-t, Cai Z-Y, Zhao J-Y, Miao F, et al. Targeting the Epithelial-to-Mesenchymal Transition in Cancer Stem Cells for a Better Clinical Outcome of Glioma. Technology in Cancer Research & Treatment. 2020; 19:1533033820948053.
- Ludwig K, Kornblum HI. Molecular markers in glioma. J Neurooncol. 2017;134(3):505-12.
- Lv D, Lu L, Hu Z, Fei Z, Liu M, Wei L, et al. Nestin Expression Is Associated with Poor Clinicopathological Features and Prognosis in Glioma Patients: an Association Study and Meta-analysis. Mol Neurobiol. 2017;54(1):727-35.
- Ma Q, Long W, Xing C, Chu J, Luo M, Wang HY, et al. Cancer Stem Cells and Immunosuppressive Microenvironment in Glioma. Front Immunol. 2018; 9:2924.
- Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 1998;273(22):13375-8.
- Magaña-Maldonado R, Chávez-Cortez EG, Olascoaga-Arellano NK, López-Mejía M, Maldonado-Leal FM, Sotelo J, et al. Immunological Evasion in Glioblastoma. Biomed Res Int. 2016; 7487313.
- Majchrzak-Celińska A, Misiorek JO, Kruhlenia N, Przybyl L, Kleszcz R, Rolle K, et al. COXIBs and 2,5-dimethylcelecoxib counteract the hyperactivated Wnt/β-catenin pathway and COX-2/PGE2/EP4 signaling in glioblastoma cells. BMC Cancer. 2021;21(1):493.
- Malmström A, Grønberg BH, Marosi C, Stupp R, Frappaz D, Schultz H, et al. Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. Lancet Oncol. 2012;13(9):916-26.
- Mao P, Joshi K, Li J, Kim SH, Li P, Santana-Santos L, et al. Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc Natl Acad Sci U S A. 2013;110(21):8644-9.
- Maraka S, Janku F. BRAF alterations in primary brain tumors. Discov Med. 2018;26(141):51-60.
- Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A, et al. Raf family kinases: old dogs have learned new tricks. Genes Cancer. 2011;2(3):232-60.
- Matsuo M, Yoshida N, Zaitsu M, Ishii K, Hamasaki Y. Inhibition of human glioma cell growth by a PHS-2 inhibitor, NS398, and a prostaglandin E receptor subtype EP1-selective antagonist, SC51089. J Neurooncol. 2004;66(3):285-92.
- Mattei V, Santilli F, Martellucci S, Delle Monache S, Fabrizi J, Colapietro A, et al. The Importance of Tumor Stem Cells in Glioblastoma Resistance to Therapy. Int J Mol Sci. 2021;22(8).
- Maurya, D.K. Colony CountJ: A User-Friendly Image J Add-on Program for Quantification of Different Colony Parameters in Clonogenic Assay. J. Clin. Toxicol. 2017, 7, 2161
- McBain C, Lawrie TA, Rogozińska E, Kernohan A, Robinson T, Jefferies S. Treatment options for progression or recurrence of glioblastoma: a network meta-analysis. Cochrane Database Syst Rev. 2021;5(1):Cd013579.
- McNamara C, Mankad K, Thust S, Dixon L, Limback-Stanic C, D'Arco F, et al. 2021 WHO classification of tumours of the central nervous system: a review for the neuroradiologist. Neuroradiology. 2022;64(10):1919-50.
- Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, et al. High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res. 2002;8(5):1167-71.
- Molinaro AM, Taylor JW, Wiencke JK, Wrensch MR. Genetic and molecular epidemiology of adult diffuse glioma. Nat Rev Neurol. 2019;15(7):405-17.
- Mondal S, Bhattacharya K, Mandal C. Nutritional stress reprograms dedifferention in glioblastoma multiforme driven by PTEN/Wnt/Hedgehog axis: a stochastic model of cancer stem cells. Cell Death Discov. 2018; 4:110.

- Montaldi AP, Godoy PR, Sakamoto-Hojo ET. APE1/REF-1 down-regulation enhances the cytotoxic effects of temozolomide in a resistant glioblastoma cell line. Mutat Res Genet Toxicol Environ Mutagen. 2015; 793:19-29.

- Mostofa AG, Punganuru SR, Madala HR, Al-Obaide M, Srivenugopal KS. The Process and Regulatory Components of Inflammation in Brain Oncogenesis. Biomolecules. 2017;7(2).

- Munoz JL, Rodriguez-Cruz V, Greco SJ, Nagula V, Scotto KW, Rameshwar P. Temozolomide induces the production of epidermal growth factor to regulate MDR1 expression in glioblastoma cells. Mol Cancer Ther. 2014;13(10):2399-411.

- Nagaraju GP, El-Rayes BF. Cyclooxygenase-2 in gastrointestinal malignancies. Cancer. 2019;125(8):1221-7.

- Nakahara Y, Okamoto H, Mineta T, Tabuchi K. Expression of the Wilms' tumor gene product WT1 in glioblastomas and medulloblastomas. Brain Tumor Pathol. 2004;21(3):113-6.

- Nakazawa MS, Keith B, Simon MC. Oxygen availability and metabolic adaptations. Nat Rev Cancer. 2016;16(10):663-73.

- Nandakumar P, Mansouri A, Das S. The Role of ATRX in Glioma Biology. Front Oncol. 2017;7:236.

- Nasrolahi A, Azizidoost S, Radoszkiewicz K, Najafi S, Ghaedrahmati F, Anbiyaee O, et al. Signaling pathways governing glioma cancer stem cells behavior. Cell Signal. 2023;101:110493.

- Nduom EK, Hadjipanayis CG, Van Meir EG. Glioblastoma cancer stem-like cells: implications for pathogenesis and treatment. Cancer J. 2012 Jan-Feb;18(1):100-6.

- Newcomb EW, Cohen H, Lee SR, Bhalla SK, Bloom J, Hayes RL, et al. Survival of patients with glioblastoma multiforme is not influenced by altered expression of p16, p53, EGFR, MDM2 or Bcl-2 genes. Brain Pathol. 1998;8(4):655-67.

- O'Neill CP, Gilligan KE, Dwyer RM. Role of Extracellular Vesicles (EVs) in Cell Stress Response and Resistance to Cancer Therapy. Cancers (Basel). 2019;11(2).

- O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Sci Transl Med. 2017;9(399).

- Ohtsuka J, Oshima H, Ezawa I, Abe R, Oshima M, Ohki R. Functional loss of p53 cooperates with the in vivo microenvironment to promote malignant progression of gastric cancers. Sci Rep. 2018;8(1):2291.

- Oliver L, Olivier C, Vallette FM. Prostaglandin E 2 plays a major role in glioma resistance and progression. Translational Cancer Research. 2016: S1073-S7.

- Olympios N, Gilard V, Marguet F, Clatot F, Di Fiore F, Fontanilles M. TERT Promoter Alterations in Glioblastoma: A Systematic Review. Cancers (Basel). 2021;13(5).

- Oprita A, Baloi S-C, Staicu G-A, Alexandru O, Tache DE, Danoiu S, et al. Updated Insights on EGFR Signaling Pathways in Glioma. International Journal of Molecular Sciences. 2021;22(2):587.

- Orasanu CI, Aschie M, Deacu M, Mocanu L, Voda RI, Topliceanu TS, et al. Morphogenetic and Imaging Characteristics in Giant Cell Glioblastoma. Current Oncology. 2022;29(8):5316-23.

- Osborn AG, Louis DN, Poussaint TY, Linscott LL, Salzman KL. The 2021 World Health Organization Classification of Tumors of the Central Nervous System: What Neuroradiologists Need to Know. AJNR Am J Neuroradiol. 2022;43(7):928-37.

- Ostermann S, Csajka C, Buclin T, Leyvraz S, Lejeune F, Decosterd LA, et al. Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients. Clin Cancer Res. 2004;10(11):3728-36.

- Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE, et al. The epidemiology of glioma in adults: a "state of the science" review. Neuro Oncol. 2014;16(7):896-913.

- Ostrom QT, Cioffi G, Waite K, Kruchko C, Barnholtz-Sloan JS. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2014-2018. Neuro Oncol. 2021 Oct 5;23(12 Suppl 2): iii1-iii105.

- Ostrom QT, Patil N, Cioffi G, Waite K, Kruchko C, Barnholtz-Sloan JS. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2013-2017. Neuro Oncol. 2020;22(12 Suppl 2): iv1-iv96.

- Osuka S, Van Meir EG. Overcoming therapeutic resistance in glioblastoma: the way forward. J Clin Invest. 2017;127(2):415-26.

- Pai SG, Carneiro BA, Mota JM, Costa R, Leite CA, Barroso-Sousa R, et al. Wnt/beta-catenin pathway: modulating anticancer immune response. J Hematol Oncol. 2017;10(1):101.

- Palumbo P, Lombardi F, Siragusa G, Dehcordi SR, Luzzi S, Cimini A, et al. Involvement of NOS2 Activity on Human Glioma Cell Growth, Clonogenic Potential, and Neurosphere Generation. Int J Mol Sci. 2018;19(9).

- Palumbo P, Miconi G, Cinque B, Lombardi F, La Torre C, Dehcordi SR, et al. NOS2 expression in glioma cell lines and glioma primary cell cultures: correlation with neurosphere generation and SOX-2 expression. Oncotarget. 2017;8(15):25582-98.

- Pang LY, Hurst EA, Argyle DJ. Cyclooxygenase-2: A Role in Cancer Stem Cell Survival and Repopulation of Cancer Cells during Therapy. Stem Cells Int. 2016; 2048731.

- Park CM, Park MJ, Kwak HJ, Moon SI, Yoo DH, Lee HC, et al. Induction of p53-mediated apoptosis and recovery of chemosensitivity through p53 transduction in human glioblastoma cells by cisplatin. Int J Oncol. 2006;28(1):119-25.

- Parney IF. Basic concepts in glioma immunology. Adv Exp Med Biol. 2012; 746:42-52.

- Patil N, Kelly ME, Yeboa DN, Buerki RA, Cioffi G, Balaji S, et al. Epidemiology of brainstem high-grade gliomas in children and adolescents in the United States, 2000-2017. Neuro Oncol. 2021;23(6):990-8.

- Peden AE, Franklin RC, Queiroga AC. Epidemiology, risk factors and strategies for the prevention of global unintentional fatal drowning in people aged 50 years and older: a systematic review. Inj Prev. 2018;24(3):240-7.

- Pellerino, A., Caccese, M., Padovan, M. *et al.* Epidemiology, risk factors, and prognostic factors of gliomas. *Clin Transl Imaging. 2022;* **10**, 467–475

- Perez A, Huse JT. The Evolving Classification of Diffuse Gliomas: World Health Organization Updates for 2021. Curr Neurol Neurosci Rep. 2021;21(12):67.

- Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat. 2007;28(6):622-9.

- Pontén F, Jirström K, Uhlen M. The Human Protein Atlas--a tool for pathology. J Pathol. 2008;216(4):387-93.

- Pritchard-Jones K. The Wilms tumour gene, WT1, in normal and abnormal nephrogenesis. Pediatr Nephrol. 1999;13(7):620-5.

- Pu D, Yin L, Huang L, Qin C, Zhou Y, Wu Q, et al. Cyclooxygenase-2 Inhibitor: A Potential Combination Strategy with Immunotherapy in Cancer. Front Oncol. 2021; 11:637504.

- Pyrko P, Soriano N, Kardosh A, Liu YT, Uddin J, Petasis NA, et al. Downregulation of survivin expression and concomitant induction of apoptosis by celecoxib and its non-cyclooxygenase-2-inhibitory analog, dimethyl-celecoxib (DMC), in tumor cells in vitro and in vivo. Mol Cancer. 2006; 5:19.

- Qian Z, Ren L, Wu D, Yang X, Zhou Z, Nie Q, et al. Overexpression of FoxO3a is associated with glioblastoma progression and predicts poor patient prognosis. Int J Cancer. 2017;140(12):2792-804.

- Qiu J, Shi Z, Jiang J. Cyclooxygenase-2 in glioblastoma multiforme. Drug Discov Today. 2017;22(1):148-56.

- Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423-37.

- Quezada C, Torres Á, Niechi I, Uribe D, Contreras-Duarte S, Toledo F, et al. Role of extracellular vesicles in glioma progression. Mol Aspects Med. 2018; 60:38-51.

- Rajaratnam V, Islam MM, Yang M, Slaby R, Ramirez HM, Mirza SP. Glioblastoma: Pathogenesis and Current Status of Chemotherapy and Other Novel Treatments. Cancers (Basel). 2020;12(4).

- Ramos AD, Magge RS, Ramakrishna R. Molecular Pathogenesis and Emerging Treatment for Glioblastoma. World Neurosurg. 2018;116:495-504.

- Rich JN, Hans C, Jones B, Iversen ES, McLendon RE, Rasheed BK, et al. Gene expression profiling and genetic markers in glioblastoma survival. Cancer Res. 2005;65(10):4051-8.

- Ricklefs F, Mineo M, Rooj AK, Nakano I, Charest A, Weissleder R, et al. Extracellular Vesicles from High-Grade Glioma Exchange Diverse Pro-oncogenic Signals That Maintain Intratumoral Heterogeneity. Cancer Res. 2016;76(10):2876-81.

- Riera-Domingo C, Audigé A, Granja S, Cheng WC, Ho PC, Baltazar F, et al. Immunity, Hypoxia, and Metabolism-the Ménage à Trois of Cancer: Implications for Immunotherapy. Physiol Rev. 2020;100(1):1-102.

- Rodríguez-Camacho A, Flores-Vázquez JG, Moscardini-Martelli J, Torres-Ríos JA, Olmos-Guzmán A, Ortiz-Arce CS, et al. Glioblastoma Treatment: State-of-the-Art and Future Perspectives. Int J Mol Sci. 2022;23(13).

- Rominiyi O, Vanderlinden A, Clenton SJ, Bridgewater C, Al-Tamimi Y, Collis SJ. Tumour treating fields therapy for glioblastoma: current advances and future directions. Br J Cancer. 2021;124(4):697-709.

- Rosso L, Brock CS, Gallo JM, Saleem A, Price PM, Turkheimer FE, et al. A new model for prediction of drug distribution in tumor and normal tissues: pharmacokinetics of temozolomide in glioma patients. Cancer Res. 2009;69(1):120-7.

- Rouzer CA, Marnett LJ. Structural and Chemical Biology of the Interaction of Cyclooxygenase with Substrates and Non-Steroidal Anti-Inflammatory Drugs. Chem Rev. 2020;120(15):7592-641.

- Sampetrean O, Saya H. Characteristics of glioma stem cells. Brain Tumor Pathol. 2013;30(4):209-14.

- Schwartzbaum J, Ahlbom A, Malmer B, Lönn S, Brookes AJ, Doss H, et al. Polymorphisms associated with asthma are inversely related to glioblastoma multiforme. Cancer Res. 2005;65(14):6459-65.

- Senhaji N, Squalli Houssaini A, Lamrabet S, Louati S, Bennis S. Molecular and Circulating Biomarkers in Patients with Glioblastoma. Int J Mol Sci. 2022;23(13).

- Sharifzad F, Ghavami S, Verdi J, Mardpour S, Mollapour Sisakht M, Azizi Z, et al. Glioblastoma cancer stem cell biology: Potential theranostic targets. Drug Resist Updat. 2019; 42:35-45.

- Shen W, Li Y, Tang Y, Cummins J, Huard J. NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. Am J Pathol. 2005;167(4):1105-17.

- Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Rev Immunol. 2011;11(11):762-74.

- Shi G, Li D, Fu J, Sun Y, Li Y, Qu R, et al. Upregulation of cyclooxygenase-2 is associated with activation of the alternative nuclear factor kappa B signaling pathway in colonic adenocarcinoma. Am J Transl Res. 2015;7(9):1612-20.

- Shi J, Zhang L, Shen A, Zhang J, Wang Y, Zhao Y, et al. Clinical and biological significance of forkhead class box O 3a expression in glioma: mediation of glioma malignancy by transcriptional regulation of p27kip1. J Neurooncol. 2010;98(1):57-69.

- Shinojima N, Tada K, Shiraishi S, Kamiryo T, Kochi M, Nakamura H, et al. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. Cancer Res. 2003;63(20):6962-70.

- Shono T, Tofilon PJ, Bruner JM, Owolabi O, Lang FF. Cyclooxygenase-2 expression in human gliomas: prognostic significance and molecular correlations. Cancer Res. 2001;61(11):4375-81.

- Simmons ML, Lamborn KR, Takahashi M, Chen P, Israel MA, Berger MS, et al. Analysis of complex relationships between age, p53, epidermal growth factor receptor, and survival in glioblastoma patients. Cancer Res. 2001;61(3):1122-8.

- Singh N, Miner A, Hennis L, Mittal S. Mechanisms of temozolomide resistance in glioblastoma - a comprehensive review. Cancer Drug Resist. 2021;4(1):17-43.

- Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature. 2004;432(7015):396-401.

- Skaga E, Skaga I, Grieg Z, Sandberg CJ, Langmoen IA, Vik-Mo EO. The efficacy of a coordinated pharmacological blockade in glioblastoma stem cells with nine repurposed drugs using the CUSP9 strategy. J Cancer Res Clin Oncol. 2019;145(6):1495-507.

- Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008;10(12):1470-6.

- Śledzińska P, Bebyn MG, Furtak J, Kowalewski J, Lewandowska MA. Prognostic and Predictive Biomarkers in Gliomas. Int J Mol Sci. 2021;22(19).

- Smith C, Ironside JW. Diagnosis and pathogenesis of gliomas. *Curr Diagn Pathol*. 2007;13:180–180

- Spinelli C, Montermini L, Meehan B, Brisson AR, Tan S, Choi D, et al. Molecular subtypes and differentiation programmes of glioma stem cells as determinants of extracellular vesicle profiles and endothelial cell-stimulating activities. J Extracell Vesicles. 2018;7(1):1490144.

- Stark AM, van de Bergh J, Hedderich J, Mehdorn HM, Nabavi A. Glioblastoma: clinical characteristics, prognostic factors and survival in 492 patients. Clin Neurol Neurosurg. 2012;114(7):840-5.

- Steiner HH, Karcher S, Mueller MM, Nalbantis E, Kunze S, Herold-Mende C. Autocrine pathways of the vascular endothelial growth factor (VEGF) in glioblastoma multiforme: clinical relevance of radiation-induced increase of VEGF levels. J Neurooncol. 2004;66(1-2):129-38.

- Stepanenko AA, Chekhonin VP. On the Critical Issues in Temozolomide Research in Glioblastoma: Clinically Relevant Concentrations and MGMT-independent Resistance. Biomedicines. 2019;7(4).

- Stoyanov GS, Lyutfi E, Georgieva R, Georgiev R, Dzhenkov DL, Petkova L, et al. Reclassification of Glioblastoma Multiforme According to the 2021 World Health Organization Classification of Central Nervous System Tumors: A Single Institution Report and Practical Significance. Cureus. 2022;14(2):e21822.

- Strojnik T, Røsland GV, Sakariassen PO, Kavalar R, Lah T. Neural stem cell markers, nestin and musashi proteins, in the progression of human glioma: correlation of nestin with prognosis of patient survival. Surg Neurol. 2007;68(2):133-43; discussion 43-4.

- Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol. 2009;10(5):459-66.

- Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005;352(10):987-96.

- Soubannier V, Stifani S. NF-κB Signalling in Glioblastoma. Biomedicines. 2017 Jun 9;5(2):29. doi: 10.3390/biomedicines5020029

- Sui H, Zhou S, Wang Y, Liu X, Zhou L, Yin P, et al. COX-2 contributes to P-glycoprotein-mediated multidrug resistance via phosphorylation of c-Jun at Ser63/73 in colorectal cancer. Carcinogenesis. 2011;32(5):667-75.

- Sun WH, Su H, Zhang LJ, Shao Y, Xu HC, Zhang T, et al. [Effects of gastrin receptor antagonist and cyclooxygenase-2 inhibitor on proliferation and apoptosis of gastric cancer cell]. Zhonghua Yi Xue Za Zhi. 2006;86(4):250-4.

- Sunayama J, Sato A, Matsuda K, Tachibana K, Watanabe E, Seino S, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A, Kitanaka C. FoxO3a functions as a key integrator of cellular signals that control glioblastoma stem-like cell differentiation and tumorigenicity. Stem Cells. 2011 Sep;29(9):1327-37.

- Suwala AK, Koch K, Rios DH, Aretz P, Uhlmann C, Ogorek I, et al. Inhibition of Wnt/beta-catenin signaling downregulates expression of aldehyde dehydrogenase isoform 3A1 (ALDH3A1) to reduce resistance against temozolomide in glioblastoma in vitro. Oncotarget. 2018;9(32):22703-16.

- Tafani M, Di Vito M, Frati A, Pellegrini L, De Santis E, Sette G, et al. Pro-inflammatory gene expression in solid glioblastoma microenvironment and in hypoxic stem cells from human glioblastoma. J Neuroinflammation. 2011;8:32.

- Talhaoui I, Matkarimov BT, Tchenio T, Zharkov DO, Saparbaev MK. Aberrant base excision repair pathway of oxidatively damaged DNA: Implications for degenerative diseases. Free Radic Biol Med. 2017;107:266-77.

- Tamimi AF, Juweid M. Epidemiology and Outcome of Glioblastoma. In: De Vleeschouwer S, editor. Glioblastoma. Brisbane (AU): Codon Publications

- Copyright: The Authors.; 2017.

- Tan AC, Ashley DM, López GY, Malinzak M, Friedman HS, Khasraw M. Management of glioblastoma: State of the art and future directions. CA Cancer J Clin. 2020;70(4):299-312.

- Taylor JW, Schiff D. Treatment considerations for MGMT-unmethylated glioblastoma. Curr Neurol Neurosci Rep. 2015;15(1):507.

- Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018;7(1):1535750.

- Tompa M, Kalovits F, Nagy A, Kalman B. Contribution of the Wnt Pathway to Defining Biology of Glioblastoma. Neuromolecular Med. 2018;20(4):437-51.

- Tong D, Liu Q, Wang LA, Xie Q, Pang J, Huang Y, et al. The roles of the COX2/PGE2/EP axis in therapeutic resistance. Cancer Metastasis Rev. 2018;37(2-3):355-68.

- Tong N, He Z, Ma Y, Wang Z, Huang Z, Cao H, et al. Tumor Associated Macrophages, as the Dominant Immune Cells, Are an Indispensable Target for Immunologically Cold Tumor-Glioma Therapy? Front Cell Dev Biol. 2021; 9:706286.

- Torp SH, Solheim O, Skjulsvik AJ. The WHO 2021 Classification of Central Nervous System tumours: a practical update on what neurosurgeons need to know-a minireview. Acta Neurochir (Wien). 2022;164(9):2453-64.

- Torrisi F, Alberghina C, D'Aprile S, Pavone AM, Longhitano L, Giallongo S, et al. The Hallmarks of Glioblastoma: Heterogeneity, Intercellular Crosstalk and Molecular Signature of Invasiveness and Progression. Biomedicines. 2022;10(4).

- Tran B, Rosenthal MA. Survival comparison between glioblastoma multiforme and other incurable cancers. J Clin Neurosci. 2010;17(4):417-21.

- Trejo-Solís C, Serrano-Garcia N, Escamilla-Ramírez Á, Castillo-Rodríguez RA, Jimenez-Farfan D, Palencia G, et al. Autophagic and Apoptotic Pathways as Targets for Chemotherapy in Glioblastoma. Int J Mol Sci. 2018;19(12).

- Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell. 1998;93(5):705-16.

- Tuettenberg J, Grobholz R, Korn T, Wenz F, Erber R, Vajkoczy P. Continuous low-dose chemotherapy plus inhibition of cyclooxygenase-2 as an antiangiogenic therapy of glioblastoma multiforme. J Cancer Res Clin Oncol. 2005;131(1):31-40.

- Valtorta S, Lo Dico A, Raccagni I, Gaglio D, Belloli S, Politi LS, et al. Metformin and temozolomide, a synergic option to overcome resistance in glioblastoma multiforme models. Oncotarget. 2017;8(68):113090-104.

- van Linde ME, Brahm CG, de Witt Hamer PC, Reijneveld JC, Bruynzeel AME, Vandertop WP, et al. Treatment outcome of patients with recurrent glioblastoma multiforme: a retrospective multicenter analysis. J Neurooncol. 2017;135(1):183-92.

- Velpula KK, Guda MR, Sahu K, Tuszynski J, Asuthkar S, Bach SE, et al. Metabolic targeting of EGFRvIII/PDK1 axis in temozolomide resistant glioblastoma. Oncotarget. 2017;8(22):35639-55.
- Vuong HG, Altibi AMA, Duong UNP, Ngo HTT, Pham TQ, Fung KM, et al. BRAF Mutation is Associated with an Improved Survival in Glioma-a Systematic Review and Meta-analysis. Mol Neurobiol. 2018;55(5):3718-24.
- Wang D, Dubois RN. Eicosanoids and cancer. Nat Rev Cancer. 2010;10(3):181-93.
- Wang H, Xu T, Jiang Y, Xu H, Yan Y, Fu D, et al. The challenges and the promise of molecular targeted therapy in malignant gliomas. Neoplasia. 2015;17(3):239-55.
- Wang S, Chen C, Li J, Xu X, Chen W, Li F. The CXCL12/CXCR4 axis confers temozolomide resistance to human glioblastoma cells via up-regulation of FOXM1. J Neurol Sci. 2020;414:116837.
- Wang TJ, Huang MS, Hong CY, Tse V, Silverberg GD, Hsiao M. Comparisons of tumor suppressor p53, p21, and p16 gene therapy effects on glioblastoma tumorigenicity in situ. Biochem Biophys Res Commun. 2001;287(1):173-80.
- Wang X, Chen JX, Liu JP, You C, Liu YH, Mao Q. Gain of function of mutant TP53 in glioblastoma: prognosis and response to temozolomide. Ann Surg Oncol. 2014;21(4):1337-44.
- Wang Z, Sun D, Chen YJ, Xie X, Shi Y, Tabar V, et al. Cell Lineage-Based Stratification for Glioblastoma. Cancer Cell. 2020;38(3):366-79.e8.
- Weller M, Butowski N, Tran DD, Recht LD, Lim M, Hirte H, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. Lancet Oncol. 2017;18(10):1373-85.
- Weller M, Felsberg J, Hartmann C, Berger H, Steinbach JP, Schramm J, et al. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. J Clin Oncol. 2009;27(34):5743-50.
- Wen PY, Packer RJ. The 2021 WHO Classification of Tumors of the Central Nervous System: clinical implications. Neuro Oncol. 2021;23(8):1215-7.
- Wenger A, Carén H. Methylation Profiling in Diffuse Gliomas: Diagnostic Value and Considerations. Cancers (Basel). 2022;14(22).
- Westphal M, Maire CL, Lamszus K. EGFR as a Target for Glioblastoma Treatment: An Unfulfilled Promise. CNS Drugs. 2017;31(9):723-35.
- Wickström M, Dyberg C, Milosevic J, Einvik C, Calero R, Sveinbjörnsson B, et al. Wnt/β-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signalling prevents chemoresistance. Nat Commun. 2015; 6:8904.
- Wu M, Guan J, Li C, Gunter S, Nusrat L, Ng S, et al. Aberrantly activated Cox-2 and Wnt signaling interact to maintain cancer stem cells in glioblastoma. Oncotarget. 2017;8(47):82217-30.
- Wuebben EL, Rizzino A. The dark side of SOX2: cancer - a comprehensive overview. Oncotarget. 2017;8(27):44917-43.
- Würth R, Bajetto A, Harrison JK, Barbieri F, Florio T. CXCL12 modulation of CXCR4 and CXCR7 activity in human glioblastoma stem-like cells and regulation of the tumor microenvironment. Front Cell Neurosci. 2014; 8:144.
- Xie H, Simon MC. Oxygen availability and metabolic reprogramming in cancer. J Biol Chem. 2017;292(41):16825-32.
- Xu K, Zhang Z, Pei H, Wang H, Li L, Xia Q. FoxO3a induces temozolomide resistance in glioblastoma cells via the regulation of β-catenin nuclear accumulation. Oncol Rep. 2017;37(4):2391-7.
- Yamaguchi F, Saya H, Bruner JM, Morrison RS. Differential expression of two fibroblast growth factor-receptor genes is associated with malignant progression in human astrocytomas. Proc Natl Acad Sci U S A. 1994;91(2):484-8.

- Yan Y, Xu Z, Dai S, Qian L, Sun L, Gong Z. Targeting autophagy to sensitive glioma to temozolomide treatment. J Exp Clin Cancer Res. 2016; 35:23.
- Yang K, Niu L, Bai Y, Le W. Glioblastoma: Targeting the autophagy in tumorigenesis. Brain Res Bull. 2019; 153:334-40.
- Yang K, Wu Z, Zhang H, Zhang N, Wu W, Wang Z, et al. Glioma targeted therapy: insight into future of molecular approaches. Mol Cancer. 2022;21(1):39.
- Yao M, Lam EC, Kelly CR, Zhou W, Wolfe MM. Cyclooxygenase-2 selective inhibition with NS-398 suppresses proliferation and invasiveness and delays liver metastasis in colorectal cancer. Br J Cancer. 2004;90(3):712-9.
- Yekula A, Yekula A, Muralidharan K, Kang K, Carter BS, Balaj L. Extracellular Vesicles in Glioblastoma Tumor Microenvironment. Front Immunol. 2019; 10:3137.
- Yin CL, Lv SQ, Chen XY, Guo H. The role of glioma stem cells in glioma tumorigenesis. Front Biosci (Landmark Ed). 2014 Jan 1;19(5):818-24. doi: 10.2741/4249.
- Yu HP, Wang X-l, Su Y, Li Y-y, Li F, Xu S-q, editors. Anti-proliferation and Apoptosis Induced by Selective COX-2 Inhibitor in Human Esophageal Squamous Carcinoma Cells. The Journal of Applied Research 2005 Vol.5, 1.
- Zhang Y, Dube C, Gibert M, Jr., Cruickshanks N, Wang B, Coughlan M, et al. The p53 Pathway in Glioblastoma. Cancers (Basel). 2018;10(9).
- Zhao H, Wu L, Yan G, Chen Y, Zhou M, Wu Y, et al. Inflammation and tumor progression: signaling pathways and targeted intervention. Signal Transduct Target Ther. 2021;6(1):263.
- Zhou J, Schmid T, Frank R, Brüne B. PI3K/Akt is required for heat shock proteins to protect hypoxia-inducible factor 1alpha from pVHL-independent degradation. J Biol Chem. 2004;279(14):13506-13.
- Zong H, Verhaak RG, Canoll P. The cellular origin for malignant glioma and prospects for clinical advancements. Expert Rev Mol Diagn. 2012;12(4):383-94.

GRATITUDE AND ACKNOWLEDGEMENTS

I just arrived at the end of a truly life-changing experience. It was the most challenging passage of my life, full of sacrifices but certainly the most beautiful and critical of my professional career. In the following part, I express my gratitude and acknowledgments all the people near me during these years.

I will be forever thankful for my supervisor, Prof.ssa Paola Palumbo. The discussions with her, the exchange of ideas, and her feedback have been invaluable. Her unconditional professional guidance and support during these years made me succeed. She will be my inspiration as an example of excellence and the best role model for a scientific researcher and mentor.

Thank you to my doctoral colleague and friend, Francesca, who was always ready to help me in numerous ways. Many thanks to her and the other colleagues from the laboratory for all the collaborative work and their friendly support. I never felt like a 'foreigner.'

This Ph.D. degree was only possible with the corporation between the two universities. I gratefully acknowledge the Rector of the University of Elbasan, Prof. Skender Topi, and Prof. Maria Grazia Cifone for the excellent opportunity to conduct my Ph.D. studies in a European country.

I would also like to thank my mom and dad for the unconditional love, support, and constant encouragement I have received over the years.

To conclude, this thesis is dedicated to my husband, Leonard, and my sons, Leonis and Luis! These past three years have been a challenging academic and personal ride. I want to apologize for the missed time with my family and thank my husband for the unlimited support, for taking care of our children, and for sticking by my side every moment. Without him, indeed, I would not have had the courage to embark on this pathway. I love you!

Mitilda