Cheila Martins Brito
Licenciada em Biologia

Clinical implications of *PIK3CA* mutations in gliomas molecular subgroups

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientadora: Doutora Marta Sofia Pojo Sousa, Instituto Português de Oncologia
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Resumo

Os gliomas são os tumores malignos mais comuns e letais do sistema nervoso central. Em 2016, a classificação da organização mundial de saúde (OMS) incluiu as mutações no gene *IDH* e a codeleção 1p/19q como critérios de diagnóstico para os gliomas. Contudo, novos biomarcadores de diagnóstico, prognostico e resposta à terapia são necessários. Assim, as mutações no gene *PIK3CA* foram recentemente descritas como constitutivas, tornando-se um potencial alvo terapêutico.

O objetivo deste trabalho foi clarificar a relevância clínica das mutações no gene *PIK3CA* de acordo com a nova classificação da OMS, assim como o impacto de vários biomarcadores no diagnóstico, prognóstico e resposta à terapia em 437 amostras de gliomas.

A análise multivariada demonstrou que os grupos moleculares de gliomas têm maior valor de prognóstico que os histológicos (P<0.001). As deleções no gene *PTEN* constituem fatores de pior prognóstico em astrocitomas *IDH wildtype,* e de melhor prognóstico em GBM *IDH wildtype*. Contrariamente, a amplificação no gene *EGFR* e as mutações no gene *TERT* não tiveram impacto na sobrevivência dos doentes. Foi verificado que a amplificação no gene *EGFR* tem valor preditivo de resposta à radioterapia (P=0.007).

As mutações no gene *PIK3CA* foram mais frequentes em oligodendrogliomas (10%). H1047R e E542K foram as mutações mais comuns nos restantes subgrupos moleculares. Foram identificadas 3 variantes patogénicas não descritas no exão 20 (c.3112T>C, c.2988T>C, c.3040C>T) e uma polimórfica (c.3210A>G). Foi identificado, pela primeira vez, o polimorfismo rs45455192 (16%-24%) nos diferentes subgrupos moleculares, contudo sem valor de prognóstico. A análise das recidivas de gliomas mostrou que as mutações neste gene constituem eventos precoces mantidos durante a progressão tumoral.

Este estudo mostrou que a classificação molecular constitui um método preciso de previsão do *outcome* clínico e que as mutações no gene *PIK3CA* são pouco frequentes em gliomas, contudo parecem ser importantes na progressão tumoral.

Palavras – Chave: Gliomas, *PIK3CA*, mutações, classificação da OMS 2016, alvo terapêutico

Abstract

Gliomas are the most common and lethal malignant tumors of central nervous system. In 2016, World Health Organization (WHO) classification included *IDH* mutations and 1p/19q codeletion as a diagnostic criteria to define gliomas. However new biomarkers of diagnosis, prognosis and response to therapy are needed. In this context, *PIK3CA* mutations have been described as constitutive mutations seeming to be a good therapeutic target.

Our objective was to clarify the clinical importance of *PIK3CA* mutations according to the 2016 WHO classification, as well as the impact of several biomarkers on diagnosis, prognosis and response to therapy in 437 glioma samples.

According to the multivariate analysis performed, gliomas molecular subgroups have higher prognostic value than histological subgroups (P<0.001). *PTEN* deletions were considered prognostic factors of poor outcomes in astrocytomas *IDH* wildtype, while in GBM *IDH* wildtype were associated with better prognosis. On opposite, *EGFR* amplification and *TERT* mutations had no impact in the overall survival of patients. We verified that *EGFR* amplification had a predictive value of response to radiotherapy (P=0.007).

PIK3CA mutations were most common in *IDH* mutant + 1p/19q codeletion (oligodendrogliomas) (10%). H1047R and E542K were the most frequent mutations identified in the remaining gliomas molecular subgroups. Importantly, we found 3 unreported pathogenic variants in exon 20 of *PIK3CA* (c.3112T>C, c.2988T>C, c.3040C>T) and one polymorphic variant (c.3210A>G). For the first time, it was identified the rs45455192 polymorphism (16% - 24%) in the different gliomas molecular subgroups, although this polymorphism did not showed prognostic value. The recurrences analysis demonstrated that *PIK3CA* mutations constitute early events maintained during tumor progression.

Overall, this study showed molecular classification is a more accurate method to predict clinical outcome and despite *PIK3CA* mutations being present at low frequency in gliomas, they seem to be important for tumor progression.

Keywords: Gliomas, *PIK3CA*, mutations, 2016 WHO classification, therapeutic target

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List of abbreviations, symbols and conventions

A - Adenine
ALT - Alternative Lengthening of Telomeres
amp - amplification
ATRX - Alpha-Thalassemia Syndrome X-linked
bp - Base pairs
CIC - Capicua transcriptional repressor
C - Cytosine
CNS - Central Nervous System
COSMIC - Catalogue of Somatic Mutations in Cancer
CRISPR- CAS9 - Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9 nuclease
CRT - Chemoradiotherapy
DAXX- Death-associated protein 6
del - deletion
DNA - Deoxyribonucleic acid
dNTP's- Deoxynucleotide triphosphates
ddNTP's- Dideoxynucleotide triphosphates
EDTA - Ethylene Diamine Tetraacetic Acid
EGF - Epidermal Growth Factor
EGFR - Epidermal Growth Factor Receptor
ERK - Extracellular signal- Regulated Kinase
F- Female
FTB - Flow-through buffer
FUBP1- Far upstream element binding protein- 1
G - Guanine
GBM - Glioblastoma
HGMB - Human Gene Mutation Database
IDH - Isocitrate Desidrogenase
IPOLFG - Instituto Português de Oncologia de Lisboa Francisco Gentil
JNK - Jun K-terminal Kinase
M- Male
MAPK - Mitogen Activated Protein Kinase
Mdm2 - murine double minute 2
***MGMT* -** O-6-methylguanine-DNA methyltransferase
$MgCl_2$ - Magnesium Chloride
min - minutes
mTOR - Mammalian Target of Rapamycin
mut- mutation

n - number of samples

NA - *Not available*

NaAc - Sodium Acetate

NaCl - Sodium Chloride

NADP - Nicotinamide Adenine Dinucleotide Phosphate

NADPH - Reduced Nicotinamide Adenine Dinucleotide Phosphate

NOS - Not Otherwise Specified

p- short arm

***P* -** P-value

p110- catalytic subunit

p85 - regulatory subunit

PCR - Polymerase chain reaction

PCV - Procarbazine, lomustine, and vincristine

PDK - Phosphoinositide – dependent kinases

PH - Pleckstrin Homology

PI3K - Phosphatidylinositol 3-kinase

PIK3CA - Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PIP3 - Phosphatidylinositol 3,4,5 – triphosphate

PIP2 - Phosphatidylinositol 4,5- bisphosphate

PTEN - Phosphatase and Tensin homolog deleted on chromosome 10

q- long arm

QT - Chemotherapy

RAS -Rat Sarcoma Virus Homolog

RT - radiotherapy

SDS - Sodium Dodecyl Sulfate

sec - seconds

SIFT- Sorting Intolerant From Tolerant

SNP - Single Nucleotide Polymorphism

STAT - Signal Transducer and Activator of Transcription proteins

Std. - Standard

T- Thymidine

Ta - annealing temperature

TBE - Tris/Borate/EDTA

TE - Tris - EDTA buffer

TET - Ten-Eleven Translocation

TERT- Telomerase Reverse Transcriptase Promoter

TP53 - Tumor protein

TMZ- Temozolomide

TrisHCL - Tris(hydroxymethyl)aminomethane hydrochloride

UNG - Uracil - N –glycosylase

3'UTR - Untranslated region

VEP - Variant Effect Predictor

WHO - World Health Organization

wt - wildtype

X - stop codon

1. Introduction

1. Principles of carcinogenesis

Cancer is one of the major public health problems worldwide, being the second leading cause of death affecting countries with different income levels (World Health Organization (WHO), 2018). Recently, it was predicted the total number of new cancer cases diagnosed will increase around 70% over the next two decades paralleled by an increasing number of deaths (Montagnana and Lippi, 2017).

Carcinogenesis is described as a multistep process resulting from the influence of genetic and environmental factors, which drive to the progressive transition of a normal cell to a neoplastic state, due to the acquisition of several hallmarks (Hanahan and Weinberg, 2000). The hallmarks of cancer correspond to eight distinctive and complementary capabilities, purposed in 2000 and then complemented in 2011 by Hanahan and Weinberg (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

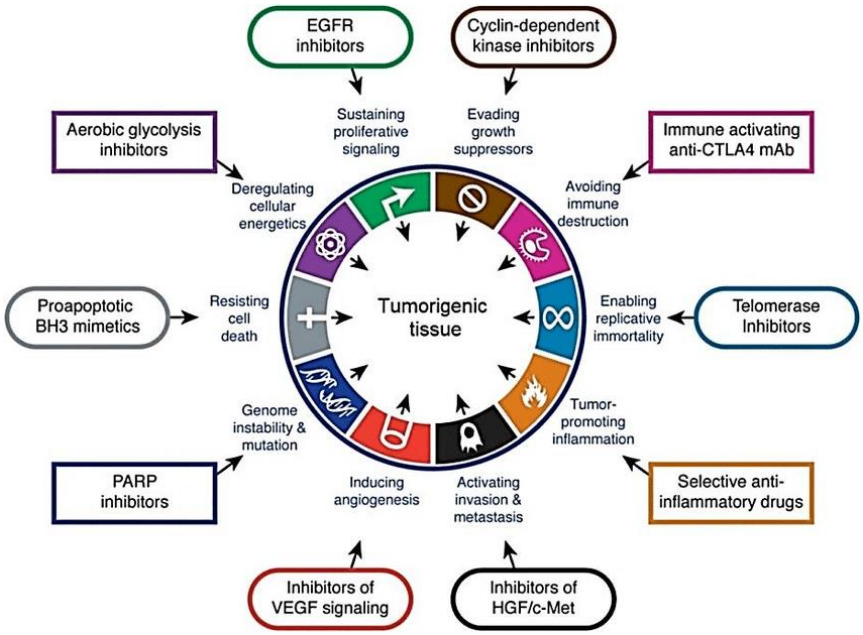


Figure 1.1 – Schematic representation of the 10 hallmarks of cancer and their respective targeting therapies. The scheme highlights the drugs which could interfere with each of the capabilities needed for tumor growth and progression. These therapeutic approaches correspond to strategies purposed by many investigators in an attempt to block the cancer progression, based on their transversal features. Adapted from Hanahan, D., (2011) *Cell*, *144*(5), 646-674.

Firstly in 2000, it was described six hallmarks of cancer to understand the features shared between the different types of neoplasms that explain the transition of normal cells into a neoplastic state (Hanahan and Weinberg, 2000). Sustaining the proliferative signaling, evading growth suppressors, resistance to cell death , enabling replicative immortality, capability to induce angiogenesis and activating invasion and metastasis were the six hallmarks of cancer defined (Figure 1.1) (Hanahan and Weinberg, 2000). These were the physiological changes noticed during the development of the malignant tumors, which play important roles in the malignant growth of cancer cells.

Furthermore, it was also mentioned two important enabling characteristics: genome instability

and tumor promoting inflammation (Hanahan and Weinberg, 2000). The breakdown in one or several genes belonging to the repair machinery and the increased sensitivity to mutagenic agents are the main reasons for the genome instability (Salk *et al*., 2010). Lately, it was also reported tumor associated inflammatory response stimulates tumorigenesis and tumor progression, because inflammation release bioactive molecules that facilitate invasion and metastasis (Colotta *et al*., 2009).

Then in 2011, Hanahan and Weinberg, introduced two new emerging hallmarks which are, capability to reprogram energy of metabolism and evade immune destruction (Figure 1.1), to contextualize the hallmarks according to the conceptual progress and recent advances on the last decade (Hanahan and Weinberg, 2011). Otto Warburg verified for the first time, that even in the presence of oxygen, cancer cells can reprogram their glucose metabolism and consequently the way how to produce energy, through glycolysis (Warburg, 1956a; Warburg, 1956b). In addition, the immune destruction is another barrier that tumor cells need to overcome, because the immune system is the primary entity responsible for the detection and elimination of cancer cells, preventing tumor formation (Hanahan and Weinberg, 2011).

Currently, these hallmarks continue to be an important source to understand the cancer biology, although this knowledge has been expanded and updated over the years.

Recently, the concept of tumorigenesis has been described as tissue and cell type specific, suggesting that this process is influenced by a context (Schneider *et al.,* 2017). Many studies have been investigated common therapeutic targets between the different types of tumors, but according to this statement those kinds of treatments are increasingly useless (Seshacharyulu *et al*., 2012; Zhang, 2012; Dillon and Miller, 2014). Even at the intratumoral level there is a high heterogeneity of cells, it is believed that cancer cells act as communities and that this cooperative behavior of subclones can influence disease progression (Tabassum and Polyak, 2015). The big challenge is to understand how these communities interact with each other, and how these dynamics change according to the tumor microenvironment. It is necessary to study these events and all the mechanisms by which cancer evolves to develop efficient therapies.

2. The tumors of Central Nervous System

The concept of brain tumors refers to a mixed group of neoplasms originating from intracranial tissues and meninges with degrees of malignancy ranging from benign to malignant (McKinney, 2004). Brain cancer constitutes a complex and heterogeneous group of tumors responsible for 3% of cancer cases worldwide (Miranda-Filho *et al*., 2016). Over time, the incidence of these tumors has increased and differs according to gender, age, race, ethnicity, and geography (Miranda-Filho *et al*., 2016; Ostrom *et al*., 2015). These tumors are rare when compared with other types of cancers, although they constitute an important source of morbidity and mortality (Miranda-Filho *et al*., 2016; De Robles *et al*., 2014). Recent studies have reported that primary brain malignancies represent only 1.4% of all cancers in adults, this incidence increases when benign and metastatic brain tumors are considered, which are more common (Vargo, 2017).

In the pediatric oncology field, the tumors of central nervous system (CNS) are the most

common solid tumors, representing more than 20% of all pediatric cases (Peris-Bonet *et al*., 2006). The annual incidence of pediatric brain tumors is estimated to be approximately 2.9 and 5.05/100,000 children in Europe and in the United States (US), respectively (Ostrom *et al*., 2015). Thus, it is possible to understand that pediatric and adult central nervous system tumors are two distinct realities, differing significantly not only in the epidemiology but also in the type of malignancies (Bondy *et al.*, 2008; Santos *et al*., 2016; Lannering *et al*., 2009).

Several types of CNS malignancies are described such as meningiomas, gliomas, medulloblastomas, choroid plexus tumors, germ cell tumors, neuronal and mixed neuronal - glial tumors, melanocytic tumors, lymphomas, between others (Louis *et al.,* 2016). Considering the set of benign tumors, meningiomas correspond to the most frequent type of brain tumor in the adult population (Wiemels *et al.,* 2010). On the other hand, the incidence of medulloblastomas, ependymomas and pilocytic astrocytoma decreases with age, since these neoplasms are more prevalent in children than in adults (Santos *et al*., 2016; Lannering *et al*., 2009; Bauchet *et al*., 2008; Alexiou *et al*., 2011).

In addition, epidemiological studies in many parts of the world have reported that brain tumors occur more frequently in men than in women, exhibiting a male: female incidence ratio that range from 1.5:1 to 3:1 (Sun *et al*., 2015; Sun e Rubin, 2012; Sun *et al*., 2014). Regarding risk factors, the exposure to moderate - high dose ionizing radiation is the single unequivocal environmental risk factor established for brain tumors (Thompson *et al*., 1994; Braganza *et al*., 2012). Besides this, another types of pathologies could elevate the tendency to develop brain tumors (Reilly, 2009).

3. Gliomas

Gliomas are among the most common malignant primary tumors of the CNS in adults, representing 81% of all malignant brain tumors (Ostrom *et al*., 2014; Ferris *et al*., 2017). This group of tumors comprises a great clinical, histological and genetic heterogeneity.

The term glioma defines any tumor that arises from the transformation of glial cells, mainly astrocytes, oligodendrocytes and ependymal cells or its precursors (Canoll and Goldman, 2008). Astrocytes are star shaped cells which represent the most abundant fraction of glial cells in the adult brain (Figure 1.2). These cells are involved in a wide range of functions: formation of the blood – brain barrier, detoxification of toxic compounds, modulation of neuronal damages, physical and metabolic support to neurons, buffering of neurotransmitter levels, maintenance of CNS homeostasis and participation in the formation of synapses (Purves *et al*., 2012). Astrocytomas derived from astrocytes, which constitute the supportive tissue of neurons.

Oligodendrocytes are myelin producing cells that insulate neurons, leading to the formation of myelin sheaths, allowing a rapid saltatory conduction of the electric impulse in neurons (Figure 1.2) (Purves *et al*., 2012). Oligodendrogliomas arrive from oligodendrocytes, being the third most common type of glioma, representing approximately 4.2% of all primary brain tumors (Mørk *et al*., 1985).

Additionally, there is another type of glioma which also derives from astrocytes although demonstrates an increased level of pathogenesis, such tumors are called glioblastomas (GBM). Importantly, these tumors represent more than 60% of all primary brain tumors in adults, occurring in 2-

3 cases per 100000 in Europe and North Europe (Jemal *et al*., 2010; Rock *et al*., 2012**)**. GBM constitute the most common type of malignant brain tumors, accounting for more than half of all newly diagnosed gliomas (Reifenberger *et al*., 2016). Despite the efforts to reach efficient therapies against GBM, it continues to be the most aggressive and lethal brain tumor associated with a dismal prognosis. Around 95% of these tumors are located in cerebral hemispheres, while few percent emerge from cerebellum and brainstem (Nakada *et al*., 2011). The patients with GBM usually have a median overall survival of 12 to 15 months from diagnosis (Stupp *et al*., 2005; Koshy *et al*., 2011).

The other group of glial cells is responsible for coating the brain ventricles and the central canal of the spinal cord. Ependymal cells can also facilitate the cerebrospinal fluid movement (Purves *et al*., 2012). Microglial cells are mainly derived from hematopoietic precursor cells, being categorized as a type of macrophage, capable of removing cellular *debris* from sites of injury, controlling pathogen invasion and tissue damage by inducing an inflammatory response (Purves *et al*., 2012). These cells seem to contribute to glioma growth and invasion, having tumor-supporting roles which result from the effect of glioma derived molecules (Li and Graeber, 2012). Thus, these complex and heterogeneous tumors can arrive from different types of brain cells, when they are exposed to damages. After being exposed to damages, the glial cells have the capacity to proliferate and propagate the lesion (Purves *et al*., 2012). This is the reason why gliomas are the most common malignant type of brain tumors.

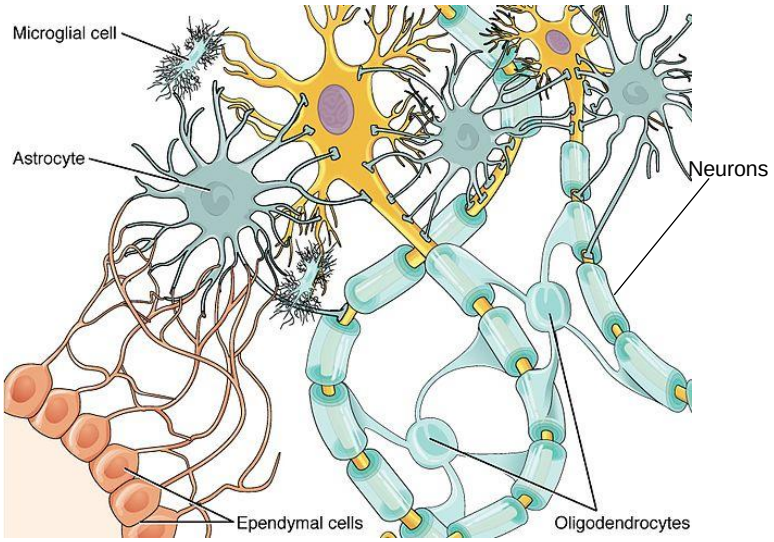


Figure 1.2 – Schematic representation of the glial cell types interacting with neurons. It is represented the myelin sheathes formed by oligodendrocytes and the metabolic and physic support function of astrocytes to neurons. Ependymal cells have essentially a structural function, forming cellular compartments, as evidenced in the figure. Adapted from https://courses.lumenlearning.com/wm-biology2/chapter/glial-cells/.

4. Molecular alterations of adult gliomas

Over the last decades, it has been found molecular alterations frequently present in gliomas, some of them are referred as playing a central role as biomarkers for diagnosis and the others as an important complement to the diagnosis and prognosis (van den bent *et al*. 2017). Even if these genes do not confer additional information about patient's clinical features, they contribute to the understanding of the tumor development. In this section are mentioned the main molecular alterations found in gliomas.

4.1. Isocitrate Dehydrogenase (IDH)

The isocitrate dehydrogenase (IDH) is a nicotinamide adenine dinucleotide phosphate (NADP) dependent enzyme that catalyzes the oxidative decarboxylation of isocitrate to form α-ketoglutarate in the tricarboxylic acid cycle producing reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Keys and McAlister-Henn, 1990). IDH1 is located within the cytoplasm and peroxisomes and IDH2 is located in the mitochondria. In addition, *IDH* mutations lead to the production of altered isocitrate dehydrogenases forms, which convert α-ketoglutarate into 2-hydroxyglutarate (Dang *et al*., 2009). Under these conditions the oncometabolite 2-hydroxyglutarate is produced and the levels of α-ketoglutarate decrease.

Furthermore, 2-hydroxyglutarate is responsible by the inhibition of α-ketoglutarate dependent dioxygenases such as ten-eleven translocation (TET) family, 5-methylcytosine hydroxylases and the Jumonji- C-domain-containing histone-lysine demethylases (Xu *et al*., 2011). This metabolic alteration lead to a global methylated state of CpG islands, which consequently induce the methylation of *MGMT* gene promoter and a better response to chemotherapy. Another possible hypothesis is that 2-hydroxyglutarate inhibits the α-ketoglutarate- dependent allkB homolog DNA repair enzymes, inducing the accumulation of DNA damages, which sensitize cells to the alkylating agents (Wang *et al*., 2015).

In gliomas, it has been identified two types of *IDH* mutations (in the *IDH1* and *IDH2* genes) and all of them are missense mutations involving a single amino acid change at arginine 132 (R132) of *IDH1* or the analogous residue in *IDH2* (R172) (Balss *et al*., 2008). The amino acidic alteration occurs in the active site of the enzyme, impairing the isocitrate binding. The *IDH2* mutations are relatively rare compared to the *IDH1* R132H mutations that represent 90% of all *IDH* mutations (Balss *et al*., 2008). In summary, *IDH* mutations are associated with a favorable prognosis of gliomas and it is speculated that they could select patients who would benefit from chemotherapy.

4.2. 1p/19q Codeletion

In addition to *IDH* mutations, also 1p/19q codeletion is an important alteration for the molecular characterization of gliomas (Louis *et al*, 2016). In the majority of cases, this alteration is caused by a balanced translocation whereas the whole short arm of chromosome 1 and the whole long arm of chromosome 19 are lost (Griffin *et al*., 2006). The loss of 1p/19q was described for the first time in 1994 by Julia Reifenberger, who considered this codeletion as a typical alteration of oligodendroglial tumors that appear as early events in their development (Reifenberger *et al*., 1994). Presently, it is clear the

association between 1p/19q codeletion and the genesis of oligodendrogliomas (Louis *et al.,* 2016). Besides this, it has been noticed that 1p/19q codeled tumors are associated with a better response to chemotherapy and consequently to patient's better prognosis, however the mechanism involved in this increased sensitivity is unclear (Griffin *et al*., 2006).

Furthermore, other molecular alterations have been investigated in order to understand their association with oligodendrogliomas and 1p/19q loss. For example, capicua transcriptional repressor (*CIC*) located at the long arm of chromosome 19, has been described as frequently mutated in oligodendrogliomas (69% of the cases analyzed) (Yip *et al*., 2011). In other studies it is also mentioned that all *CIC* mutations occur in tumors 1p/19q codeleted, and *IDH* mutated (Sahm *et al*., 2012).

Far upstream element binding protein- 1 (*FUBP1*) located at the short arm of chromosome 1, is another gene that sometimes appear mutated in oligodendrogliomas (Yip *et al*., 2011). However, the *CIC* mutations are much more common than *FUBP1* mutations in oligodendrogliomas, and usually *FUBP1* mutations only appear in tumors *CIC* mutated (Sahm *et al*., 2012). However, the correlation of *FUBP1* mutations and 1p/19q codeletion is unclear.

4.3. Alpha-Thalassemia*/* Mental Retardation Syndrome X-linked (ATRX)

The *ATRX* gene was identified for the first time in patients with x-linked mental retardation syndrome characterized by psychomotor difficulties and facial dimorphism (Gibbons *et al*., 1995). It is known that *ATRX* encode a histone chaperone protein involved in chromatin remodeling and transcription through the formation of a complex with DAXX (death-associated protein 6) responsible by the inclusion of H3.3 histone proteins into the telomeric regions (Goldberg *et al*., 2010). The disruption of this complex lead to the alternative lengthening of telomeres (ALT) and genomic instability (Lovejoy *et al*., 2012).

In gliomas, *ATRX* alterations are present in around 70% of *IDH* mutated tumors from astrocytic lineage, although *ATRX* loss and 1p/19q codeletion seem to be mutually exclusive (Kannan *et al*., 2012). This fact suggests that *ATRX* analysis may help to differentiate the astrocytic from oligodendroglial lineages. Furthermore, it has been described an association between *ATRX* alterations, *TP53* mutations and *ALT* phenotype in astrocytic tumors (Liu *et al*., 2012). Patients with astrocytomas containing *ATRX* loss and *IDH* mutated have prolonged overall survival, seeming that *ATRX* loss defines a subgroup of astrocytic tumors with favorable prognosis (Wiestler *et al*., 2013). In GBM, *ATRX* loss is identified in a reduced percentage of tumors, and it seems that its role is not specific (Nandakumar *et al*., 2017).

Therefore, *ATRX* loss is an important alteration to validate the diagnosis of astrocytomas, and it may be useful in doubtful cases to distinguish the different gliomas subgroups (Louis *et al.,* 2016).

4.4. Tumor Protein (TP53)

TP53 is a tumor suppressor gene that encodes a transcriptional factor responsible for regulating cell cycle, impairing the proliferation of damaged cells with oncogenic features. TP53 protein is involved in a wide range of cellular processes such as DNA repair, apoptosis, cell cycle arrest, senescence and genome stability (Gillet *et al*., 2014). Mutations in *TP53* are the most common genetic alterations among

human cancers and its inactivation lead to tumor cells invasion, proliferation and survival (Muller and Vousden, 2013).

Moreover, these mutations are present in 70% of astrocytomas, more specifically in 95% of *IDH* mutated tumors without 1p/19q codeletion (Takami *et al*., 2014). However these alterations are also found in other gliomas subgroups even in a low percentage. Currently, this gene is not analyzed for routine diagnosis of gliomas for two reasons: it is correlated with distinct gliomas entities, so by itself cannot discriminate the glioma sample and because this is a long gene, becoming difficult its analysis recurring to the conventional molecular techniques (van den bent *et al*., 2017). *TP53* mutations as well as *ATRX loss* are two molecular alterations, analyzed only in particular situations when the diagnosis of gliomas need to be confirmed, assuring the correct validation and differentiation of the tumor type.

4.5. Telomerase Reverse Transcriptase (TERT)

Telomerase Reverse Transcriptase (*TERT)* Promoter is a gene involved in telomerase activation, being responsible for the generation of its catalytic subunit (Bryan and Cech, 1999). Telomerase activity is important to maintain telomeres length, since this large multicomponent reverse transcriptase is able to recognize, bind and elongate telomeres (Bryan and Cech, 1999). However, telomerase expression is reduced in the most normal tissues (Kim *et al*., 1994), except for cells that contain high rates of self-renewal such as intestinal epithelium and hematopoietic stem cells (Chiu *et al*., 1996).

Nevertheless, it is described that tumor cells can proliferate indefinitely, maintaining the length of their telomeres, due to increased levels of telomerase activity or by ALT (Kim *et al*, 1994; Bell *et al*., 2016; Amorim *et al*., 2016). In addition, *TERT* promoter mutations were associated with increased *TERT* expression and consequently elevated levels of telomerase activity due to recruiting transcriptional factors that usually did not bind to *TERT* promoter (Bell *et al.,* 2016 Amorim *et al*., 2016).

Around 80-90% of gliomas have *TERT* promoter mutations, suggesting that this is the main mechanism of telomerase activation (Bollam *et al*., 2018). Over 85% of GBM *IDH* wildtype and 77% of GBM *IDH* mutated have *TERT* promoter mutations (Lee *et al*., 2017). In addition, oligodendrogliomas also have an elevated percentage of *TERT* promoter mutations in approximately 97% of cases (Lee *et al*., 2017). The incidence of *TERT* promoter mutations in the astrocytoma group is less common, 20% in anaplastic astrocytomas *IDH* wildtype and 4.4% in anaplastic astrocytomas *IDH* mutated (Lee *et al*., 2017). All these percentages are distinct between the different studies, because of that it is needed a large cohort of well classified and characterized samples according to the 2016 WHO classification to establish the standard incidences. In gliomas as in many types of cancer, the most common mutations in *TERT* are C228T and C250T map -124 and -146 bp upstream of *TERT* ATG site (Huang *et al*., 2013).

There is a great interest in understanding the impact of these mutations on the prognosis of patients with gliomas. *TERT* promoter mutations did not showed statistically significant association with the overall survival of patients with GBM (Eckel-Passow *et al*., 2015). On the other hand, it is speculated that these mutations could be correlated with better prognosis in *IDH* mutated grade II and III tumors (Kim *et al*., 2018; Yang *et al*., 2016). When it was analyzed the overall survival of patients with grade II and III gliomas based on *IDH* status, *TERT* promoter mutations were associated with a decreased overall survival in *IDH* wildtype gliomas. Nevertheless, they reveal a positive correlation with the overall

survival of *IDH* mutated gliomas (Vuong *et al*., 2017). Therefore, Eckel - Passow reported that grade II and III gliomas patients with *IDH* and *TERT* promoter wildtype have worse overall survival compared to patients with *IDH* and *TERT* promoter mutated or *IDH* mutated alone, but showed greater overall survival compared with patients only with *TERT* promoter mutation (Eckel-Passow *et al*., 2015). Based on these results, *TERT* promoter mutations could be used as a biomarker in association with *IDH* analysis to predict patient's prognosis. In the future, it is crucial new studies to ensure the viability of these observations.

4.6. Epidermal Growth Factor Receptor (EGFR)

Epidermal Growth Factor Receptor (EGFR*)* also designated as *HER1* or *ERBB1*, is a transmembrane glycoprotein belonging to the *HER* superfamily of receptor tyrosine kinases (Downward *et al*., 1984). The EGFR activation occurs through the binding of epidermal growth factor (EGF) ligands and growth factors to its extracellular domain. This connection between the ligand and the extracellular domain leads to the dimerization of EGFR and consequently to a conformational change that activates the intracellular domain of the receptor. Once the intracellular domain is phosphorylated, the receptor activates PI3K/Akt (protein kinase B), ERK and JAK/STAT signaling pathways to ensure cell survival (Downward *et al*., 1984; Yarden and Pines, 2012). These pathways regulate a wide range of cellular processes such as proliferation, apoptosis, angiogenesis, metabolism, protein synthesis, autophagy, cell migration and differentiation (Hobbs *et al*., 2012). *EGFR* amplification is a structural alteration that determines the deregulation of all these cellular processes, inducing the constitutive activation of downstream signaling pathways that enhance tumor growth, migration, angiogenesis and metastatic spread. Therefore, *EGFR* constitutes a proto-oncogene, whose structural alterations are present in different types of cancers (Hobbs *et al*., 2012). *EGFR* amplification is a relatively common alteration identified in approximately 40%-50% of all the GBM cases (Libermann *et al*., 1985; Wong *et al.,* 1987; Decker, 1990), although this amplification is predominant in GBM *IDH* wildtype (Sturm *et al.,* 2012).

There is no unanimity regarding the prognostic value of *EGFR* amplifications in GBM, some studies have documented that *EGFR* amplifications in GBM do not have prognostic value (Chen *et al*., 2015; Quan *et al*., 2005) others have reported that these alterations are associated with unfavorable outcomes (Shinojima *et al.,* 2003). Thus, the independent prognostic role of *EGFR* was not clearly proved.

Despite this, EGFR has been noticed as a putative therapeutic target, based on the frequency of *EGFR* mutations and its identification as an upstream trigger of cell signaling cascades deregulation (Padfield *et al*., 2015; Reardon *et al*., 2014). Lately, tyrosine kinase inhibitors targeting EGFR or their constitutively activated forms (Caraglia *et al.*, 2006) have been created with the aim of impairing the constitutive activation of downstream pathways. Surprisingly in GBM, these therapies have been inefficient mainly due to the compensatory mechanisms triggered by cancer cells (Westphal *et al*., 2017; Taylor *et al*., 2012). GBM can overcome the inhibition of EGFR through distinct compensatory mechanisms such as: Receptor Tyrosine Kinase (RTK) co-activation, *PTEN* mutations, enhanced immunosuppression, increased expression of anti-apoptotic proteins and efflux of tyrosine kinase

inhibitors (Taylor *et al*., 2012). In the future it will be crucial to study new alternatives to inhibit the compensatory mechanisms developed in GBM, improving the efficiency of these therapies.

4.7 Phosphatase and Tensin homolog (PTEN)

Phosphatase and Tensin homolog (*PTEN)* deletions are frequent in gliomas, particularly in 30%-40% of GBM cases (McLendon *et al*., 2008; Verhaak *et al.,* 2010). The inactivation of this gene could result from genomic alterations such as the entire or partial chromosomal loss, specific allelic loss and inactivating mutations (Srividya *et al*., 2010). These alterations are not restrict to CNS malignancies but also to other types of tumors such as breast, kidney and lung cancers (Simpson and Parsons, 2001). *PTEN* is an important tumor suppressor gene localized in chromosome 10, which is involved in the phosphoinositol – 3 - Kinase (PI3K) pathway, the most mutated signaling pathway during gliomas development (Maehama e Dixon, 1998). This gene encode a phosphatase that converts phosphatidylinositol 3, 4, 5 – triphosphate (PIP3) into phosphatidylinositol 4,5- bisphosphate (PIP2), having the opposite function of PI3K. PTEN functions as a regulator of PIP3 levels in cells, to prevent the excessive activation of Akt, and consequently the deregulation of all the intracellular mechanisms necessary to cell survival and homeostasis (Yang *et al*., 2017).

The impact of *PTEN* deletion in patient's prognosis is not well established, the results obtained seem to be controversial (Srividya *et al*., 2010; Carico *et al*., 2012). *PTEN* deletion was one of the first cytogenetic alterations introduced in gliomas diagnosis due to its high frequency and importance as an identifying feature (McLendon *et al*., 2008). *PTEN* deletion by itself is present in a higher percentage than all the other *PTEN* mutations in gliomas (Srividya *et al*., 2010). The data about these alterations are controversial, different authors are not sure about the clinical relevance of *PTEN* alterations or if they should be routinely assessed (van den Bent *et al*., 2017).

4.8. O-6-methylguanine-DNA methyltransferase (MGMT)

In addition to the biomarkers used to establish the diagnosis and characterization of gliomas, there are biomarkers that allow the prediction of the most appropriate therapies to be applied after surgery (Reifenberger *et al*., 2016). O-6-methylguanine-DNA methyltransferase *(MGMT)* promoter methylation is a predictive biomarker of benefit from alkylating-agents chemotherapy mainly in patients with *IDH* wildtype gliomas, particularly in elderly patients (aged ≥70 years) (Laperriere *et al.,* 2013; Wick *et al*., 2012; Reifenberger *et al.,* 2016).

MGMT is a DNA repair enzyme, which repairs the DNA damages caused by temozolomide, by removing the alkyl group from guanine residues and transferring them to its active site (Rivera *et al.,* 2009). Thus, MGMT levels are directly correlated with the DNA reparation capacity provided by this enzyme, and consequently with the sensitivity of cancer cells to temozolomide treatment. For example, high levels of MGMT prevent the cytotoxic effects of temozolomide, exerting a protective role against this drug, whereas reduced levels of MGMT expression enhance the temozolomide effect (Baer *et al*., 1993). For many years, the motif underlying the decreased in *MGMT* expression was unknown, until an epigenetic event was found to regulate its expression, causing the *MGMT* silencing through methylation

of CpG islands of its promoter (Watts *et al*., 1997). Therefore, *MGMT* promoter methylation leads to decreased levels of MGMT, maintaining the alkyl groups in DNA, which is directly associated with a greater responsiveness of tumors to alkylating agents (Esteller *et al*., 2000; Watts *et al*., 1997).

Additionally, high levels of *MGMT* promoter methylation are significantly correlated with prolonged overall survival in patients with GBM treated with neoadjuvant temozolomide (Hegi *et al*., 2004; Chinot *et al*., 2007) and increased progression free survival in patients with gliomas treated upfront with temozolomide (Friedman *et al*., 1998). Around 40% of GBM *IDH* wildtype have hypermethylation of *MGMT* promoter, suggesting that these patients have a better response to temozolomide (Wick *et al*., 2014). In GBM *IDH* mutated, *MGMT* promoter methylation is present in the majority of cases, predicting a favorable prognosis, but this predictive biomarker cannot differentiate which therapy, radiotherapy or temozolomide chemotherapy, is better to be administered in patients with gliomas (Wick *et al*., 2013). In gliomas grade II, it was also noticed that *MGMT* methylated tumors respond better to temozolomide treatment applied neoadjuvant, being a good predictor of favorable progression free survival (Everhard *et al*., 2006). Overall, many studies have shown high levels of *MGMT* methylation in patients with gliomas predict prolonged survival time compared to patients with *MGMT* unmethylated (Li *et al*., 2017).

Thus, the molecular analysis to determine the percent of *MGMT* promoter methylation is an important indicator in the determination of patients who would benefit from temozolomide chemotherapy. This predictive biomarker helps to estimate the possible behavior of patients when exposed to therapy and how the course of disease will be.

5. The evolution of WHO Classification for adult gliomas based on molecular characterization

The international classification of human tumors published by World Health Organization (WHO) since 1956, intended to establish a classification and grade system for brain tumors that could be accepted and used worldwide (Louis *et al*., 2007). In the 2007 WHO classification a histological grading system was implemented as a form of predicting the behavior of each neoplasm. The histopathological grading established a hierarchic organization of brain tumors according to the malignancy level visualized by the pathologist (Kleihues *et al*., 1993). The malignancy scale goes from grade I to IV, whereas grade I is associated with low levels of anaplasia and better prognosis and grade IV is correlated with the highest levels of anaplasia and worst prognosis (Louis *et al*., 2007).

Oligoastrocytomas were an entity described in 1993 WHO classification as “tumors showing a conspicuous mixture of 2 distinct neoplastic cell types resembling the tumor cells in oligodendroglioma and diffuse astrocytoma” (Kleihues *et al*., 1993). This group of tumors, also designated as mixed gliomas, are brain tumors originate from both oligodendrocytes and astrocytes. The oligoastrocytoma classification has been severely contested because there are no immunohistochemical markers and molecular genetic alterations to distinguish this group of tumors from diffuse astrocytomas or oligodendrogliomas (Kleihues *et al*., 2002). It is not possible to establish the accurate diagnosis of oligoastrocytomas without confusing them with other glioma entities.

The 2007 WHO classification for brain tumors is defined as a histopathological classification, because it was based on the morphological appearance of tumors cells and on the microscopic

similarities with their origin cells (Louis *et al*., 2007). For many years, this methodology has been used as the gold standard to analyze the cells morphology and their degree of differentiation (van den Bent *et al.,* 2017).

However, the long-term application of 2007 WHO classification demonstrated some weaknesses and doubts in the differentiation of glioma entities. Thus, it seems that the histopathological classification was no longer meeting the current clinical needs due to different reasons (van den Bent *et al.,* 2017). Firstly, this classification was based on the interobserver variability, mainly in grade II and III gliomas differentiation (van den Bent, 2010). The lack of restrictive criteria, establishing the differentiation between the grades of gliomas constitute a great concern. Additionally, the oligoastrocytoma concept has been severely contested owing the necessity to validate the differences between this entity and astrocytomas and oligodendrogliomas (Sahm *et al*., 2014).

Based on the limitations demonstrated by the 2007 classification, several studies have highlighted the importance of introducing molecular alterations in the diagnosis and classification of brain tumors (Foote *et al*., 2015). During the last two decades, most studies, have defended that molecular alterations associated with histological data confers a more accurate classification and a better prediction of clinical outcome compared to the histological classification alone. This association between histological and molecular features is considered because brain tumors have a complex origin and may appear similar in terms of histology but have different underlying molecular profiles, which is only seen through molecular genetics (Louis *et al*., 2016).

All this insight lead to the 2016 WHO classification for brain tumors that finally includes molecular alterations in the diagnosis of gliomas, reinforcing the importance of molecular biomarkers in gliomas classification (Louis *et al*., 2016). This new classification breaks the century-old principle of diagnosis based entirely on microscopy. Presently, the new classification includes *IDH* mutations and 1p/19q codeletion as central biomarkers to define glioma subgroups (Louis *et al*., 2016).

In this perspective, based on the 2016 WHO classification astrocytomas were subdivided into *IDH*-wildtype and *IDH*-mutated. The great majority (70-80%) of astrocytomas correspond to the *IDH* mutated category (Balss *et al*, 2008; Hartmann *et al*., 2009). It has been described that the *IDH* mutated subgroup seems to be correlated with a better prognosis in both astrocytoma grades compared to *IDH* wildtype subgroup (Louis *et al*., 2016).

Oligodendrogliomas are now characterized by the presence of a codeletion in the 1p and 19q chromosomal arms combined with a mutation in the *IDH* gene family (Louis *et al*., 2016).

Additionally, GBM the most aggressive malignant type of glioma, was subdivide into GBM *IDH* wildtype and *GBM IDH* mutated. The GBM *IDH* mutated subgroup represents approximately 10% of all the GBM cases and usually appear in younger patients, having a better outcome compared to GBM *IDH* wildtype subgroup (Parsons *et al*.,2008; Nobusawa *et al*., 2009, Ohgaki and Kleihues, 2013; Louis *et al*., 2016). This subgroup of GBM corresponds to the secondary GBM that progressed from astrocytomas. On the opposite, GBM *IDH* wildtype subgroup account for 90% of all GBM cases and usually appear in older patients (55 years), being the new designation for the primary GBM (Ohgaki and Kleihues, 2013; Louis *et al*., 2016).

It is important to highlight that in the 2016 WHO classification the concept of mixed oligoastrocytoma disappear, remaining only oligoastrocytomas (NOS) cases in which the molecular analysis was not concluded or was inconclusive, becoming impossible to include these gliomas in the appropriate subgroup (Louis *et al*., 2016).

In addition to the biomarkers referenced above, other molecular alterations such as *ATRX* and *TP53* mutations were considered in the 2016 WHO classification of gliomas (Louis *et al*., 2016). The molecular analysis of these two biomarkers can be done to confirm the astrocytoma diagnosis, however this analysis is not mandatory and by itself is not enough to determine the final diagnosis of an astrocytoma. Thus, the new classification included *IDH* mutations and 1p/19q codeletion as part of the standard diagnosis, which means that gliomas diagnosis depends on the analysis of these alterations, being the two most important biomarkers of this classification (Louis *et al*., 2016). The remaining molecular alterations referenced (*ATRX and TP53*) are complementary and may be useful for define and characterize the gliomas groups, particularly in doubtful cases (Figure 1.3).

Many other studies have been developed to investigate biomarkers which could confer some additional information about the prognosis of patients and ultimately their response to therapy. The introduction of a biomarker in a universal classification requires many studies to assure the viability and veracity of its impact. Because of this, many genes frequently mutated in gliomas such as telomerase reverse transcriptase promoter (*TERT*), epidermal growth factor receptor (*EGFR*) and phosphatase and tensin homologue (*PTEN*) have been studied (van den Bent *et al.,* 2017). These genes which are not currently present in the new classification may have some importance to the routine diagnostics, to understand the overall picture, even if they are not essential for any diagnosis. This brings the problem of deciding, which genes should be routinely analyzed, which genes are optional but give additional information and which genes do not have clinical significance(van den Bent *et al.,* 2017).

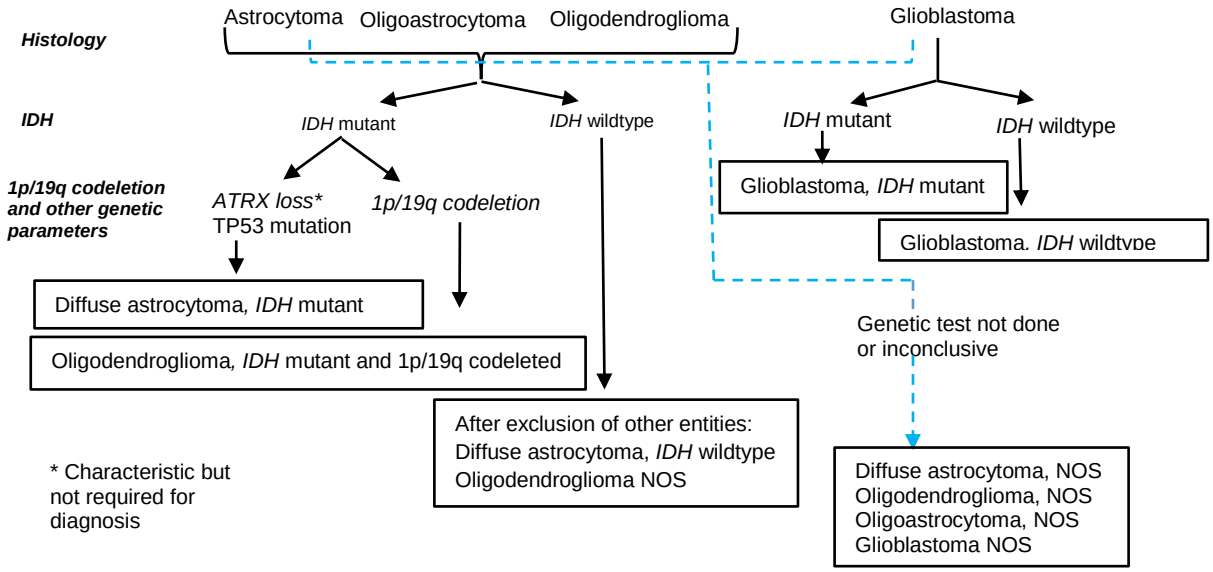


Figure 1.3 - **Schematic representation of 2016 WHO classification for brain tumors, with the specific criteria to the molecular definition of each subgroup of gliomas**. *IDH* mutations, 1p/19q codeletion, *ATRX* and *TP53* mutations are the main biomarkers featured in this new classification. Adapted from Louis, D. et.al (2016). *Acta Neuropathologica*, 131(6),803-820

6. Importance of new biomarkers for gliomas classification

Additional biomarkers will be needed to better understand the prognostic role of these alterations in gliomas and for stratify these very complex and heterogeneous tumor entities. In this perspective, it is important to discover new possible pathways and targets that could be helpful to determine better choices of treatments (Louis *et al*., 2016).

In this context, it seems that are missing biomarkers that for example could clearly distinguish the astrocytomas group from the GBM group and molecular alterations that may help to characterize the GBM *IDH* mutated subgroup and GBM *IDH* wildtype subgroup, in an attempt to create subdivisions in these lethal group of tumors. GBM is the most common type of malignant brain tumors associated with fatal outcomes, so characterizing these tumors in subgroups that can impart additional information about patients is a big step.

In addition to *IDH* mutations, it would be interesting to identify molecular alterations that could characterize and divide astrocytomas, in order to create subgroups that would provide some additional information about their aggressiveness. The need of new biomarkers is evidenced in the 2016 WHO classification for brain tumors, since some categories are defined as NOS (not otherwise specified), because the molecular markers available are not enough to achieve the most correct glioma diagnosis (Louis *et al*., 2016).

In sum, it is crucial to study additional biomarkers that may help to clarify and define the different subtypes of gliomas, understanding the impact of these molecular alterations in patient's diagnosis, prognosis and response to therapy.

6.1. *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha)

In this context, it was already known the importance of *PIK3CA* in the aggressiveness of different types of tumors. *PIK3CA* mutations are frequently identified in patients with breast cancer 25%-40% (Samuels and Velculescu, 2004), endometrial 36% (Oda *et al*., 2005) and colon cancer 32% (Samuels *et al*., 2004). Nevertheless in gliomas, the percentage of *PIK3CA* mutations is not well established, which means that there is no concordance between the different studies.

According to Samuel *et al*. these mutations were present in 27% (4/15) of GBM (Samuels *et al*., 2004), although Broderick *et al*. found only 5% (5/105) of *PIK3CA* mutations in its GBM cohort (Broderick *et al*., 2004). Many other works, reported discrepant percentages ranging from 5% to 30% of *PIK3CA* mutations in GBM (Gallia *et al*., 2006; Hartmann *et al*., 2005; Verkaak *et al*., 2010; Lee *et al*., 2017). The differences between the frequencies obtained could result from the variable number of samples used to perform the *PIK3CA* mutational analysis, some of these studies used reduced sampling, considering we are working with a highly heterogeneous type of tumor. This variation detected in frequencies is essentially predominant in the studies on GBM, because the analysis of *PIK3CA* in oligodendrogliomas and astrocytomas has been less explored. However, for oligodendrogliomas the percentages stablished until now, also ranged between 2% (3/66) (Hartmann *et al*., 2006) and 14% (3/21) (Broderick *et al*.,

2004). In addition, Broderick and co-authors were the only group to define the percentage of these oncogenic mutations in anaplastic astrocytomas, which was around 3% (1/31) (Broderick *et al*., 2004).

PIK3CA is a gene with 21 exons located on the long arm of chromosome 3 (3q26) which encodes the p110α catalytic subunit of class IA PI3K lipid kinases (Volinia *et al*., 1994). PI3K are important enzymes involved in the PI3K/Akt signaling pathway, which regulate many cellular activities such as proliferation, angiogenesis, growth, motility and survival (Katso *et al*., 2001). These enzymes are organized in three main classes (I, II and III) based on its structure and substrate affinity (MacDougall, *et al*., 1995). The class I is subdivided into class IA and Class IB, the first includes p110α, p110β and p110δ catalytic subunits and the second contains p110γ catalytic subunit (Kang *et al*., 2006). The class IA of PI3K enzymes is the most studied, being the main focus due to its impact on tumorigenesis. Moreover, this class is characterized by the formation of a heterodimeric complex between a catalytic subunit (p110) and a regulatory subunit (p85) (Fry *et al.,* 1992). The *PIK3CA* gene contains a regulatory subunit binding domain (p85), which allows the integration of signals from the receptor to the catalytic subunit (Samuels, 2004). Furthermore, this gene also contains a RAS binding domain important to perform the ERK signaling pathway activation, helical and catalytic domains (Samuels, 2004).

According to Samuel *et al*., the most *PIK3CA* mutations occurred in two small clusters in the helical (exon 9) and kinase (exon 20) domains. (Samuels *et al*., 2004). Exon 9 and 20 of *PIK3CA* are defined in distinct types of cancer as the main observed hotspots of *PIK3CA* mutations (Broderick *et al*., 2004; Samuels *et al*., 2004). Many articles referred E542K and E545K as the two most frequent mutations found in exon 9 of *PIK3CA* and H1047R and H1047Y as the main *PIK3CA* mutations found in exon 20 (Broderick *et al*., 2004; Samuels *et al*., 2004; Gallia *et al*., 2006).

Previously, it was documented that *PIK3CA* mutations led to the constitutive activation of class IA PI3K kinases. However, the mechanisms by which mutations in exon 9 and exon 20 of *PIK3CA* induce the PI3K gain of function are distinct. *PIK3CA* mutations in exon 9 induce the gain of function in a Ras-GTP binding dependent manner, while exon 20 mutations are dependent from the conformational change induced by the regulatory subunit (Zhao and Vogt, 2008). Moreover, the mutations in exon 20 interfere with the kinase domain of *PIK3CA*, inducing the independence of *PI3K* from the upstream signaling. On the other hand, the helical domain seems to be the local of interaction between the regulatory and the catalytic subunit, suggesting that mutations in exon 9 could block the connection between the both subunits, inhibiting the effect of the regulatory subunit (Miled *et al*., 2007).

Overall, the exons 9 and 20 *of PIK3CA* have important roles in the functionality of p110α, explaining why the mutations in these spots are the most reported.

6.2. PI3K/Akt signaling pathway

Currently, class I PI3K kinases are known to be controlled by distinct receptor tyrosine kinases which, when activated by upstream signals, stimulate phosphorylation of PIP2 in PIP3 (Lai *et al.,* 2015). During this process increased levels of PIP3 are generated, which are accumulated at the intracellular medium (Cantley, 2002). Simultaneously, there is a phosphatase (PTEN) that antagonizes the activity of PI3K converting PIP3 in PIP2, to regulate the PIP3 levels. *PTEN* is a tumor suppressor gene with an

important role in the regulation of PI3K/Akt upstream and downstream signaling (Maehama and Dixon, 1998). When the PI3K/Akt signaling pathway is regulated, some PIP3 is accumulated near the plasmatic membrane of cell, leading to the recruitment of proteins, such as Akt, which contains a lipid binding domain, Pleckstrin Homology (PH) (Lai *et al.,* 2015). The Akt protein is recruited by PIP3, but only becomes active after being phosphorylated by phosphoinositide – dependent kinases (PDK) (Vara *et al*., 2004). After activation, Akt phosphorylates effector proteins, such as mammalian target of rapamycin (mTOR) and murine double minute 2 (Mdm2), regulating innumerable cell activities including proliferation, apoptosis, autophagy, angiogenesis, protein synthesis and metabolism (Vara *et al*., 2004). In figure 1.4, it is represented all the PI3K/AKT signaling pathways and its main players.

Therefore, to prevent the development of tumors it is important to maintain the regulation of PI3K/Akt signaling pathway. The deregulation of this pathway is mainly associated with *PTEN*, *PIK3CA* and tyrosine kinases receptors genetic alterations (Lai *et al.,* 2015). The tyrosine kinase receptors alterations, such as *EGFR* amplification, induce the constitutive activation of the downstream signaling pathway. *PTEN* inactivation, due to mutations or deletions, stimulates an excessive accumulation of PIP3 in the intracellular medium due to the loss of its ability to regulate PIP3 levels (Lai *et al.,* 2015). *PIK3CA* mutations lead to conformational changes in the p110α catalytic subunit, becoming its associated PI3K independent on upstream signaling, making them constitutively active (Lai *et al.,* 2015). In these three situations, there is an excessive accumulation of PIP3 which culminates in the over activation of Akt, causing an excessive increase in proliferation and angiogenesis and a decrease in apoptosis.

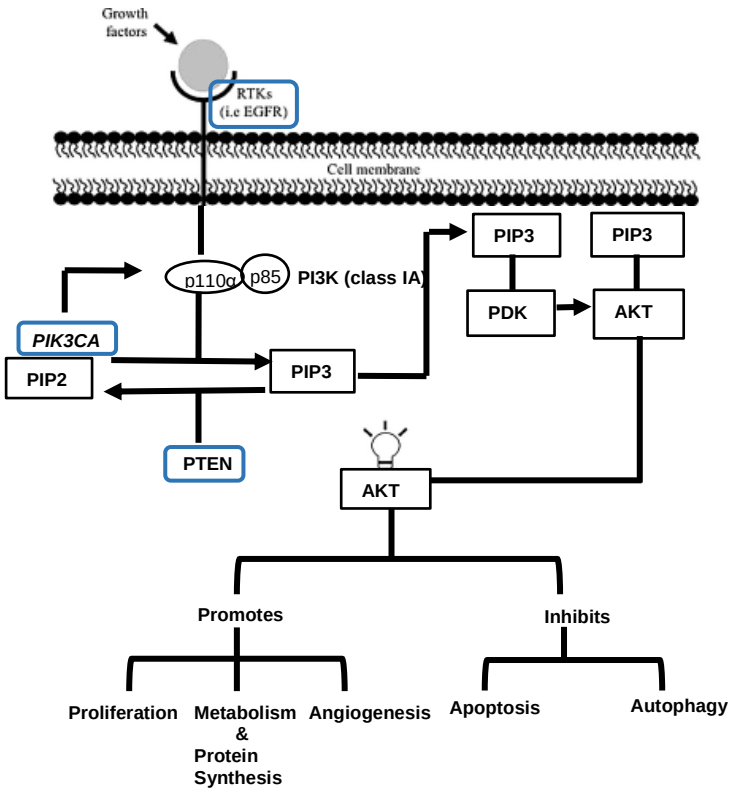


Figure 1.4 - Overview of PI3K/Akt signaling pathway and their downstream effects. The blue boxes indicate the three main genes (*EGFR, PIK3CA and PTEN*) involved in the deregulation of this pathway. The regulation of this enzymatic cascade is crucial to prevent the development of tumors. Adapted from Kai, L., et al. (2015) *Journal Of Clinical Pathology*, *68*(4), 253-257.

6.3. Why *PIK3CA* could be a good therapeutic target?

The PI3K/Akt signaling pathway is one of the most mutated pathways during the development of gliomas (Mao *et al*., 2012). Therefore, all the efforts should be done to efficiently target possible molecules involved in this important pathway. Several clinical trials have been performed to generate drugs targeting *EGFR,* when this receptor is mutated. Although as *EGFR* is located upstream of this pathway, its inhibition has not been successful due to compensatory mechanisms developed by cancer cells (Westphal *et al*., 2017). In this point of view, maybe *PIK3CA* could be a potential target, since it is located more downstream compared to *EGFR*.

Recently, oncogenic mutations of *PIK3CA* have been referred as possible initial events in GBM, which means that these mutations could be an important player in GBM initiation (Lee *et al*., 2017). Additionally, *PIK3CA* mutations were referred as associated with a role in tumor multiplicity and in the heterogeneous patterns of drug-response (Lee *et al*., 2017). Furthermore, these mutations were defined as clonal mutations shared by all sectors of the tumor (Lee *et al*., 2017). Altogether these new data, seem to highlight the role of *PIK3CA* in GBM. Nevertheless, it is not clear if these mutations remain during GBM progression or if they are passenger mutations with an important role in the initiation of the tumor. Neither, its concrete role in response to therapy. In addition, the impact of these alterations in the other groups of gliomas is unknown, as well as in gliomas recurrences.

In a previous pioneer study, Verhaak and co-authors reported that *PIK3CA* mutations were found mainly in the GBM proneural subtype, which is associated with *IDH* mutations, and which have no survival advantage from aggressive treatment protocols (Verhaak *et al*., 2010) .Besides this, in 2015 The Cancer Genome Atlas Research Network, also showed that this gene is mutated in 20% of low grade gliomas with an *IDH*-mutation and 1p/19q codeletion (Brat*., et al*., 2015).

Recently, it has been developed several inhibitors targeting *PI3K/Akt* signaling pathway members. The problem is that the majority of inhibitors target PI3K kinases (Lai *et al.,* 2015). As mentioned previously, PI3K are enzymes included in three classes and all of them play vital roles in cellular activities, which means that the general inhibition of these enzymes could cause adverse effects on healthy cells. In this perspective, inhibitors targeting specifically the p110α catalytic subunit could maximizes their therapeutic potential in patients with *PIK3CA* mutations without causing the side effects of interfering with other PI3K classes (Lai *et al.,* 2015). For example, alpelisib (BYL719) is a specific inhibitor to *PIK3CA* protein*,* which was tested in breast cancer and currently is in phase II clinical trials (Massacesi *et al*., 2016). According to this statements, *PIK3CA* mutations may be a positive predictor biomarker for the clinical use of *PIK3CA*-selective inhibitors (Fritsch *et al*., 2014).

Based on this data, *PIK3CA* mutations could be a potential therapeutic target because of their uniform distribution in the tumor, possible early emergence, and involvement in the most mutated signaling pathway in gliomas and specificity, sparing cells from adjacent toxicities caused by treatments.

In the last decade, *PIK3CA* has been studied to understand its possible role in the development of tumors. However, the impact of this gene in gliomas has been analyzed according to the 2007 WHO classification. Until this moment, the investigators are unable to certainly refer which glioma molecular subtype is more correlated with *PIK3CA* mutations, or rather, these mutations occur more frequently.

Until now, the difficulties in achieving a concordant *PIK3CA* mutational frequency are related to sample size, which introduces a great variability between the different studies (Samuels, 2004; Broderick *et al*., 2004; Gallia *et al*., 2006; Hartmann *et al*., 2005; Wen *et al*., 2012; Lee *et al*., 2017). As we know, gliomas are relatively rare tumors, becoming difficult to obtain a cohort with a considerable number of samples. In addition, despite the number of samples, the major concern was to acquire gliomas samples well characterized and classified, which was not easy using the 2007 WHO rules (van den Bent *et al.,* 2017; van den Bent, 2010; Sahm *et al*., 2014; Louis et al., 2007). Thus, the proper classification used also potentiate this variation in the frequencies of *PIK3CA* mutations.

Since the new classification has been stablished, several questions were raised to finally understand the possible role of this gene as a biomarker. Presently, it is not clear, what is the role of *PIK3CA* mutations in the prognosis of diffuse gliomas with codeletion and /or *IDH* mutations, neither its role in GBM *IDH* wildtype versus GBM *IDH* mutated. It would be interesting, analyze to which subgroup of GBM these mutations are most associated. Furthermore, it is unknown if *PIK3CA* mutations have prognostic value in the different gliomas subtypes or if these alterations could be associated with a preferential response to therapy.

It is increasingly believed that the behavior of a tumor is explained not only by a single molecular alteration in a gene but by the interaction between different alterations in distinct genes (Selleck *et al.,* 2017; Kraus, 2018). In this perspective, it is unknown the possible correlation between the mutations in *PIK3CA* and alterations in other genes. It would be useful, understand if there is any correlation between the onset of these mutations and *PTEN* deletion, *EGFR* amplification, *TERT* mutations, *1p/19q* codeletion and *MGMT* methylation. Additionally, *PIK3CA* mutations are possibly early events in the development of tumors (Lee *et al*., 2017), although it is not clear the role of these mutations in gliomas clonal expansion. Importantly, the question that remains to answer, is will these mutations remain during tumor progression and relapses or are they passenger mutations.

In sum, until now it is unknown the role of *PIK3CA* mutations in gliomas stratification according with the new 2016 WHO classification, as well as its role in the prognosis and response to treatment of glioma molecular subgroups. In the future, this gene could be another important candidate to understand the behavior of gliomas.

2. Objectives

Considering the role of *PIK3CA* mutations in several types of cancer as well as their relevance as a putative druggable target, here, we propose to clarify the clinical impact of *PIK3CA* mutations in the molecular subgroups of gliomas. In this work, we intend to analyze the importance of *PIK3CA* mutations in gliomas using a cohort of 437 gliomas referred at the Instituto Português de Oncologia Francisco Gentil de Lisboa, from 2011 to 2016, the most were molecularly characterized for *PTEN* loss, *EGFR* amplification, *IDH* mutation and *TERT* promoter mutations, 1p/19q codelection, and *MGMT* methylation.

The specific aims are:

i) to reorganize the gliomas samples according to the new 2016 WHO classification;

ii) to analyse the impact of the 2016 WHO classification in the reclassification of gliomas cohort;

iii) to evaluate the impact of many biomarkers, such as *IDH*, *TERT*, *PTEN*, *EGFR*, *MGMT* and 1p/19q codeletion in diagnosis, prognosis and response to therapy on the Portuguese cohort of gliomas;

iv) to analyse the impact of *PIK3CA* mutations on the molecular stratification according with WHO 2016 classification;

v) to evaluate the impact of *PIK3CA* mutations on the prognosis, diagnosis, aggressiveness and response to therapy in molecular subgroups of gliomas;

vi) to investigate the potential correlation between *PIK3CA* mutations and other biomarkers such as: *TERT* mutations, *PTEN* loss, *EGFR* amplification, and *MGMT* methylation;

vii) to study the role of these mutations in a group of 33 recurrences samples of GBM;

3. Material and Methods

1. Study population

Firstly, we elaborated a database of adult glioma samples, diagnosed from 2011 to 2016, referred to the Cytogenetics Lab of Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG). Initially, we had 486 primary glioma samples, although in this work we included only 419 primary tumors, 15 first recurrences and 3 second recurrences samples. These 67 primary samples were excluded for two reasons: lack of material to perform the molecular analysis and/or because they were histologically or molecularly doubtful cases with an uncertain diagnosis. For example cases with 1p or 19q codeletion or *IDH* wildtype with 1p/19q codeletion.

Additionally, the glioma cohort was reclassified and organized according the 2016 WHO classification, considering the *IDH* mutations and 1p/19q codeletion as central biomarkers. Furthermore, this characterization also included the genetic analysis of several markers which are frequently altered in gliomas such as: *TERT* mutations, *MGMT* methylation, *PTEN* deletion and *EGFR* amplification. The majority of this genetic analysis was previously performed by the Cytogenetic group.

The database also included clinical information from patients: age, sex, treatments administered, recurrences, overall survival (OS), and follow-up. Tumor samples were received as fresh tissue or paraffin-preserved tissue for DNA extraction.

Table 3.1. Glioma samples. This table represents all the gliomas samples studied.

Variable	No	Types of gliomas			
		GBM (n)	Oligodendrogliomas (n)	Astrocytomas (n)	Recurrences (n)
Number of samples	437	256	49	109	23 (+10)
Age (years)	Median	63.0	48.5	42.0	43.0
	Minimum	17.0	27.0	16.0	24.0
	Maximum	87.0	76.0	80.0	66.0
Sex	Male	168	34	64	11
	Female	88	15	45	4
Adjuvant therapy	Received	226	30	78	15
	None	19	1	12	0
	No data	11	18	19	0

n- Number of samples per group; +10 – samples already included in the astrocytoma group

2. DNA extraction

The DNA extraction from frozen tissues was performed using the conventional method of phenol-chloroform (MP Biomedicals; MercK). Initially, the tumor piece was cut in a fragment with approximately 27mm^3 using a scalpel blade. Firstly, the fragment suffered mechanical digestion, and then chemical digestion after exposed to 600µl of lysis buffer (10mM TrisHCl, 400mM NaCl, 2 mM EDTA), SDS 10% (Invitrogen™, Thermo Scientific) and proteinase K 20mg/µl (Sigma P6556) at 56º (Eppendorf, Thermomixer compact). After the enzymatic digestion, phenol (MP Biomedicals) and chloroform (MercK) (1:1) were added to the samples.

Next, the DNA was precipitated after addition of sodium acetate (3M NaAc, pH 5.2) and ethanol 100% (Sigma - Aldrich) at -20ºC overnight. This protocol ends with a centrifugation of 14000 rpm for 30 minutes (*eppendorf Centrifuge*, raio do rotor= 180mm) and removal of the supernatant with subsequent drying of the pellet at 37ºC. Finally, the DNA was dissolved in TE (DNA Hydratation Solution, QIAGEN) in an appropriate volume according to the amount of DNA extracted.

From tissues fixed in formaldehyde and preserved in paraffin the extraction was performed using the QIAGEN's Gene Read ™ DNA FFPE Kit. The procedure begins with the desparaffinization of 3 sections of tissue with approximately 10µm at 56ºC (Eppendorf, Thermomixer compact). The second step corresponds to the enzymatic digestion, performed through the addition of RNAse-free water, FTB buffer and proteinase K at 56ºC (QIAGEN). The time required to achieve the complete enzymatic digestion of the tissue could be different between the samples, depending on the amount of biological material inserted into the sections. The process ends with a sequence of successive steps that aim to purify and wash the DNA, using a column QIAmp MinElute. In the last step, the Uracil - N –glycosylase (UNG) hydrolyzes the N-glycosidic bond between the deoxyribose sugar and uracil in DNA containing deoxyuridine in place of thymidine repairing the possible regions of DNA damaged and the addition of RNase A (100mg/ml) was used to ensure the absence of vestigial RNA. The DNA was precipitated in ethanol 100% and AL buffer (QIAGEN), then washed and eluted in TE buffer (DNA Hydratation Solution, QIAGEN).

Additionally, for some samples used during this project the DNA was extracted recurring to an automatized process by Maxwell® RSC Instrument (Promega), using the RSC DNA FFPE kit (Promega). The Maxwell extraction allows obtaining a high-quality nucleic acid purified with minimal steps from paraffin tissues. This methodology is more efficient, fast, and produces DNA in appropriate conditions to be used in other techniques. The protocol used followed the instructions of Promega.

After extraction the DNA quantification was evaluated through a spectrophotometric method using Nanodrop 2000 (Thermo Scientific). However, it was necessary to load a 0.8% agarose gel electrophoresis to analyze DNA quality. After the DNA quantification, it was performed a dilution for 80ng/µl in all samples used to evaluate the mutational status of *PIK3CA, IDH1* and *TERT* promoter.

3. Polymerase Chain Reaction (PCR)

The PCR technique was used to amplify specific regions of *PIK3CA*, *IDH1* and *TERT* using the PCR standard program.

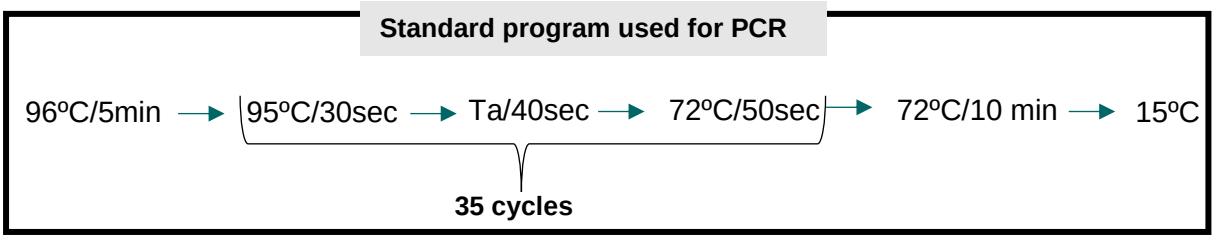


Figure 3.1 – Standard program used for PCR amplification of several genes in brain tumors. Ta, represents the annealing temperature, which is variable and calculate according to the fragment that we are interested in study. The time of each step is represented in seconds (sec) and minutes (min). The scheme represents the different steps (denaturation, annealing and extension) that occur during a PCR, repeated 35 times.

The PCR reactions were performed for a final volume of 12.5 µl, using 1µl of DNA 80ng/µl. The PCR mix contained: sterilized water (LABESFAL), 50mM $MgCl_2$ (Invitrogen™ ,Thermo Scientific), 10x Hi-Fi Buffer (Invitrogen™ ,Thermo Scientific), 0.2 mM deoxynucleotides (dNTP's; Invitrogen™ Thermo Scientific), 10pmol primer forward and primer reverse (Invitrogen™, Thermo Scientific) and 1U Taq polymerase Platinum (Invitrogen™, Thermo Scientific).

In this project our interest regions were the coding exons 9 and 20 of *PIK3CA*. To perform the analysis of these exons it was used 4 sets of primers, two of them targeting the exon 20 (271bp), one target exon 9 and the other set of primers targeting the exon 9 pseudogene found in chromosome 22 (Baker *et al*., 2012). The exon 9 contains 125 bp, which means that only one set of primers would be enough to amplify this region. The primers used in the PCR reactions targeting *PIK3CA* are described in table 3.2.

Table 3.2 - Sequence of the *PIK3CA, IDH1* and *TERT* promoter primers used for PCR reactions

Primers	Gene	Exon	Forward Primer	Reverse Primer
1	*PIK3CA*	9	5' GCTTTTTCTGTAAATCATCTGTG 3'	5' CATGCTGAGATCAGCCAAATTC 3'
2	*PIK3CA*	9*	5' TCCTCTCTCTGAAATCACTGA 3'	5' ACATGCTGAGATCAGCCAAAT 3'
3	*PIK3CA*	20	5' GCAAAGACCTGAAGGTATTAACAT 3'	5' GGGTCTTTCGAATGTATGCAA 3'
4	*PIK3CA*	20	5' ATGATGCTTGGCTCTGGAAT 3'	5' GGTCTTTGCCTGCTGAGAGT 3'
5	*IDH1*	4	5' CGGTCTTCAGAGAAGCCATT 3'	5' GCAAAATCACATTATTGCCAAC3'
6	*TERT*	1	5' GCACAGACGCCCAGGACCGCGCT 3'	5' TTCCCACGTGCGCAGCAGGACGCA 3'

Exon 9 Pseudogene*

The PCR conditions used for each pair of primers, as well as, its length is represented in table 3.3.

Table 3.3- PCR conditions used for each pair of primers targeting *PIK3CA, IDH1 and TERT*. In this table is represented the length of the fragment amplified for each pair of primers, the concentration of $MgCl_2$ used and the annealing temperature used in each reaction.

Primers	Fragment length (bp)	[$MgCl_2$] (mM)	T_a (ºC)
1	286	50mM	61
2	131	50 mM	58
3	205	50mM	61
4	268	50mM	61
5	129	50mM	56
6	196	50mM	69.5

The PCR reactions were realized in a Veriti termocycler (Applied Biosytems). The 4 sets of primers were used to perform the mutational analysis of *PIK3CA* directed to exon 9 and 20 in 437 gliomas samples. Additionally, the PCR reactions efficiency and success were controlled through an agarose gel 2% horizontal electrophoresis.

3.1. Enzymatic digestion /purification

It was used an enzymatic method to purify each PCR product, using two distinct enzymes: Exonuclease I 20U/ul (Thermo Scientific) and FastAP Thermosensitive Alkaline Phosphatase 1U/ul (Thermo Scientific). The enzymatic reaction included 15 minutes at 37ºC the optimal temperature for the action of both enzymes, followed by 15 minutes at 85ºC to inactivate them and stop the reaction.

4. Sequencing

To determine the *PIK3CA*, *IDH1* and *TERT* promoter sequence of interest, we recurred to automatic sequencing and followed the protocol purposed by Big Dye ™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

After all the procedure the samples were introduced in the automatic sequencer ABI Prism™3130 Genetic Analyzer (Applied Biosystems) to analyze the mutations presence.

4.1. Analysis of the functional impact of the variants identified

When we identified new variants that were not described in Ensembl, Catalogue of Somatic Mutations in Cancer (COSMIC) and The Human Gene Mutation Database (HGMB), it was performed *in silico* analysis. The analysis *in silico* predicts the possible impact of these new variants on the structure and function of the protein encoded, determining the eventual benign or pathogenic role of them. In here, it was used 3 informatics tools to perform this prediction: MutationTaster

(http://www.mutationtaster.org), Polyphen (http://genetics.bwh.harvard.edu/pph2) and Variant Effect Predictor (https://www.ensembl.org/info/docs/tools/vep/index.html).

The Polyphen predicts the effect of a substitution from one amino acidic residue for another in the structure and function of the encoded protein. This program calculates the probability of that amino acidic change having a pathogenic or benign effect, according to the sequence and location of the substitution in cause.

The Variant Effect Predictor, also designated as VEP, determines the effect of single nucleotide polymorphisms, substitutions, insertions, deletions or structural variants on genes, transcripts and protein sequences. This software provides extra options that include the prediction of the variant effect taking into account other *in silico* analysis performed by Polyphen and SIFT. Overall, this software provides a complete analysis comparing the data from different databases.

The MutationTaster performs a global analysis of the conservation, location, and structure at the site of the alteration. However, unlike the other programs this allows predicting the effect of insertions and deletions up to 12bp and possible changes in splicing sites. All the programs provide an overall estimation of the genetic alteration effect (pathogenic or benign) in the protein, based on a statistical analysis. MutationTaster is the most complete software, since includes the analysis of distinct types of alterations.

5. Statistical Analysis

The primary endpoint used was overall survival, defined as the time from the glioma diagnosis to the patient death or loss of follow-up. Survival analysis was done using Kaplan-Meier estimator and the log-rank test for group comparison. In addition, to the univariate analysis performed through the log-rank test, the survival data were also studied using a multivariate analysis with Cox regression proportional hazard model. The multivariate analysis allows to study the independent association of the biomarkers of interest and molecular groups with overall survival, while controlling for potential confounders such as age, sex and treatment.

The Fisher exact test was used to determine the association between categorical variables, mainly for analyze associations between the presence of *PIK3CA* mutations and other genetic alterations. All tests were two-sided and we considered a significance level of 5%. The statistical analysis applied here was performed using IBM SPSS Statistics 21.0.

4. Results

1. Impact of the 2016 WHO classification in gliomas characterization

Initially, we organized and characterized the glioma cohort according to the 2016 WHO classification, stratifying the glioma samples based on these new rules. The Table 4.1 shows the differences between the numbers of primary samples obtained applying the 2007 and 2016 WHO classifications.

Table 4.1- Effect of the 2016 WHO classification in the subdivision of gliomas samples

	Subtype and Number of glioma samples				
WHO classification	GBM	Oligodendrogliomas (Grade II/III)	Astrocytomas (Grade I/II/III)	Oligoastrocytomas	NOS
2007	256	82 (52/30)	60 (11/21/28)	53	4
2016	256	49	109	0	41

NOS – Glioma samples not included in the groups formed

In our cohort as expected, the 2016 WHO classification had an impact in the reorganization of the major subgroups of gliomas, namely in the oligodendrogliomas and astrocytomas subgroups, leading to the reduction of the number of oligodendrogliomas and an increase in the number of astrocytomas samples, compared to the 2007 WHO classification (Table 4.1). However, this new classification did not change the number of GBM samples, already established using the 2007 WHO classification. The oligoastrocytoma concept disappear, being these samples distributed by the remaining classes of gliomas according to the new molecular classification. Nevertheless, 41 samples were not included in the major subgroups of gliomas because they do not fulfill the established requirements (Table 4.1). This result highlights the need for new biomarkers to perform gliomas diagnosis.

Table 4.2 –Prevalence of gliomas molecular subgroups characterized according to the 2016 WHO classification. It is represented the molecular subgroups of GBM, Astrocytomas and *IDH* mutant +1p/19q codeletion (Oligodendrogliomas) analyzed during this study according to the *IDH* and 1p/19q codeletion analysis.

Molecular Subgroups	Number of samples	% Gliomas
GBM	256	61.8 (256/414)
***IDH* mutated**	11	2.7 (11/414)
***IDH* wildtype**	245	59.1 (245/414)
Astrocytomas	109	26.3 (109/ 414)
***IDH* mutated**	56	13.5 (56/414)
***IDH* wildtype**	53	12.8 (53/414)
IDH mutant + 1p/19q codeletion*	49	11.9 (49/414)
Total	414	100 (414/414)

*Oligodendroglioma

In Table 4.2 is represented the number of primary gliomas samples included in each molecular subgroup, based essentially on the analysis of the *IDH* mutations and 1p/19q codeletion for all the cases, according to the 2016 WHO classification. GBM samples corresponded to 61.8% of the entire cohort

analyzed, of which 59.1% were GBM *IDH* wildtype and the remaining 2.7% were GBM *IDH* mutated. The astrocytoma subgroup was the second most prevalent and the oligodendroglioma subgroup was the least incident in the cohort.

After the subdivision of these groups based on the 2016 WHO classification, it was important to re-characterize the 5 molecular subgroups according with age, sex, and whether some type of adjuvant therapy was administered to patients. In Table 4.3, we performed the descriptive analysis of the 5 glioma molecular subgroups.

Table 4.3 – **Patients characterization based on the molecular subgroup established.**

Variable	No	Gliomas Molecular subgroups				
		GBM *IDH* wildtype	GBM *IDH* mutated	Oligo[a] (*IDH* mutant +1p/19q codeletion)	Astrocytoma *IDH* wildtype	Astrocytoma *IDH* mutated
Number of samples	414	245	11	49	53	56
Age of diagnosis (years)	Median	63.0	44.0	48.5	52.0	38.0
	Minimum	18.0	17.0	27.0	16.0	23.0
	Maximum	87.0	59.0	76.0	80.0	66.0
Sex	Male	163	5	34	31	33
	Female	82	6	15	22	23
	Ratio (M/F)	2:1	0.8:1	2:1	1.4:1	1.4:1
Adjuvant Therapy	Receveid	211	11	30	33	45
	None	19	0	1	8	4
	No data	15	0	18	12	7

[a]Oligodendroglioma

In this cohort, as expected, was observed a great difference in the median age of patients with GBM *IDH* wildtype and GBM *IDH* mutated, as well as in astrocytomas *IDH* wildtype in comparison with astrocytomas *IDH* mutated (Table 4.3). These results are in agreement with all previous works which reported *IDH* mutated gliomas arise in younger patients compared to *IDH* wildtype tumors (Parsons *et al*., 2008; Nobusawa *et al*., 2009, Ohgaki and Kleihues, 2013; Louis *et al*., 2016).

Our results also indicated a higher prevalence of gliomas in men of the molecular subgroups analyzed (1.4:1 - 2:1), except for the GBM *IDH* mutated subgroup. This discrepant result may have occurred because of the small number of samples included in the GBM *IDH* mutated subgroup (n=11). Overall these results are in concordance with the established by 2016 WHO.

2. Survival analysis based on the 2007 and 2016 WHO classifications

Then, we performed survival curves using glioma subgroups classified according the 2007 and 2016 WHO classifications, in order to analyze the overall survival patterns across the distinct groups.

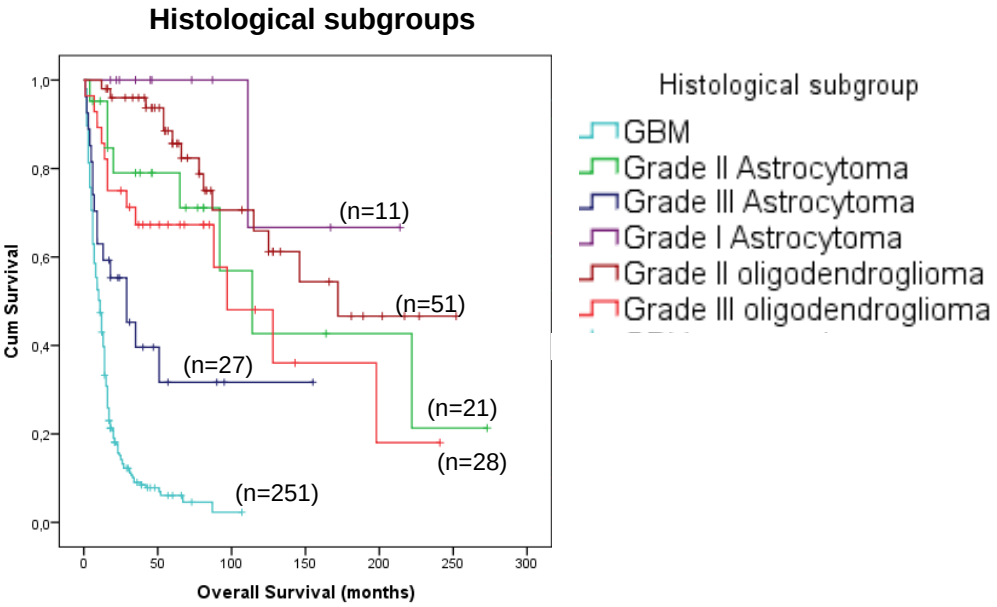


Figure 4.1 – Kaplan-Meier Curves of overall survival for the subgroups of gliomas established according to the 2007 WHO classification: GBM (n=251), Grade III Astrocytomas (n=27), Grade II Astrocytoma (n=21), Grade I astrocytomas (n=11), Grade II oligodendroglioma (n= 51), Grade III oligodendroglioma (n=28).

Table 4.4 – Medians for the survival time of each glioma histological subgroup.

	N	Median (months)	
Histological subgroups		Estimate	Std. Error
Grade II Oligodendroglioma	51	172.00	.
Grade III Oligodendroglioma	28	97.00	24.34
GBM	251	11.00	1.02
Grade I Astrocytoma	11		
Grade II Astrocytoma	21	114.00	26.28
Grade III Astrocytoma	27	29.00	10.99
Overall	389	16.00	0.84

Our results, are in accordance with the studies previously published (Burkhard *et al*., 2003; Louis *et al*.,2007) which referred grade I astrocytomas as the histological subgroup associated with a prolonged overall survival, being the only group considered as “benign” in the scale of malignancy defined by the 2007 WHO classification (Louis *et al.*, 2007) (Figure 4.1). Into the malignant gliomas set, grade II oligodendrogliomas were the subgroup with better prognosis. As expected the GBM was the group associated with the poorer outcomes, followed by grade III astrocytomas (Figure 4.1 and Table 4.4).

Patients with grade II gliomas had a prolonged survival compared to patients with grade III gliomas. It is notorious that oligodendrogliomas (grade II and III) are associated with an increased overall survival compared to astrocytomas (grade II and III).

Posteriorly we performed the survival analysis for the gliomas reclassified based on the molecular criteria, in order to confirm differences in the patients prognosis based on molecular classification (Figure 4.2 and Table 4.5).

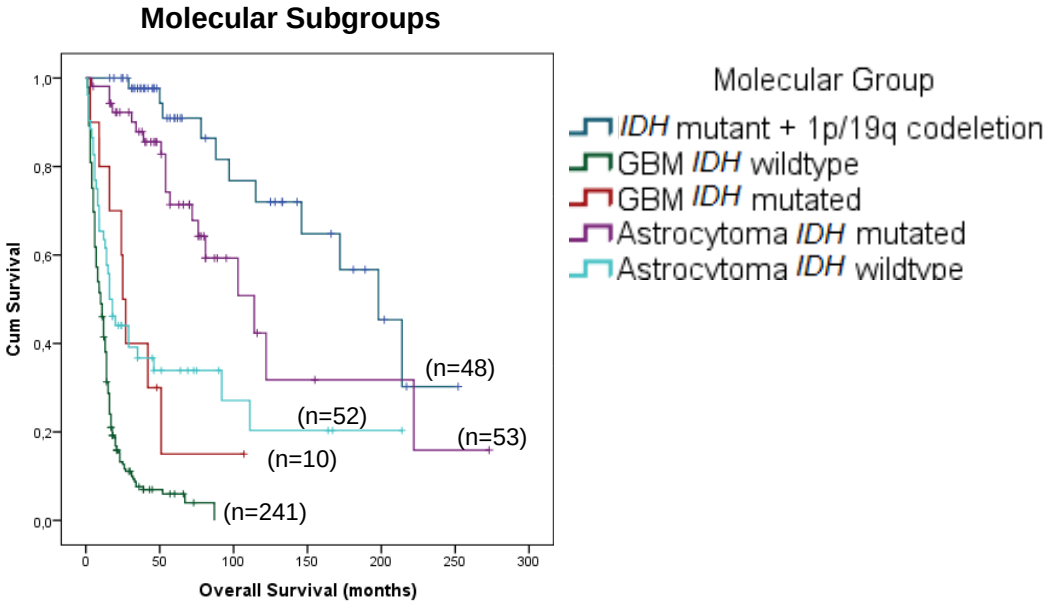


Figure 4.2 – Kaplan-Meier Curves of overall survival for the glioma molecular subgroups established according to the 2016 WHO classification: *IDH* mutant + 1p/19q codeletion (n=48), Astrocytoma *IDH* mutated (n=53), Astrocytoma *IDH* wildtype (n= 52), GBM *IDH* mutated (n= 10) and GBM *IDH* wildtype (n=241).

Table 4.5 – Medians for the survival time of each glioma molecular subgroup.

Molecular Subgroups of gliomas	N	Median (months)	
		Estimate	Std. Error
***IDH* mutant + 1p/19q codeletion**	48	198.00	22.29
GBM *IDH* wildtype	241	10.00	1.02
GBM *IDH* mutated	10	25.00	2.37
Astrocytoma *IDH* mutated	53	114.00	23.22
Astrocytoma *IDH* wildtype	52	16.00	2.96
Overall	404	16.00	0.81

The molecular stratification showed similar survival curves when compared with the histological classification. The *IDH* mutant + 1p/19q codeletion (oligodendroglioma) subgroup continues to be the malignant group associated with better prognosis (Table 4.5), however this molecular subgroup had a higher median overall survival (198 months) compared to the oligodendrogliomas histological subgroups (172 and 97 months) (Table 4.4), which reinforces the relevance of molecular classification.

In addition, this new classification allowed the subdivision of GBM and astrocytomas in subgroups according to the *IDH* mutational analysis, which clearly divided this two groups into 2 subgroups with distinct overall survival (Figure 4.2). GBM *IDH* wildtype are associated with poor outcomes, compared to GBM *IDH* mutated, which mean *IDH* mutations are correlated with better prognosis in both astrocytomas and GBM, as mentioned in the WHO 2016 statements.

In Table 4.6, it was used the Cox Regression Hazard model to determine if the molecular and histological subgroups formed were associated with the overall survival of patients after adjustment for gender, age at diagnosis and treatment.

Table 4.6 – Univariate and Multivariate Cox regression for 2007 and 2016 WHO classifications.

Variable	N	Univariate analysis			N	Multivariate analysis*		
		Hazard ratio	95% CI	P Value		Hazard ratio	95% CI	P Value
Molecular Subgroup								
GBM *IDH* wildtype	241	20.96	10.89 – 40.37	<0.001	230	20.55	9.18 -46.00	<0.001
GBM *IDH* mutated	10	7.90	3.11 – 20.06	<0.001	10	12.41	4.33–35.57	<0.001
Astrocytoma *IDH* wildtype	52	7.03	3.53 – 14.01	<0.001	41	8.61	3.62 - 20.49	<0.001
Astrocytoma *IDH* mutated	53	2.05	0.96 – 4.39	0.064	45	2.82	1.18 - 6.77	<0.001
IDH mutant + 1p/19q codeletion (oligo[a])	48		Reference		33		Reference	
Histologic Subgroup								
GBM	251	39.77	5.51 – 286.95	<0.001	240	35.73	4.60– 277.46	0.001
Grade II astrocytoma	21	4.49	0.56 – 36.03	0.157	15	4.58	0.54 – 38.55	0.162
Grade III astrocytoma	27	14.01	1.85 – 106.15	0.011	23	16.23	2.01– 127.64	0.008
Grade II Oligo[a]	51	2.65	0.35 – 20.17	0.347	41	2.18	0.27 – 17.70	0.466
Grade III Oligo[a]	28	5.80	0.76 – 44.36	0.091	21	4.87	0.59 – 39.91	0.141
Grade I astrocytoma	11		Reference		9		Reference	

[a] oligodendroglioma;*Multivariate analysis performed controlling the following independent variables: age, gender, treatment.

These results demonstrated the prognostic impact of the molecular classification, all the molecular subgroups showed a statistically significant association with the overall survival of patients after adjustment for gender, age and treatment.

Grade I and II astrocytomas and grade II oligodendrogliomas, correspond to histological groups without significant prognostic impact according to the statistical test performed.

3. Study of the importance of *EGFR* amplification, *PTEN* deletion, *TERT* mutations and *MGMT* methylation for gliomas diagnosis and prognosis

In addition, to the *IDH* mutations and 1p/19q codeletion, other biomarkers such as: *TERT, PTEN, EGFR and MGMT* are mentioned as additional biomarkers associated with gliomas aggressiveness and development. Here we also evaluated its impact in gliomas diagnosis, prognosis and response to therapy, since these analyzes have been controversial among different studies.

3.1. The percentage of *TERT* mutations, *PTEN* deletions, *EGFR* amplification, *MGMT* methylation in each glioma molecular subgroup

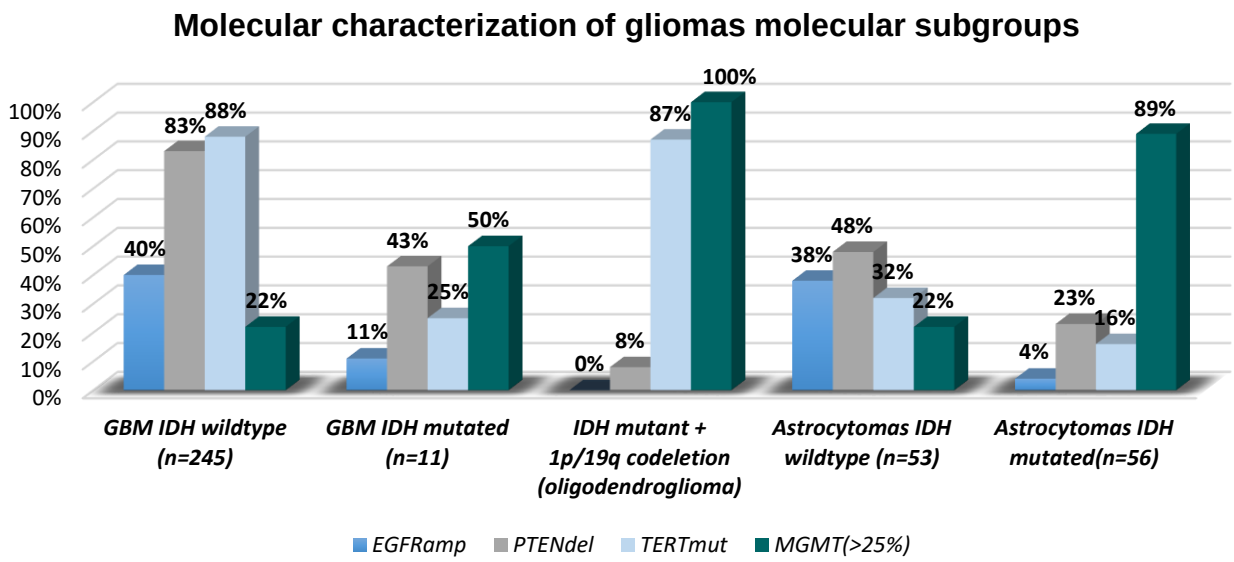


Figure 4.3 - Frequency of *EGFR* amplification (amp), *PTEN* deletion (del), *TERT* mutations (mut) and *MGMT* methylated in GBM *IDH* wildtype, GBM *IDH* mutated, *IDH* mutant + 1p/19q codeletion (oligodendrogliomas), Astrocytomas *IDH* wildtype and Astrocytomas *IDH* mutated.

These results showed *EGFR* amplification is more prevalent in gliomas *IDH* wildtype, whether GBM or astrocytomas, in comparison with gliomas *IDH* mutated. In the *IDH* mutant + 1p/19q codeletion subgroup was not detected any *EGFR* amplifications (Figure 4.3).

PTEN deletions were more frequent in the GBM *IDH* wildtype subgroup (83%). However, a high percentage of these alterations were also found in GBM *IDH* mutated and astrocytomas *IDH* wildtype (43% and 48% respectively), suggesting that its frequency arise with the subgroup aggressiveness. In the remaining molecular subgroups (*IDH* mutant +1p/19q codeletion subgroup and astrocytomas *IDH* mutated) the frequency of *PTEN* deletions is lower, although this is a genetic alteration distributed by the 5 distinct molecular subgroups (Figure 4.3).

The *TERT* promoter mutations have an elevated prevalence in gliomas. In this cohort, *TERT* mutations were predominant in GBM *IDH* wildtype and *IDH* mutant + 1p/19q codeletion (oligodendrogliomas). These mutations are dispersed across the 5 molecular subgroups, which is in agreement with the results reported by Lee *et al.* (Lee *et al*., 2017).

Furthermore, the percentage of *MGMT* methylated samples was higher mainly in *IDH* mutant +1p/19q codeletion (oligodendrogliomas), astrocytomas *IDH* mutated and GBM *IDH* mutated samples. As expected, *MGMT* methylation is most frequent in *IDH* mutated gliomas subgroups, since these mutations induce a generalized state of DNA methylation.

In Table 4.7 is demonstrated the ratios and the total of samples analyzed to obtain the frequencies of each biomarker across the distinct molecular subgroups of gliomas. The distinct number of samples analyzed for each molecular subgroup, is correlated with technical difficulties.

Table 4.7 – Ratios used to calculate the percentages of *EGFR* amplification, *PTEN* deletion, *TERT* mutations, and *MGMT* methylated.

Molecular Subgroup	N	*EGFR*amp No.(%)	*PTEN*del No.(%)	*TERT*mut No.(%)	*MGMT*(>25%) No.(%)
GBM *IDH* wildtype	245	90/226 (40)	187/225 (83)	107/122(88)	52/235(22)
GBM *IDH* mutated	11	1/9 (11)	3/7 (43)	1/4 (25)	5/10 (50)
***IDH* mutant + 1p/19q codeletion (oligodendroglioma)**	49	0/48 (0)	3/48 (8)	20/23 (87)	48/48 (100)
Astrocytomas *IDH* wildtype	53	15/40 (38)	20/42 (48)	7/22 (32)	11/50 (22)
Astrocytomas *IDH* mutated	56	2/53 (4)	11/53 (23)	4/25 (16)	49/55 (89)

3.2. Prognostic impact of *EGFR* amplification, *PTEN* deletion, *TERT* mutations and *MGMT* methylation

In here we intend to analyze the prognosis effect of the distinct genetic alterations in the molecular subgroups of gliomas, where there is a significant representation of them. In the *IDH* mutant + 1p/19q codeletion (oligodendrogliomas), due to the low incidence of each genetic alteration (Figure 4.3 and Table 4.7), the number of samples obtained was reduced to determine the prognostic value for each biomarker.

3.2.1. *EGFR* amplification

In this study, was verified for the first time that *EGFR* amplification did not have prognostic value in GBM *IDH* wildtype as well as in astrocytomas *IDH* wildtype subgroups (Figure 4.4). Until now, most of the studies performed this analysis using the histological classes, explaining the controversial results obtained (Chen *et al*., 2015; Quan *et al*., 2005; Shinojima et *al.,* 2003; Aibaidula *et al.,* 2017).

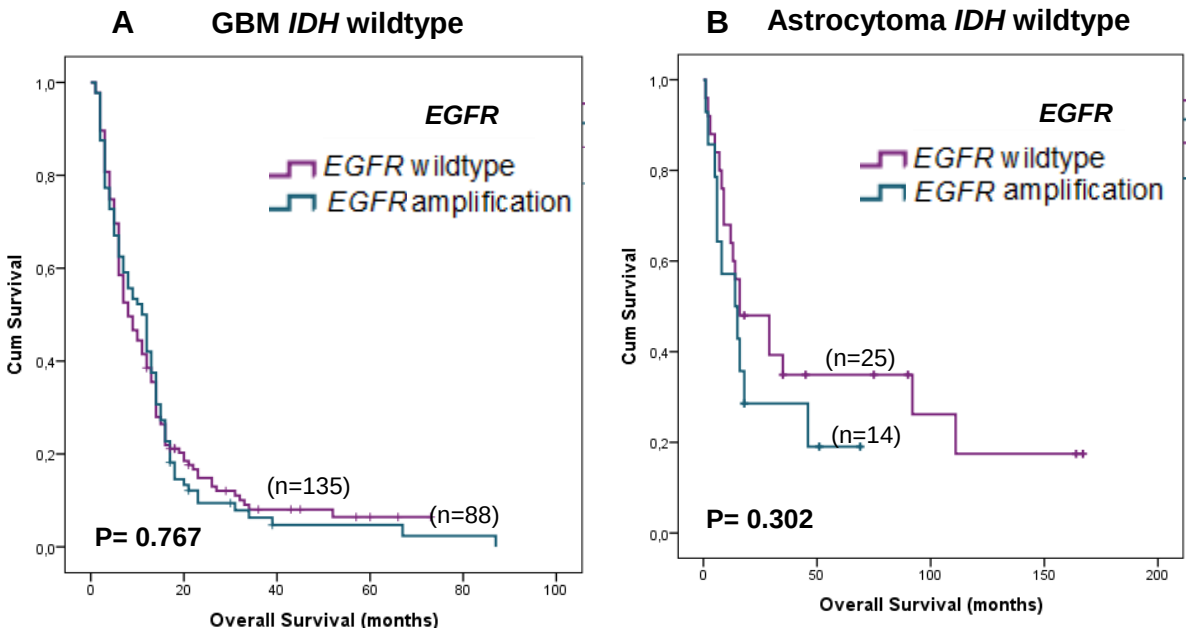


Figure 4.4 – Kaplan – Meier curves of overall survival to determine the *EGFR* amplification effect in patients with GBM *IDH* wildtype (A) and Astrocytomas *IDH* wildtype (B). The analysis was performed in: (A) GBM *IDH* wildtype *EGFR* wildtype (n= 135) and *EGFR* amplified (n= 88), (B) Astrocytomas *IDH* wildtype *EGFR* wildtype (n= 25) and *EGFR* amplified (n=14)

3.2.2 - *TERT* mutations

In GBM *IDH* wildtype patients, *TERT* mutations did not have prognostic value (Figure 4.5). This could be explained by the disproportion between the *TERT* wildtype (n=16) and *TERT* mutated (n=104) subgroups (Figure 4.5). Eckel-Passow *et al*., reported that *TERT* promoter mutations in GBM *IDH* wildtype group were not statistically associated with poorer outcomes, which is in agreement with our results (Eckel-Passow *et al*., 2015). However, our findings differ from those reported by Lee *et al*., who referred *TERT* promoter mutations as a prognostic factor of poor outcome in patients with GBM *IDH* wildtype (Lee *et al*., 2017).

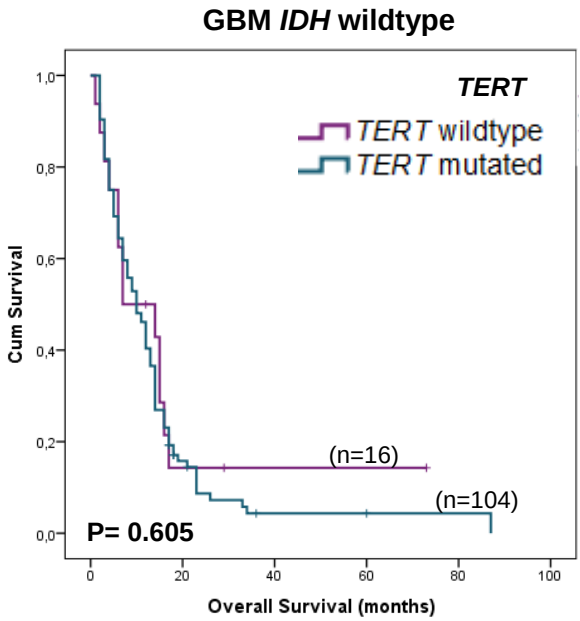
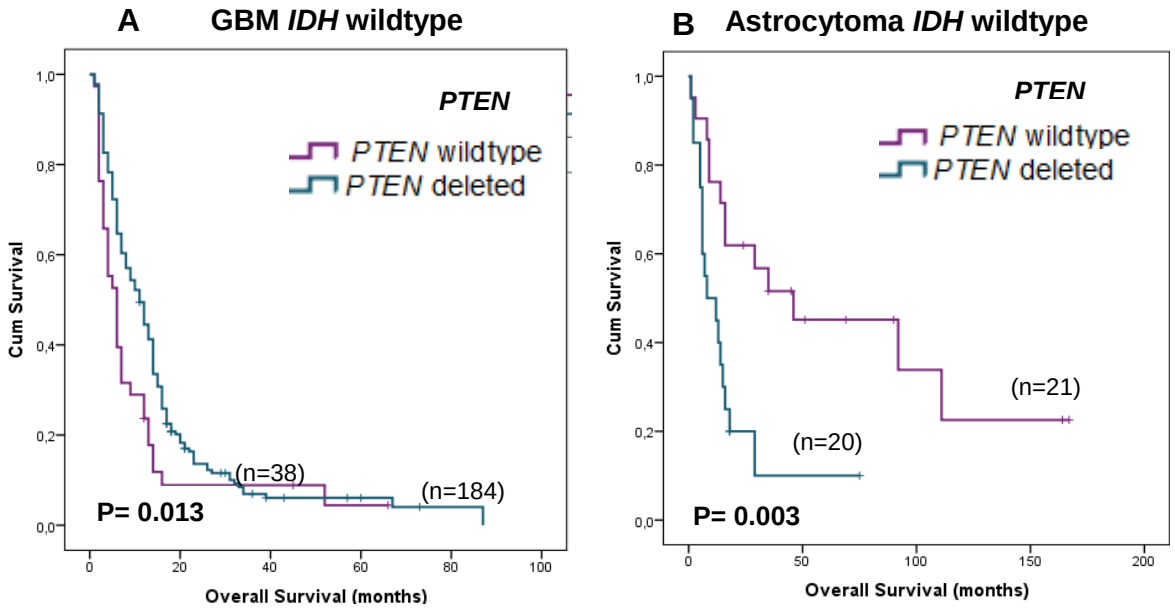


Figure 4.5 – Kaplan – Meier survival analysis to determine *TERT* mutations prognostic impact in patients with GBM *IDH* wildtype. The analysis was performed in: GBM *IDH* wildtype *TERT* wildtype (n=16) and *TERT mutated* (n=104).

3.2.3. *PTEN* deletion

PTEN deletions in patients with GBM *IDH* wildtype are significantly associated with a prolonged overall survival (Figure 4.6). However, in patients with astrocytomas *IDH* wildtype, these genetic alterations have an opposite effect, seeming to be strongly correlated with poor outcomes. This dual role was verified in this study for the first time, maybe due to the most existent works used the histological classes to analyze the prognostic value of *PTEN* deletions. Additionally, in astrocytomas *IDH* mutated, *PTEN* deletions were not associated with the patients' overall survival, although this group has a limited number of samples.



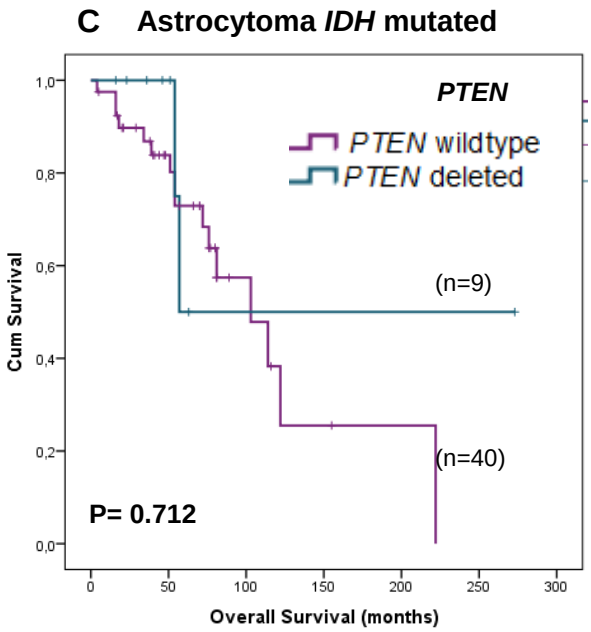


Figure 4.6 – Kaplan – Meier survival analysis to determine *PTEN* prognostic impact in patients with GBM *IDH* wildtype (A) astrocytomas *IDH* wildtype and (B) Astrocytomas *IDH* mutated (C). The analysis was performed in: (A) GBM *IDH* wildtype *PTEN* wildtype (n=38) and *PTEN deleted* (n=184), (B) astrocytomas *IDH* wildtype *PTEN* wildtype (n=21) and *PTEN* deleted (n=20), (C) astrocytomas *IDH* mutated *PTEN* wildtype (n=40) and *PTEN* deleted (n=9).

3.2.4. *MGMT* Methylation

MGMT methylation, a well-known predictive biomarker, also in our cohort demonstrate to be a good prognostic factor. Patients with GBM *IDH* wildtype *MGMT* methylated are associated with a prolonged overall survival, seeming that *MGMT* methylation is an indicator of good prognosis (Figure 4.7)

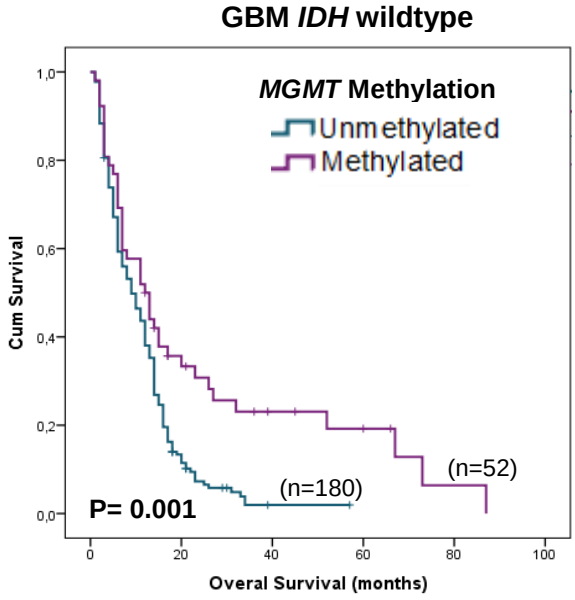


Figure 4.7 – Kaplan – Meier survival analysis to determine *MGMT* methylation prognostic impact in patients with GBM *IDH* wildtype. The analysis was performed in: (A) GBM *IDH* wildtype *MGMT* methylated (n=52) and *MGMT* unmethylated (n=180).

3.3. The effect of *EGFR* amplification, *PTEN* deletion and *MGMT* methylation in response to therapy

Then we analyzed the predictive value of these biomarkers, in response to therapy of GBM *IDH* wildtype patients. It is important to determine whether these alterations confer some additional information about the response to chemoradiotherapy and radiotherapy, the main adjuvant therapies administered in patients with gliomas.

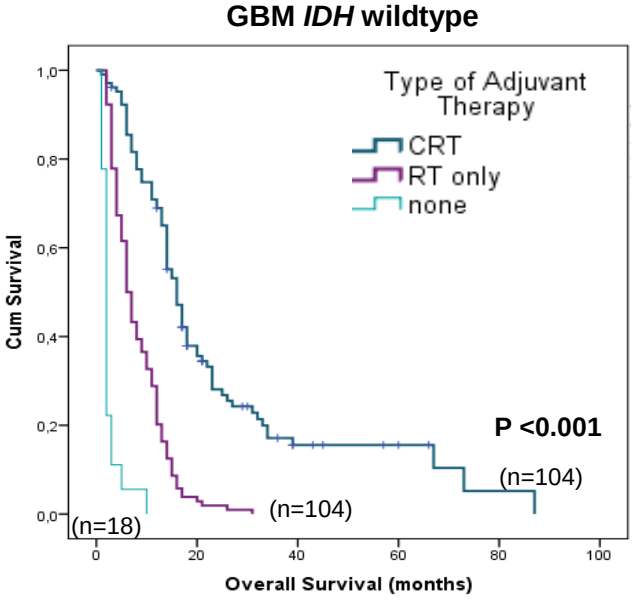


Figure 4.8 – Kaplan – Meier survival analysis categorized according to the type of adjuvant therapy administered in patients with GBM *IDH* wildtype. The analysis was performed using patients exposed to chemoradiotherapy – CRT (n=104), Radiotherapy – RT (n=104) and no treatment – none (n=18).

In Figure 4.8, is shown the standard of response to adjuvant therapies demonstrated by GBM *IDH* wildtype patients. As expected, the chemoradiotherapy is associated with a prolonged overall survival in comparison with radiotherapy alone, which is in agreement with previous studies reporting that chemotherapy with TMZ in addition to radiotherapy confers a survival benefit to GBM patients (Stupp *et al*., 2005).

In this analysis, no patient received only chemotherapy (TMZ), explaining why this category do not appear in figure 4.8.

3.3.1. *EGFR* amplification

As show in Figure 4.9, *EGFR* amplification did not have a predictive value for response to chemoradiotherapy. However, it seems that patients with *EGFR* amplification have a better response to radiotherapy compared with *EGFR* wildtype patients (Figure 4.9 (B)). For the first time, this result suggests, *EGFR* amplification could be a predictor of benefit from radiotherapy, which was unexpected since some studies reported *EGFR* amplification as a predictor of radioresistance in GBM (Barker *et al*., 2000; Sarkaria *et al*., 2006).

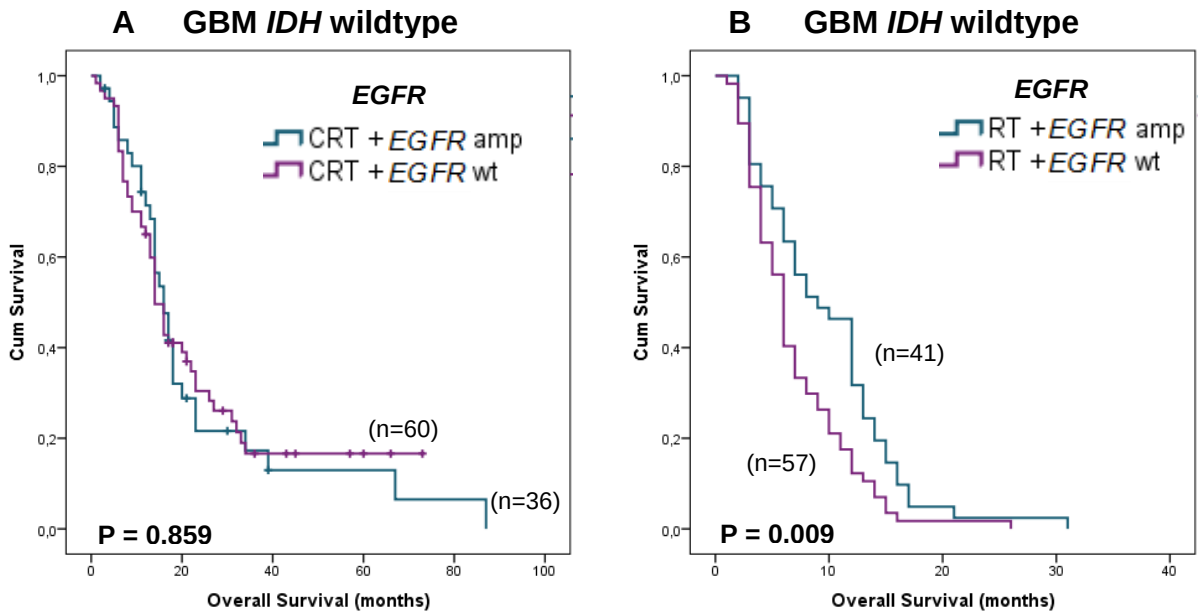


Figure 4.9 – Kaplan – Meier survival estimates of overall survival according to the *EGFR* status and random assignment to Chemoradiotheraphy (A) or Radiotherapy (B) in patients with GBM *IDH* wildtype. The analysis was performed using: CRT + *EGFR* amplified (amp) (n= 36), CRT + *EGFR* wildtype (wt) (n=60), RT + *EGFR* amplified (n= 41) and RT + *EGFR* wildtype (n=57) patients.

3.3.2. *PTEN* deletion

The *PTEN* deletion effect on response to therapy was only evaluated for patients exposed to radiotherapy, since the most cases of patients exposed to chemoradiotherapy were *PTEN* deleted. *PTEN* deletion did not have predictive value for response to radiotherapy in GBM *IDH* wildtype, which could be correlated with the disproportion between both subgroups.

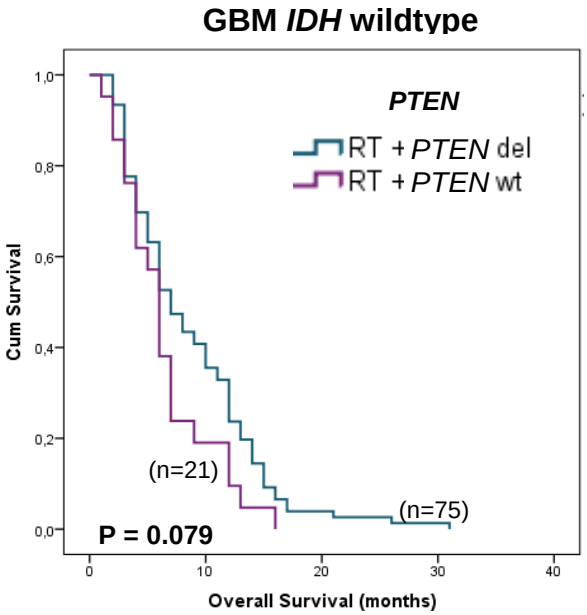


Figure 4.10 – Kaplan – Meier survival estimates of overall survival according to the *PTEN* status and random assignment to Radiotherapy in patients with GBM *IDH* wildtype. The analysis was performed using: RT + *PTEN* deleted (n= 75) and RT + *PTEN* wildtype (n=21) patients.

3.3.3. *MGMT* methylation

As expected, in our results *MGMT* methylated samples are associated with an improved response to chemoradiotherapy compared to *MGMT* unmethylated samples. *MGMT* is an important predictor of response to chemotherapy, although it is not correlated with the response to radiotherapy (Figure 4.11).

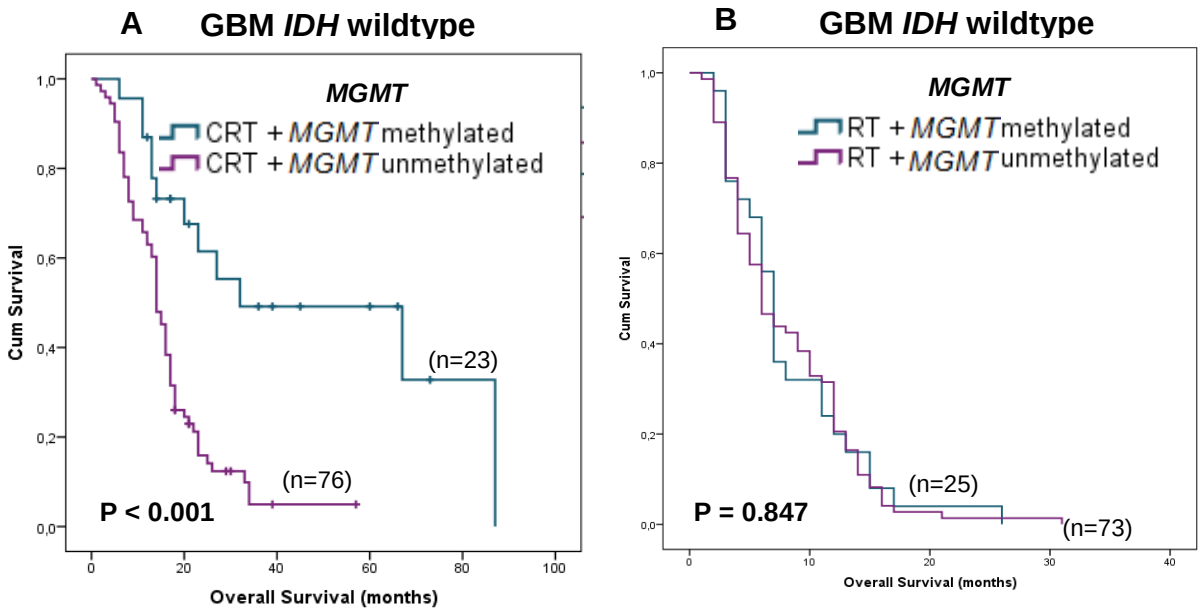


Figure 4.11 – Kaplan – Meier survival estimates of overall survival according to the *MGMT promoter methylation* status and random assignment to Chemoradiotheraphy or Radiotherapy in patients with GBM *IDH* wildtype. The analysis was performed using: CRT + *MGMT* methylated (n= 23) and CRT + *MGMT* unmethylated (n=76), RT + *MGMT* methylated (n= 25) and RT + *MGMT* unmethylated (n=73) patients.

In Kaplan Meir survival curves where the *P- value* was significant, to prove the molecular alteration impact in the prognosis of patients or response to therapy, we used a multivariate Cox Regression analysis. Hazard rates and 95% confidence intervals also were obtained for age-, gender-, treatment- adjusted stratified Cox proportional hazard models (Table 4.8).

Table 4.8 – Univariate and multivariate Cox Regression analysis of overall survival.

	N	Median Survival (months)	Univariate analysis			N	Multivariate analysis*		
			Hazard ratio	95% CI	P Value		Hazard ratio	95% CI	P Value
GBM									
IDH mutated	10	25.0	0.39	0.19 - 0.79	0.009	10	0.52	0.24 – 1.11	0.092
IDH wildtype	241	10.0		Reference		230		Reference	
Astrocytoma									
IDH mutated	52	16.0	0.30	0.17-0.54	<0.001	45	0.31	0.16 – 0.59	<0.001
IDH wildtype	51	114.0		Reference		41		Reference	
GBM *IDH* wildtype									
PTEN wildtype	38	6.0		Reference		35		Reference	
PTEN deletion	184	11.0	0.63	0.43 – 0.91	0.015	178	0.67	0.45 – 0.99	0.048
GBM *IDH* wildtype									
MGMT methylated	52	12.0	0.57	0.40 – 0.81	0.002	50	0.59	4.12 – 6.86	0.006
MGMT unmethylated	180	9.0		Reference		171		Reference	
Astrocytoma *IDH* wildtype	21	46.0		Reference				Reference	
PTEN wildtype						21			
PTEN deletion	20	8.0	3.10	1.40 – 6.84	0.005	20	4.347	1.42 – 13.29	0.010
GBM IDH wildtype									
RT + EGFR amp	41	9.0	0.60	0.40 – 0.91	0.017	41	0.558	0.37- 085	0.007
RT + EGFR wt	57	6.0		Reference		57		Reference	
GBM IDH wildtype									
CRT + Methylated	23	32.0	0.28	0.14 – 0.55	<0.001	23	0.271	0.14 -0.58	<0.001
CRT + Unmethylated	76	14.0		Reference		76		Reference	

***Multivariate analysis performed controlling the following independent variables: age, gender, treatment**

The distinct number of samples between the molecular groups analyzed could influence the P-value obtained, as well as the confidence intervals, which explains why the p-value obtained by the multivariate analysis for *IDH* in GBM was not significant (Table 4.8).

Additionally, the multivariate Cox Regression analysis showed that *IDH* mutations in astrocytomas and *MGMT* methylation in GBM *IDH* wildtype are statistically significant good prognostic factors. On the other hand, *PTEN* deletion in astrocytomas *IDH* wildtype, was associated with poorer outcomes in a statistically significant way. In GBM *IDH* wildtype *PTEN* deletion was correlated with better outcomes, although the number of *PTEN* wildtype samples used was reduced (Table 4.8). In addition, this analysis showed the predictive effect of *EGFR* amplification to radiotherapy response, as well as, *MGMT* methylated predictive effect on chemotherapy response.

4. *PIK3CA* mutational analysis

After a careful characterization of the cohort, we focused in the main goal of this work, the impact of *PIK3CA* mutations in the molecular subgroups of gliomas. This analysis was directed to exon 9 and 20 of *PIK3CA*, the main hotspots of this gene which encode the helical and catalytic domains of p110α, respectively. All the molecular alterations found in *PIK3CA* were researched in specific databases in order to understand their possible role.

Table 4.9 – Percentage of *PIK3CA* mutations identified in this in study.

***PIK3CA* mutations in gliomas**			
Molecular subgroup	**N**	**Number of mutations**	**Mutations (%)**
GBM	256	8	3%
IDH wildtype	245	7	3%
IDH mutated	11	1	9%
Oligodendrogliomas (*IDH* mutant + 1p/19q codeletion)	49	5	10%
Astrocytomas	109	7	6%
IDH wildtype	53	4	8%
IDH mutated	56	3	6%
Total	414	20	5%

N- Dimension of the sampling

The mutational analysis of *PIK3CA* showed that molecular alterations in this gene occurred more frequently (10%) in the oligodendroglioma (*IDH* mutant + 1p/19q codeletion) subgroup (Table 4.9). On the other hand, these mutations tend to appear less frequently in the GBM subgroups (3%), which constitutes the major glioma subgroup in study. However, the percentage of *PIK3CA* mutations seems to be higher in the GBM *IDH* mutated subgroup compared to the *IDH* wildtype subgroup, although it is important highlight the reduced number of GBM *IDH* mutated samples (n=11) in comparison with the elevated number of samples included in the GBM *IDH* wildtype subgroup (n=245).

Additionally, the frequency of these mutations in astrocytomas was similar for both subgroups (6% in *IDH* mutated subgroup and 8% in *IDH* wildtype subgroup).

Overall, in our cohort we detected 20 *PIK3CA* mutations corresponding to 5.00% of mutations in 414 glioma samples (Table 4.9). These percentages seem to have a different distribution by the glioma molecular subgroups compared to the studies using only the histological criteria to classify the glioma samples.

4.1. *PIK3CA* mutations already reported

Table 4.10 – Description of the *PIK3CA* mutations already reported identified in our cohort.

***PIK3CA* reported mutations**			**Molecular subgroups of gliomas**				
Exon	**Nucleotide**	**Amino acid**	**GBM *IDH* wildtype**	**GBM *IDH* mutated**	***IDH* mutant + 1p/19q codeletion ***	**Astrocytoma *IDH* wildtype**	**Astrocytoma *IDH* mutated**
20	c.2965 C>G	L989V					1
20	c.2991 C>T	L997=			1		
20	c.3140 A>G	H1047R	3	1		1	
20	c.3140 A>T	H1047L				1	
9	c.1616 C>T	P539L			1		
9	c.1624 G>A	E542K				1	1
9	c.1633 G>C	E545N	1				
9	c.1634 A>C	E545A	1				
9	c.1635 G>T	E545D			1		
9	c.1636 C>A	Q546K				1	
9	c.1637 A>C	Q546P	1				
9	c.1656 G>A	E552K			1		
	Total		6	1	4	4	2

*Oligodendroglioma

The variants represented in Table 4.10 were researched in different databases such as Ensembl, Catalogue of Somatic Mutations in Cancer (COSMIC) and The Human Gene Mutation Database (HGMB) in order to analyze their possible role and frequency. These mutations were already described and documented by other scientific groups (Broderick *et al*., 2004; Samuel *et al*., 2004; Zhao and Vogt, 2008).

The majority of the mutations identified correspond to missense alterations that led to the exchange of one amino acid by another, only the L997= is a synonymous variant. According to COSMIC L997= is a pathogenic alteration since it leads to splice site changes in the protein. The most common mutation in the cohort was the H1047R that appeared in GBM and astrocytoma samples. However, we found a higher diversity of mutations in exon 9 compared to exon 20. Overall, we identified 9 cases with *PIK3CA* mutations in exon 9 and 8 cases with mutations in exon 20.

During the mutational analysis directed to exon 9 and 20 of *PIK3CA*, we found some variants that had not yet been described. To understand the possible role of these variants, we used *in silico* analysis, which was crucial to estimate the possible effect of these variants on the p110α catalytic subunit.

Table 4.11 – Unreported variants identified in *PIK3CA* and estimation of their impact through *in silico* analysis

***PIK3CA* Unreported Variants**						
Molecular subgroup	**Exon**	**Nucleotide**	**Amino acid**	**Mutation Taster Prediction**	**Variant Effect Predictor**	**Observations**
GBM *IDH* wildtype	20	c.3112T>C	Y1038H	Disease causing	Missense variant (moderate impact)	Protein features might be affected
GBM *IDH* mutated	20	c.3210A>G	_____	Polymorphism	3'UTR variant	_____
IDH mutant + 1p/19q codeletion*	20	c.2988T>C	N996N	Disease Causing	Synonymous variant	Splice site changes
Astrocytoma *IDH* mutated	20	c.3040C>T	Q1014X	Disease Causing	Stop gained	Creates a Stop codon (changes from position 3207 to 3042)

X – Indicates a stop codon; *Oligodendroglioma

In this study, we found 4 unreported variants in exon 20 with distinct putative impacts in the protein encoded, 3 of them were considered pathogenic and the other polymorphic. It is predicted that the c.3112T>C variant has a possible pathogenic effect on the protein encoded, since this molecular alteration lead to an amino acidic change with a moderate impact (Table 4.11). In addition, the synonymous variant c.2988T>C, was estimated as disease causing, because it can determine splice site changes inducing the formation of a distinct protein. Moreover, the *in silico* analysis, indicated c.3210A>G as a polymorphic variant in the 3'UTR region, with no pathogenic effect attributed.

Furthermore, *in silico* analysis also indicated c.3040C>T as a pathogenic variant, which creates a stop codon in position 3042 instead of 3207. This variant can induce the formation of a p110 catalytic subunit slightly truncated with around less 55 amino acids. All these variants only appear one time in 4 distinct cases. The *in silico* analysis performed allow predicting the effect of these molecular alterations on the functionality of the encoded protein.

5. *PIK3CA* mutations and other biomarkers: *TERT* mutations, *PTEN* deletion, *EGFR* amplification and *MGMT* methylation

Since *PIK3CA* encodes a subunit of an important enzyme involved in the PI3K/Akt signaling pathway, it seems interesting to study the mutational status of other genes (mainly *EGFR* and *PTEN*) ,which make part of the same pathway, when *PIK3CA* is mutated. To understand if *PIK3CA* mutations are associated with the onset of other molecular alterations or if they are mutually exclusive mutations.

Table 4.12 – Clinical and molecular data from samples of *PIK3CA* mutated GBM in our cohort

Molecular Characterization of *PIK3CA* mutated GBM								
GBM *IDH* wildtype samples	**Age (years)**	**Sex (M/F)[b]**	**Overall Survival (months)**	***PIK3CA**** **Exon**	***TERT* mut**	***EGFR* amp**	***PTEN* del**	***MGMT* Methylation**
1	56	M	9	20	+	-	+	10%
2	54	F	6	20	+	-	+	32%
3	47	M	52	9	NOS[a]	-	-	42%
4	61	M	23	9	+	-	+	7%
5	53	M	18	9	NOS[a]	+	+	14%
6	28	M	14	20	-	-	+	15%
7	69	M	7	20	NOS[a]	-	-	9%
Total/Median	54	6/1	14	---------	75% (3/4)	14% (1/7)	71% (5/7)	29% (2/7)
GBM *IDH* mutated samples								
1	41	F	24	20	NOS	+	-	70%

a - cases where it was not possible perform the analysis; b- Male/ Female

(+) Present; (-) absent; *TERT* mutations (mut); EGFR amplification (amp); PTEN deletion (del);

*Fisher exact test was used to calculate the possible association between *PIK3CA* mutations and *PTEN* deletions (P=0.324)

In Table 4.12 it was performed a clinical and molecular characterization of patients with *PIK3CA* mutated GBM. In the GBM *IDH* wildtype subgroup the most cases with *PIK3CA* mutations, more specifically 71% (5/7), are positive for *PTEN* deletion. It seems that in 5 out of 7 cases of GBM *IDH* wildtype occur two events of dysregulation in the PI3K/Akt signalling pathway. However, using Fisher exact test, there was no statistically significant association between *PIK3CA* mutations and *PTEN* deletion in GBM *IDH* wildtype subgroup (P=0.324), which could be explained by the reduced number of samples.

Additionally, the *EGFR* amplification is present in only 1 out of 7 cases, so the sample number 5 is triple positive (*PIK3CA* mutated, *EGFR* amplified and *PTEN* deleted). The most *PIK3CA* mutated GBM *IDH* wildtype cases are *MGMT* unmethylated. Moreover, *TERT* mutations were only analysed for 4 *PIK3CA* mutated samples and they were present in 3 of them.

In GBM *IDH* mutated subgroup, it was found only one case with *PIK3CA* mutation, which demonstrates an opposite molecular standard compared to GBM *IDH* wildtype.

Table 4.13 – Clinical and molecular data from samples of *PIK3CA* mutated, *IDH* mutant + 1p/19q codeleted gliomas.

Molecular characterization of *PIK3CA* mutated oligodendrogliomas

IDH* mutant + 1p/19q codeletion Samples	**Age (years)**	**Sex (M/F)[b]**	**Overall Survival (months)**	**Exon**	***TERT* mut**	***EGFR* amp**	***PTEN* del**	***MGMT* Methylation**
1	42	F	181[c]	9	+	-	-	46%
2	57	M	172	20	-	-	-	46%
3	41	M	44 [c]	20	NOS[a]	-	-	43%
4	63	M	214	9	+	-	+	30%
5	62	M	166[c]	9	NOS[a]	-	-	59%
Total/Median	57	4/1	172	---	66.7% (2/3)	0% (0/0)	20% (1/5)	100% (5/5)

a - cases where it was not possible perform the analysis ; b- Male/ Female ; c- Patients that still alive

(+) Present; (-) absent; *TERT* mutations (mut); EGFR amplification (amp); PTEN deletion (del); * Oligodendroglioma

The results demonstrated in Table 4.13 shown all *PIK3CA* mutated *IDH* mutated + 1p/19q codeleted (oligodendrogliomas) are negative for *EGFR* amplification. *PTEN* deletion was identified in only 20% of the cases. In addition, the 5 cases of *PIK3CA* mutated oligodendrogliomas are *MGMT* methylated. Overall, these results are completely different from the ones obtained for the both GBM subgroups.

Table 4.14 – Clinical and molecular data from samples of *PIK3CA* mutated astrocytomas

Molecular characterization of *PIK3CA* mutated astrocytomas

Astrocytomas *IDH* wildtype samples	Age (years)	Sex (M/F)[b]	Overall Survival (months)	*PIK3CA** Exon	*TERT* mut	*EGFR* amp	*PTEN* del	*MGMT* Methylation
1	57	F	12	9	NOS[a]	-	+	15%
2	57	F	1	20	NOS[a]	+	+	33%
3	54	F	15	9	NOS[a]	+	+	28%
4	65	M	2	20	+	-	+	10%
Total/Median	57	1/3	7	--	NOS[a]	50% (2/4)	100% (4/4)	50% (2/2)
Astrocytomas *IDH* mutated samples								
1	28	F	16	9	+	-	-	23%
2	23	M	87[c]	20	NOS[a]	NOS[a]	NOS[a]	14%
3	51	F	222	20	NOS[a]	-	-	99%
Total /Median	28	1/2	87	--	(1/1)	0% (0/2)	0% (0/2)	33% (1/3)

a - cases where it was not possible perform the analysis ; b- Male/ Female; c- Patients that still alive

(+) Present; (-) absent; *TERT* mutations (mut); EGFR amplification (amp); PTEN deletion (del)

*Fisher exact test was used to calculate the possible association between *PIK3CA* mutations and *PTEN* deletions in astrocytomas *IDH* wildtype (P=0.099)

In table 4.14 it is represented the molecular and clinical characterization of *PIK3CA* mutated astrocytomas *IDH* wildtype and *IDH* mutated.

However, the 4 astrocytomas *IDH* wildtype *PIK3CA* mutated have *PTEN* deletion, and two of them also are positive for *EGFR* amplification. In GBM *IDH* wildtype, *PTEN* deletion was also present in the majority of *PIK3CA* mutated tumors (5/7) (Table 4.12). Using Fisher exact test, it was possible to verify that there was no association between *PIK3CA* mutations and *PTEN* deletion (P=0.099), although this result could be different with an increased number of *PIK3CA* mutated cases. In *IDH* wildtype subgroups, PI3K/signaling pathway seems to be more altered, with at least 2 events occurring simultaneously. These tumors appear to be more complex, including distinct molecular alterations compared to *IDH* mutated subgroups.

In addition, astrocytomas *IDH* mutated with *PIK3CA* mutations analyzed are negative for *EGFR* amplification and *PTEN* deletion. Moreover, only one of these cases has *MGMT* methylated. It seems astrocytomas *IDH* mutated are tumors less molecularly altered in comparison with the *IDH* wildtype tumors, which is in accordance with the aggressiveness and overall survival of patients.

6. Impact of *PIK3CA* mutations in patient's prognosis

In this study, we tried to understand the putative role of *PIK3CA* in the prognosis of patients with gliomas, because it was important to clarify if *PIK3CA* mutations confer additional information about this disease. However, the number of *PIK3CA* mutations found in our cohort was reduced (20/414), so it was not possible determine the prognostic value and response to therapy of these mutations in the molecular subgroups of gliomas.

7. Rs45455192 single nucleotide polymorphism (SNP)

During the mutational analysis of *PIK3CA* gene, it was identified a SNP (Rs45455192) located in the intronic region flanking the coding exon 9 of *PIK3CA*. This polymorphism was not documented yet in gliomas. In Table 4.15, we assessed the T allele frequency in the distinct glioma molecular subgroups.

Table 4.15 Frequency of Rs45455192 in glioma molecular subgroups

SNP in brain tumors			
SNP	Localization	Glioma subgroup	Polymorphism (%)
Rs45455192	Intron 8 (-55) c. 1540-55C>T	**GBM**	18.00%(46/256)
		IDH mutated	18.00% (2/11)
		IDH wildtype	18.00% (44/245)
		IDH* mutant + 1p/19q codeletion	24.00% (12/49)
		Astrocytomas	19.00%(20/109)
		IDH wildtype	21.00% (11/53)
		IDH mutated	16.00% (9/56)

***Oligodendroglioma

As shown in Table 4.15, the SNP Rs45455192 is located -55 nucleotides from coding exon 9, being the T variant found in all glioma molecular subgroups. In addition, this less common variant was identified in a similar percentage in the different subgroups of gliomas ranging from 16% to 24%, which means that the CT genotype seems to have an uniform distribution between the different gliomas types.

As the frequency of this polymorphism in the European population was estimated as 8%, and the percentage for the molecular subgroups of gliomas obtained was higher, we studied the potential impact of this polymorphism in the survival of patients with gliomas (Figure 4.12). Despite this

polymorphism had already been documented in other types of cancer, its impact on patients prognosis has never been studied until now.

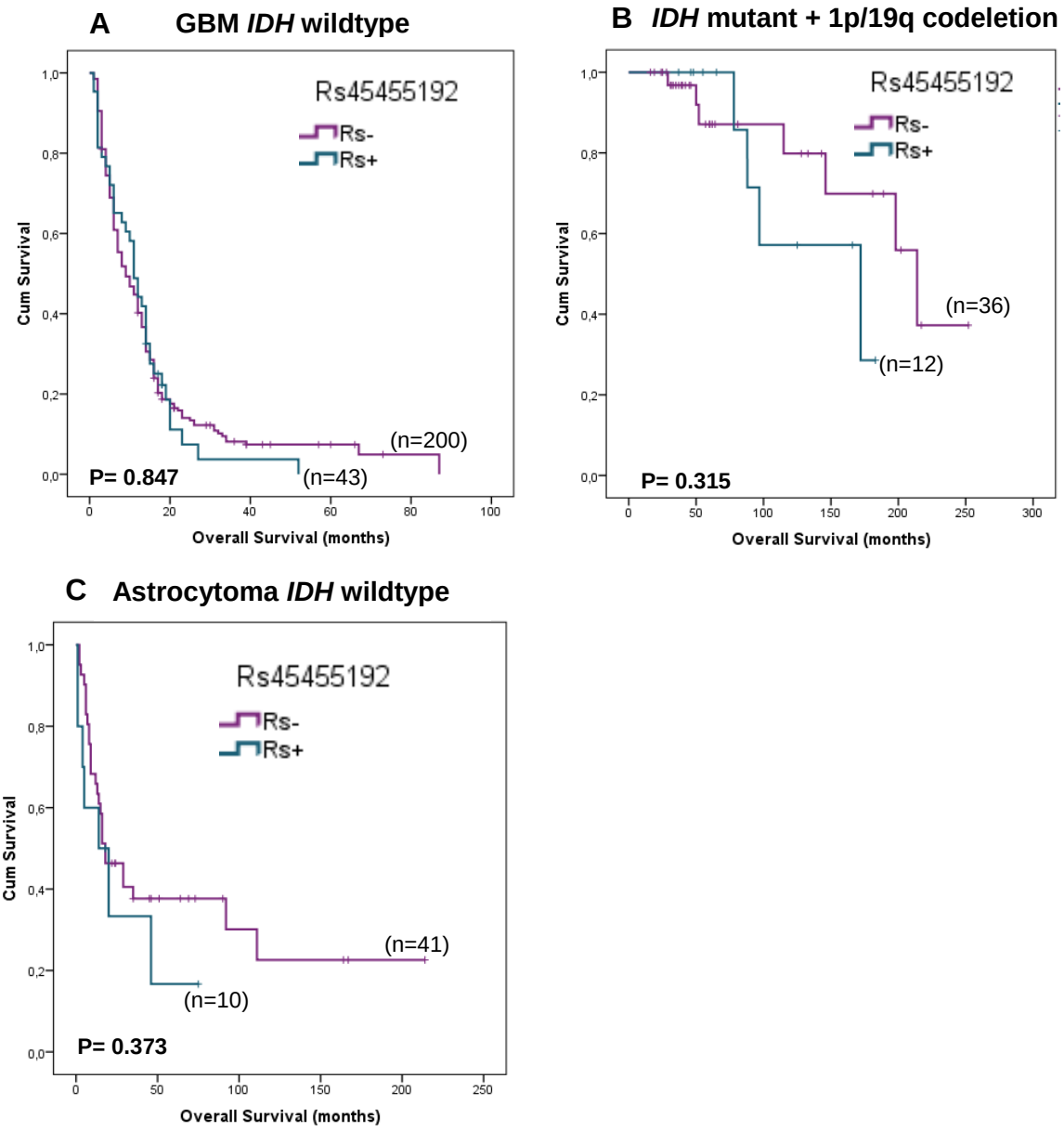


Figure 4.12 – Kaplan – Meier curves of overall survival to determine the prognostic effect of Rs45455192 in (A) GBM *IDH* wildtype – Rs+ (n= 43) and Rs- (n= 200), (B) *IDH* mutant + 1p/19q codeletion (Oligodendrogliomas) – Rs+ (n=12) and Rs- (n= 36), (C) Astrocytoma *IDH* wildtype – Rs+ (n=10) and Rs- (n=41)

No significant association was found between Rs45455192 and the overall survival of the patients included in these 3 molecular subgroups of gliomas (Figure 4.12).

In Table 4.16 is represented the respective dimension of the groups used to create these survival curves, as well as the medians calculated for GBM *IDH* wildtype, *IDH* mutant + 1p/19q codeletion (oligodendrogliomas) and astrocytomas *IDH* wildtype in the presence or absence of the less common variant (T).

Table 4.16 - Medians corresponding to the survival time established for the molecular subgroups with or without the polymorphism.

Type of glioma	N	Median (months)	
		Estimate	Std. Error
GBM *IDH* wildtype	242		
Rs45455192+	43	11.000	1.093
Rs45455192-	199	9.000	1.342
IDH mutatnt + 1p/19q codeletion (Oligodendroglioma)	48		
Rs45455192+	12	172.000	58.440
Rs45455192-	36	214.000	15.959
Astrocytoma *IDH* wildtype	52		
Rs45455192+	10	14.000	8.894
Rs45455192-	42	18.000	6.061

Rs45455192 (+) – present
Rs45455192 (-) - absent

The results represented in Table 4.16 show that despite this C to T polimorphism does not have statistically significant prognostic value (Figure 4.11), the T variant has a slightly effect on the median survival of patients with gliomas. The median survival of patients with Rs45455192 positive *IDH* mutant +1p/19q codeletion (oligodendrogliomas), and astrocytomas *IDH* wildtype is slightly reduced compared to the median survival of patients with the same tumors Rs45455192 negative.

These data are not statistically significant may be due to the number of Rs45455192 positive and negative cases in the distinct groups of gliomas analyzed. However, looking for the medians of overall survival it seems this SNP could have a trend on the patient's survival. The same trend was not shown for the GBM *IDH* wildtype subgroup. However, in this subgroup it seem to be a trend the long survival patients with tumors Rs45455192 negative (Figure 4.12 (A)), live more than long survival patients with Rs45455192 positive, although it is crucial increase the number of samples to evaluate the effect of this polymorphism.

8. *PIK3CA* mutational analysis in recurrent gliomas

Finally we analyzed the impact of *PIK3CA* mutations in gliomas recurrence, in order to detect whether these mutations are present during all stages of glioma development or whether they are passanger mutations with an important role at the initiation of the tumor. This is an important issue, since a putative treatment, using an inhibitor targeting *PIK3CA* could increase the specificity and applied in an early stage of the tumor could be more effective.

Table 4.17. *PIK3CA* mutational analysis in 15 recurrence cases analyzed

Number of cases	Primary tumor (P) Diagnosis	1ª recurrence (1ª) Diagnosis	2ª recurrence (2ª) Diagnosis	*PIK3CA* mutational status
1	Astrocytoma *IDH* wildtype	GBM *IDH* wildtype	_____	P- wildtype 1ª - wildtype
2	Astrocytoma *IDH* mutated	GBM *IDH* mutated	_____	P- c.2965C>G 1ª- c.2965C>G
3	Astrocytoma III *IDH* mutated	GBM *IDH* mutated	_____	P- wildtype 1ª - wildtype
4	Astrocytoma III *IDH* wildtype	GBM Giant cells *IDH* wildtype	_____	P- wildtype 1ª - wildtype
5	Astrocytoma II *IDH* mutated	GBM *IDH* mutated	_____	P-wildtype 1ª - wildtype
6	Astrocytoma II *IDH* wildtype	GBM *IDH* wildtype	_____	P- wildtype 1ª- wildtype
7	Astrocytoma *IDH* mutated	GBM I*DH* mutated	GBM *IDH* mutated	P- c.1633G>A; c.1664+5G>C 1ª- c.1633G>A; c.1664+5G>C 2ª- c.1633G>A; c.1664+5G>C
8	Glioma *IDH* wildtype	GBM *IDH* wildtype	_____	P- wildtype 1ª - wildtype
9	Astrocytoma *IDH* mutated	GBM *IDH* mutated	_____	P- wildtype 1ª -wildtype
10	Astrocytoma III *IDH* mutated	GBM I*DH* mutated	_____	P- wildtype 1ª - wildtype
11	Astrocytoma II *IDH* mutated	GBM *IDH* mutated with PNET component	_____	P- wildtype 1ª - wildtype
12	Astrocytoma III *IDH* wildtype	GBM *IDH* wildtype	_____	P- wildtype 1ª - wildtype
13	Astrocytoma III *IDH* wildtype	GBM *IDH* wildtype	_____	P- wildtype 1ª - wildtype
14	Glioma III *IDH* wildtype	Glioma III *IDH* wildtype	GBM *IDH* wildtype	P- wildtype 1ª – wildtype 2ª - wildtype
15	Astrocytoma II *IDH* mutated	Astrocytoma II *IDH* mutated	GBM *IDH* mutated	P- wildtype 1ª- wildtype 2ª - wildtype

Primary tumor (P); 1ª Recurrence (1ª); 2ª Recurrence (2ª)

We identified only 2 cases out of 15 (case number 2 and number 7) with *PIK3CA* mutations, as demonstrated in Table 4.17. Patient number 2 had a mutation in exon 20 of *PIK3CA (*c.2965C>G), present in the primary tumor and in the relapse. Patient number 7, had a *PIK3CA* mutation in exon 9 (c.1633G>A) and also an unreported variant in intron 9 (c.1664+5G>C) which were present in the primary tumor and also in the two relapses.

Furthermore in these two recurrent cases the *PIK3CA* mutations appear in the primary tumor, suggesting this molecular alteration could be an early event during gliomagenesis. In these glioma recurrent cases, the progression of the tumor is associated with an increased aggressiveness (astrocytoma to GBM), explaining why it is so important identify new therapeutic targets present in all the sectors of the tumor to prevent its dissemination through the residual tumor cells. Moreover, based on these results, *PIK3CA* mutations seem to be maintained during the tumor recurrences. This indicates *PIK3CA* mutations are not passenger mutations, but rather events present throughout the development of the tumor that may contribute to its aggressiveness. The *PIK3CA* mutational analysis in glioma recurrences was performed for the first time in here, despite the number of cases being reduced (n=15) these results seem to be interesting. In future, it would be important validate them in other recurrence cases.

5. Discussion

1. Impact of the 2016 WHO classification in gliomas characterization

In 2016, the classification for CNS tumors was published on Acta Neuropathologica (Louis *et al*., 2016), which brought a new insight about the gliomas' organization and characterization. In this work we reclassified the gliomas of IPOLFG cohort according to the 2016 WHO classification (Table 4.1). Based on these results, the number of *IDH* mutant + 1p/19q codeletion (oligodendrogliomas) was significantly reduced after the molecular reclassification, which could be explained by the current definition of this glioma group. In addition, the frequency of astrocytomas increased significantly (from 60 to 109), mainly due to the disappearance of the oligoastrocytoma designation (Table 4.1).

According to Tabouret *et al.*, the reclassification of the french cohort showed a similar frequency of *IDH* mutant + 1p/19q codeleted gliomas, while the number of GBM and astrocytomas diagnoses increased (Tabouret *et al*., 2016). These distinct results obtained between our study and the french cohort, are explained by the fact the most oligoastrocytomas in our study were reclassified as astrocytomas, while in the french cohort the most oligoastrocytomas were considered GBM (Tabouret *et al*., 2016). Even with the introduction of new molecular biomarkers, the oligoastrocytomas inclusion in the major groups of gliomas continue to be a difficult task. In addition, a Japanese study also reported astrocytomas and *IDH* mutant + 1p/19q codeleted (oligodendrogliomas) subgroups as the main targets of 2016 WHO classification effect, which is in agreement with our results (luchi *et al*., 2018).

In addition to these 3 major molecular subgroups of gliomas, we also identified a small group of samples (n=41) which remain to be classified, because they correspond to particular cases of gliomas with 1p or 19q codeletion or *IDH* wildtype and 1p/19q codeleted tumors (Figure 4.1). For these situations, it seems to miss additional biomarkers to differentiate the type of glioma.

As previously reported by luchi *et al*., (66%), Tabouret *et al*., (50%), Reifenberger *et al*., (50%) and Ostrom *et al*.,(45%), also in this cohort GBM constitute the most prevalent glioma type (61.8%) (luchi *et al*., 2018; Tabouret *et al*., 2016; Reifenberger *et al*., 2016; Ostrom *et al*., 2014). Additionally, GBM *IDH* wildtype corresponded to around 59.1% of GBM samples and the remaining 2.7% were GBM *IDH* mutated, being the first subtype most frequent in patients with a median age of 63 years and the second most prevalent in younger patients (median age of 44 years) (Table 4.2 and Table 4.3). These frequencies are variable between the distinct studies, although GBM *IDH* mutated were referred as approximately 10% of all GBM in 2016 WHO classification (Parsons *et al*.,2008; Ohgaki e Kleihues, 2012; Louis *et al*., 2016) which means that the percentage obtained in our cohort was slightly reduced. Moreover, the frequency of GBM *IDH* mutated analyzed by luchi and co-workers was 7.8% and Tauboret *et al*. achieved a frequency of 17.0% (luchi *et al*., 2018; Tabouret *et al*., 2016).

The prevalence of *IDH* mutant + 1p/19q codeleted gliomas (12%) (Table 4.2), is higher than the described by Louis *et al*. (5.9%) and lower than the reported by Tabouret *et al*. (32.5%). Astrocytomas *IDH* wildtype and *IDH* mutated showed similar frequencies (Table 4.2), differing from the results presented by Blass *et al*. and Hartmann *et al*., which estimated around 70% of astrocytomas *IDH* mutated (Balss *et al*., 2008; Hartmann *et al*., 2009). However, in this study the analysis of *ATRX* loss and *TP53* mutations was not performed to validate the astrocytoma diagnosis, which constitutes a

limitation. Our results showed 2016 WHO classification induce an increased prevalence of astrocytomas and a decreased number of oligodendrogliomas, being the reclassification of the oligoastrocytoma group the main cause of differences between studies of 2016 WHO classification impact.

The survival analysis performed using 2016 WHO classification, corroborate with the idea that molecular subgroups established allowed a better division of gliomas in terms of diagnosis and prognosis, separating the patients with better and poorer outcomes more easily. Our results are in accordance with the studies that described 2016 WHO classification as a more accurate method to predict the clinical outcome (Tabouret *et al.,* 2016; Louis *et al*., 2016).

In this study, as well as in previous works, grade II oligodendrogliomas were the malignant glioma group related with better prognosis (Figure 4.1 and Table 4.4) (luchi *et al*., 2018; Tabouret *et al*., 2016). On the other hand, GBM is one of the most complex and heterogeneous types of cancer, being associated with an elevated mortality and morbidity (Ostrom *et al*., 2014). Not surprisingly, this glioma group was associated with the worst prognosis (Figure 4.1 and Table 4.4) as documented by other studies (van den Bent and Chang, 2018; luchi *et al*., 2018; Tabouret *et al.,* 2016). As already reported, also in this study it was clear that using the 2007 WHO classification all the histological entities followed the principle: higher tumor grade corresponds to shorter survival (Louis *et al*., 2007).

The survival curves performed using gliomas reclassified according to the 2016 WHO classification, showed some similarities with the histological classification. *IDH* mutant + 1p/19q codeletion (oligodendrogliomas) were the molecular group associated with a prolonged overall survival (198 months) (Figure 4.2 and Table 4.5) demonstrating an increased median overall survival compared to grade II (172 months) and III oligodendrogliomas (97 months) (Figure 4.1 and Table 4.4). Griffin *et al*. and Jenkins *et al*., referred 1p/19q codeleted gliomas were associated with better prognosis compared to the non-codeleted tumors (Griffin *et al*., 2006; Jenkins *et al*., 2006). This may suggest that the new oligodendrogliomas specification can group a set of gliomas with better prognosis, as showed by our results (Figure 4.2).

The *IDH* mutational analysis divided the GBM group into two subgroups with distinct prognostic value (Louis *et al*., 2016; Wang *et al*., 2015). The *IDH* mutated subgroup is associated with a median survival of 25 months and the *IDH* wildtype subgroup with a median survival of 10 months (Figure 4.2 and Table 4.5).These results differ from the reported by Yan *et al*. (31 months for GBM *IDH* mutated and 15 months for GBM *IDH* wildtype), although are in agreement with the described by Nobusawa *et al.* (9.9 months for GBM *IDH1* wildtype and 24.0 months for GBM *IDH1* mutated).

Interestingly, the median overall survival of patients with astrocytomas *IDH* mutated is equal to the median overall survival of grade II astrocytomas (Table 4.3 and Table 4.5). This may suggest that grade II astrocytomas could be associated with better overall survival due to the most cases possess *IDH* mutations. In this cohort, both astrocytomas and GBM *IDH* mutated are associated with better prognosis as previously demonstrated by other studies (Parsons *et al*.,2008; Nobusawa *et al*., 2009, Ohgaki e Kleihues, 2012; Louis *et al*., 2016). *IDH* mutations induce the *MGMT* methylation and consequently a better response to chemotherapy (Xu *et al*., 2011). *IDH* mutated and *MGMT* methylated tumors have a better response to chemotherapy, as well as 1p/19q codeleted tumors have a better response to PCV (Griffin *et al*., 2006; Baer *et al*., 1993). The survival curves obtained for the molecular

groups of gliomas are in agreement with the survival analysis performed by Pekmezci and co-authors (Pekmezci *et al*., 2017)

The molecular classification brought an improvement in the diagnosis accuracy and patient management, determining some additional information about response to therapy. The 2016 WHO classification introduced a precise specification of gliomas compared to the previous 2007 WHO classification. This analysis also validated the dimension and representativeness of our dataset for further study of *PIK3CA* mutations.

Afterwards, we evaluated the impact of *EGFR* amplification, *PTEN* deletion, *TERT* mutations and *MGMT* methylation in addition to *IDH* mutations and 1p/19q codeletion in gliomas diagnosis, prognosis and response to therapy. These analyzes were performed to characterize our dataset and to define the putative association between these alterations and *PIK3CA* mutations in gliomas.

EGFR amplifications have been described as frequent in GBM (40-50%) (Libermann *et al*., 1985; Wong *et al.,* 1987; Decker, 1990), especially in GBM *IDH* wildtype (Sturm *et al.,* 2012). In our cohort, we observed 40% of *EGFR* amplification in GBM *IDH* wildtype and 38% in astrocytomas *IDH* wildtype, suggesting that this alteration is more frequent in *IDH* wildtype subgroups (Figure 4.3 and Table 4.7). The higher frequency of *EGFR* amplification in GBM *IDH* wildtype was also reported by other studies (Waitkus et al., 2016; Sartori *et al*., 2017).

Most of the studies performed to evaluate the *EGFR* amplification prognostic value used histological classes, rather than molecular subgroups of gliomas (Shinojima et *al.,* 2003; Chen *et al*., 2015; Quan *et al*., 2005). In here, we verified for the first time in GBM *IDH* wildtype and astrocytomas *IDH* wildtype that *EGFR* amplification has not a significant prognostic value (Figure 4.4). However, this cytogenetic alteration continues to be analyzed since contributes to the overall knowledge of the tumor.

The response to therapy analysis demonstrated that GBM *IDH* wildtype with *EGFR* amplification seems to have a better response to radiotherapy (Figure 4.9), the same did not happen with response to chemoradiotherapy. The results obtained differ from those found by the other groups, which indicate *EGFR* amplification is associated with radioresistance (Barker *et al*., 2000; Sarkaria *et al*., 2006). However, the previous observations were performed in GBM samples, in our study we analyzed the effect of *EGFR* amplification in GBM *IDH* wildtype. In addition, the presence of *IDH* mutations was described as capable of increase the cancer cells sensitivity to radiation (Li *et al*., 2012; Wang *et al.,* 2014).

PTEN deletions were transversal molecular alterations across all glioma subgroups in our cohort (Figure 4.3 and Table 4.7). However, these genetic alterations are most common in GBM *IDH* wildtype (88%) and least frequent in *IDH* mutant + 1p/19q codeletion (oligodendrogliomas) (8%), suggesting that its frequency arise with gliomas aggressiveness. *PTEN* deletions frequency and prognosis value were previously analyzed using the histological classes, instead of molecular subgroups (McLendon *et al*., 2008; Verhaak *et al.,* 2010; Srividya *et al*., 2010; Carico *et al*., 2012). In here we performed this analysis using the glioma molecular subgroups.

In addition, the multivariate analysis showed *PTEN* deletions were good prognostic factors of overall survival in GBM *IDH* wildtype (P=0.048) and prognostic factors of poor outcome in astrocytomas *IDH* wildtype (P=0.010) (Figure 4.6 and Table 4.8). Interestingly, the statistical analysis performed

corroborate with the idea of a dual role of *PTEN* deletions in GBM *IDH* wildtype and astrocytomas *IDH* wildtype which has so far not been documented (Table 4.8). *PTEN* deletion effect on GBM *IDH* wildtype, could be explained by the low number of *PTEN* wildtype samples, which lead us to purpose the validation of this result using a larger sampling. In addition, the median overall survival obtained for *PTEN* deleted GBM *IDH* wildtype was 11 months, similar to the one achieved for all the GBM group (Table 4.8). Moreover, the response to therapy analysis was only performed for patients exposed to radiotherapy, because most patients exposed to chemoradiotherapy had *PTEN* deleted (Figure 4.10). It seems that *PTEN* deleted, as a biomarker, is not predictive of GBM response to radiotherapy (Figure 4.10).

TERT promoter mutations are distributed across all the glioma molecular subgroups, with an elevated incidence in GBM *IDH* wildtype (88%) and oligodendrogliomas (87%) (Figure 4.3 and Table 4.7), which is in accordance with other studies (Lee *et al*., 2017; Louis *et al*., 2016). Recently, Eckel-Passow and co-authors reported *TERT* promoter mutations were not statistically associated with poorer outcomes in GBM *IDH* wildtype (Eckel-Passow *et al*., 2015). In our dataset *TERT* promoter mutations alone were not considered independent predictors of poor outcome in GBM *IDH* wildtype, (Figure 4.5), which is concordant with Nguyen *et al*. and Eckel-Passow *et al*. studies (Nguyen *et al*., 2017; Eckel-Passow *et al*., 2015). However, our findings differ from those described by Lee *et al*., whose results indicate *TERT* promoter mutations as a prognostic factor of poor outcome in GBM *IDH* wildtype (Lee *et al*., 2017).The response to therapy analysis was not performed due to the reduced number of *TERT* wildtype samples in the dataset, resulting from the high incidence of these mutations (Table 4.7).

MGMT methylation was also analyzed in this work, being used a cut-off of 25.00%, which is the most frequently applied to differentiate the methylated from the unmethylated glioma samples (Reifenberger *et al*., 2012; Ramalho- Carvalho *et al*., 2013). *MGMT* is a biomarker studied since many years ago, which is characterized by a predictive potential of response to chemotherapy (Baer *et al*., 1993; Watts *et al*., 1997; Esteller *et al*., 2000). In this cohort, we found the follow percentages of *MGMT* methylated samples: 25% in GBM *IDH* wildtype, 50% in GBM *IDH* mutated, 100% in oligodendrogliomas, 22% in astrocytoma *IDH* wildtype and 89% in astrocytoma *IDH* mutated (Figure 4.3 and Table 4.7).

As described the higher frequency of *MGMT* methylated samples correspond to gliomas types with *IDH* mutations (Reinfenberg *et al*., 2016). *IDH* mutations are responsible by the increased levels of 2-hydroxiglutarate, which determines the inhibition of several enzymes such as Jumonji- C-domain-containing histone-lysine demethylases (Xu *et al*., 2011). Consequently, the decreased activity of histone-lysine demethylases induces a global state of DNA methylation that cause the *MGMT* methylation (Watts *et al*., 1997). Once *MGMT* is methylated, the alkyl groups added by temozolomide are maintained leading to the formation of adducts in DNA and ultimately to the death of cancer cells (Baer *et al*., 1993).

The Kaplan-Meier survival analysis showed that *MGMT* methylated GBM *IDH* wildtype are associated with a better prognosis compared to unmethylated tumors (Figure 4.7), which was also verified by Yang and co-authors (Yang *et al*., 2015). In addition, the multivariate analysis indicates *MGMT* methylation as a good prognostic factor of overall survival in GBM *IDH* wildtype (Table 4.8).

Furthermore, the response to therapy analyzed in the GBM *IDH* wildtype group validated the role of *MGMT* methylation as a predictive biomarker of response to chemotherapy, but not as predictive biomarker of response to radiotherapy (Figure 4.11). Many studies have reported this association between *MGMT* methylation, a better response to chemotherapy and consequently a prolonged overall survival (Hegi *et al*., 2004; Chinot *et al*., 2007).

2. *PIK3CA* mutational analysis

The main goal of this project was the *PIK3CA* mutational analysis in gliomas molecular subgroups, in order to determine whether *PIK3CA* could be a good biomarker for diagnosis, prognosis and response to therapy in gliomas. We hypothesized that *PIK3CA* mutations could be important in the context of a glioma because these mutations were already described as involved in early events in the development of tumors (Lee *et al*., 2017).

Firstly, we analyzed *PIK3CA* mutations frequency in the different glioma molecular subgroups formed. For the first time it was used a great glioma cohort well stablished and characterized according with the 2016 WHO classification. We found that *PIK3CA* mutations seem to be less common in GBM subgroups than described using the histological groups. In 2004, Samuel *et al* was the first author to sequence *PIK3CA* in 15 GBM, reporting a *PIK3CA* mutational frequency of 27% (4/15) (Samuel *et al.,* 2004). This report was followed by Broderick *et al* and Hartmann *et al*. which described 5% and 14% of *PIK3CA* mutations in 105 and 70 GBM samples respectively (Broderick *et al*. 2004; Hartmann *et al*., 2005). Recently, also Lee *et al*. and co-workers referred 30% of *PIK3CA* mutations in multifocal GBM (Lee *et al*., 2017). Here we identified 3% of *PIK3CA* mutations in GBM which is a lower frequency compared with the obtained by studies mentioned previously (Table 4.9). Importantly, we used 256 GBM samples, which constitutes a larger sample size.

In this study, it was verified for the first time that *PIK3CA* mutations were more frequent in GBM *IDH* mutated (9%) compared to GBM *IDH* wildtype (3%), although it is important to highlight that the number of GBM *IDH* mutated samples was very low (1/11) (Table 4.9). In addition, the results obtained from the analysis of *PIK3CA* for the recurrent glioma group, reinforce the idea that these mutations are more common in the GBM *IDH* mutated subgroup. Since we identified *PIK3CA* mutations in 2 out of the 8 patients with GBM *IDH* mutated in the glioma recurrent group (Table 4.17). Interestingly, if we estimate the *PIK3CA* mutations frequency in all GBM *IDH* mutated samples, including the recurrent GBM *IDH* mutated samples we obtained 3 mutations in 19 cases (16%). Furthermore, our results are also in agreement with the results of Verhaark and colleagues, who define *PIK3CA* mutations as mainly present in the GBM proneural subtype, which is also characterized by the presence of *IDH* mutations (Verhaak *et al.,* 2010).

Surprisingly in *IDH* mutant + 1p/19q codeleted (oligodendrogliomas) we verified *PIK3CA* mutations in 10% of the samples which is a higher percentage compared to the described by Hartmann *et al*.(2%) and a similar percentage compared to the referred by Broderick *et al*. (14%) (Hartmann *et al*., 2005; Broderick *et al*., 2004) (Table 4.9). Interestingly, in 2010, Verhaak and colleagues not only found the *PIK3CA* mutations mainly in the GBM proneural subtype as also described this subtype of GBM as atypical due to possess a high expression of oligodendrocytic development genes (Verhaak *et al.,* 2010).

According to our results, it seems that Verhaak study included oligodendrogliomas which were wrongly classified as GBM, explaining why *PIK3CA* mutations were more prevalent in the GBM proneural subtype. In 2015, the Cancer Genome Atlas Research also showed this gene was mutated at high percentage in *IDH*-mutated gliomas with 1p/19q codelection, which correspond to the current definition of oligodendroglioma (Brat*. et al*., 2015). Our results are in accordance with the findings purposed by Verhaak and Cancer Genome Atlas. Regarding astrocytomas subgroups (*IDH* wildtype and *IDH* mutated), there was no significant differences between the frequencies of *PIK3CA* mutations (Table 4.9). In here, we verified for the first time the frequency of *PIK3CA* mutations in these 2 subgroups.

In this work 17 out of 20 mutations identified were previously described, 8 in exon 20 and 9 in exon 9 (Table 4.10). Exon 9 encodes the helical domain and exon 20 encodes the kinase domain of p110α catalytic subunit, being the main hotspots of *PIK3CA* mutations (Samuel *et al.,* 2004). The regulatory subunit of PI3K controls the enzymatic activity of the catalytic subunit, in order to regulate the phosphorylation of PIP2 in PIP3 and consequently all the PI3k/Akt signaling pathway (Vara *et al*., 2004). In addition, it was mentioned a possible interaction between the regulatory subunit of *PIK3CA* and the helical domain of the catalytic subunit, though which the regulatory subunit exert its inhibitory effect on the catalytic subunit (Miled *et al*., 2007). The mutations in the helical domain may block the connection between the helical domain of the catalytic subunit and the regulatory subunit, preventing the regulation of the PI3K action (Miled *et al*., 2007). Thus, both exons have important functions for the structural maintenance and function of p110α catalytic subunit.

The most common mutation found was H1047R in exon 20, mainly present in GBM *IDH* wildtype (Table 4.10). As these mutations in exon 20 are mainly present in GBM and astrocytoma *IDH* wildtype, the subgroups associated with poorer outcomes (Figure 4.2), we hypothesized that exon 20 mutations could be associated with an increased aggressiveness compared to exon 9 mutations. This hypothesis could be validated performing functional assays. Broderick and co-workers, also detected 3 out of the 6 exon 20 mutations in GBM (Broderick *et al*., 2004). Furthermore, other study performed functional assays to identify the functions of *PIK3CA* specific variants using breast cancer cell lines, H1047R and E545K induce the most invasive phenotypes compared to other 23 rare variants of *PIK3CA* (Dogruluk *et al*., 2015).

Despite the most frequent *PIK3CA* mutation occurred in exon 20, it was verified a great diversity of *PIK3CA* mutations in exon 9. The most frequent mutation in exon 9 was E542K (Table 4.10). There is no association between the type of mutation identified and the molecular subgroup studied. In general, the most common mutations are coincident with the ones described in other studies (Broderick *et al*., 2004; Samuel *et al*., 2004; Zhao e Vogt, 2008).

During the mutational analysis of *PIK3CA* directed to exon 9 and 20, we found 4 unreported variants, whose clinical impact was unknown. After identifying unreported variants, the most studies recur to in silico tests in order to select the rare variants which would be potentially pathogenic (Murray *et al*., 2007). The frequency and effect (benign or pathogenic) are important criteria to select a variant for a functional assay (Salgado *et al.,* 2016). The c.3040C>T and c.3112 T>C are the two most promisor unreported variants, since one is a missence mutation and the other is a nonsense mutation, both estimated as disease causing. On the other hand, c.3210A>G was estimated as a polymorphism, being

one of the variants that would not be selected for posterior functional analysis, as well as c.2988T>C, which despite being considered pathogenic variant is a synonymous one, losing importance compared to the others. However, some studies, also select synonymous variants for functional assays mainly when they are present in important domains (Dogruluk *et al*. 2015). In silico analysis allows us to have an idea of the new variants impact on the structure and function of the protein encoded. However, in future we should perform functional assays.

Then, we study if there was an association between *PIK3CA* mutations and other important and frequent genetic alterations in gliomas. As *EGFR*, *PTEN* and *PIK3CA* are involved in the PI3K signaling pathway, we analyzed the possible correlation between these genes. We performed this analysis for all the gliomas molecular subgroups, however the number of *PIK3CA* mutations found in each group was low.

In the GBM *IDH* wildtype subgroup, 71% of samples have a *PIK3CA* mutation and a heterozygous *PTEN* deletion (Table 4.12), suggesting the simultaneous occurrence of two events in the PI3K/ Akt signaling pathway. We speculate *PIK3CA* mutation could be the second event to activate this pathway, because the *PTEN* deletion is present in a heterozygous status. These two events could be determinant to constitutively activate the PI3K/Akt pathway. Until now, it was described that the main factors of dysregulation in this pathway were *PIK3CA* mutations, *PTEN* deletions, and Receptor Tyrosine Kinase (RTK) alterations by itself (Lai *et al*., 2015; Mao *et al*., 2012).

Furthermore, our results corroborate with the idea that *PIK3CA* mutations are not mutually exclusive with *PTEN* deletion in gliomas. The same was previously described for breast cancer (Péres – Tenorio *et al*., 2007). However, in diffuse large B cell lymphoma, *PTEN* deletions and *PIK3CA* mutations are mutually exclusive (Abubaker *et al*., 2007). Interestingly, only 29% (2/7) of samples had *MGMT* methylated (Table 4.12) which suggest that for this group of patients, chemotherapy would not be efficient (Baer *et al*., 1993). For these patients it would be interesting an alternative therapeutic approach.

The personalized medicine tries to apply individualized therapies based on the features of the patient's tumors, considering important all the putative targets of therapies for achieving a cure (Holland e Ene, 2015). In this perspective, it would be important to study the administration of a *PIK3CA* inhibitor to decrease the PI3K activity and consequently the Akt function in these patients where the remaining therapeutic alternatives will fail.

In GBM *IDH* mutated, the only case with *PIK3CA* mutation seems to have a molecular standard distinct from the patients with GBM *IDH* wildtype (Table 4.12). Similarly, to what happens with GBM *IDH* wildtype, it was also verified two events of dysregulation in the PI3K/Akt signaling pathway, but this time instead of *PTEN* deletion, the GBM *IDH* mutated sample had *EGFR* amplification. Once again, it would be interesting analyze the effect of these two mutations simultaneously *in vitro* and *in vivo*, to understand its role on the tumor aggressiveness. In lung adenocarcinomas, it was detected that concurrent *PIK3CA* mutations in *EGFR* mutated tumors induce poorer outcomes compared to *EGFR* mutated tumors, seeming *PIK3CA* stimulate the tumor aggressiveness (Eng *et al*., 2015). It would also be interesting to have a greater number of *PIK3CA* mutated GBM *IDH* mutated to validate this hypothesis.

Importantly in oligodendrogliomas, only one *PIK3CA* mutated tumor demonstrated *PTEN* deletion (Table 4.13), suggesting that in the most samples did not happen the 2 deregulatory events mentioned previously. However, these 2 events could be related with aggressiveness because its absence occur in the molecular group of gliomas with prolonged overall survival. It was reported that activating mutations in *PIK3CA* and partial deletion of *PTEN in* cancer both enhance the PI3K/Akt signaling pathway (Chalhoub and Baker, 2009). This hypothesis is in agreement with the findings reported for endometrial cancer, whose concomitant alterations in *PIK3CA* and *PTEN* indicating a potential additive or synergistic effect and an important role in tumorigenesis (Oda *et al*., 2005).

Furthermore, in astrocytomas *IDH* wildtype the second group associated with poor survival, all the *PIK3CA* samples had *PTEN* deletion. In addition 50% of these samples also had *EGFR* amplification (Table 4.14). These events could have a synergistic effect, justifying their presence in more aggressive tumors (Chalhoub and Baker, 2009; Oda *et al*., 2005). The results obtained for astrocytomas *IDH* wildtype are similar to the ones obtained for GBM *IDH* wildtype, as well as, the obtained for astrocytomas *IDH* mutated are similar to oligodendrogliomas (*IDH* mutant + 1p/19q codeletion). However, Fisher Exact test demonstrated that there was no statistical association between *PIK3CA* mutations, *PTEN* deletion in astrocytomas *IDH* wildtype (P=0.099). The results obtained, should be validate using a greater number of *PIK3CA* mutated gliomas.

One of the objectives of this study was to understand the impact of *PIK3CA* mutations on the gliomas stratification, as well as in the diagnosis, prognosis and response to therapy. Given the frequency of these mutations in our cohort being reduced, it was not possible to determine the value of these mutations on the prognosis and response to therapy of glioma molecular subgroups. In breast cancer *PIK3CA* prognostic value remains controversial (Harlé et al., 2013). Some articles referred these mutations as predictive of poorer outcomes (Lai *et al*., 2008 ; Mangone *et al*., 2012) and other indicate them as good prognostic factors (Kalinsky *et a*l., 2009). Additionally, most works reported *PIK3CA* mutational status is not a prognostic factor in colorectal cancer patients (Stec *et al.,* 2015; Mei *et a*l., 2016; Liao *et al*., 2012).

During the mutational analysis directed to exon 9 and 20 of *PIK3CA*, we identified Rs45455192, a SNP in intron 8. This SNP was found for the first time in 18.5% of human oral squamous carcinoma samples (Kostakis *et al*., 2010). Subsequently it was only referenced in a sample of pancreaticobiliary adenocarcinoma and there is no data about the possible impact of this variant (Weiss *et al*., 2013).

We identified this SNP in gliomas molecular subgroups ranging from 16% to 24% of samples. (Table 4.15). According to the Ensembl data, the Rs45455192 is present in 3% of the worldwide population, and specifically in the European population achieve the maximum incidence of 8%. Considering the frequency of this polymorphism in glioma molecular subgroups, we speculated that may be this polymorphism could have some importance in the prognosis of patients. Despite being an intronic SNP, it has been reported that introns also can harbor functional polymorphisms, which could influence the expression of genes (Millar *et al*., 2010; Juneau *et al*, 2006).

For this reason, we tried to understand if the polymorphism would have prognostic value in the molecular subgroups of gliomas. However, this polymorphism did not showed a significant prognostic value in gliomas molecular subgroups (Figure 4.11). Nevertheless, we observed a trend for the median

survival of oligodendrogliomas, and astrocytomas *IDH* wildtype patients with the polymorphism being slightly reduced compared to the median survival of the respective groups without the polymorphism (Table 4.16). In a type of aggressive tumor such as gliomas a SNP by itself could not be sufficient to manifest its putative pathogenic effect, being a possible explanation for the trend observed.

PIK3CA mutations were described as constitutive in the tumor, remaining present in all the sectors of the same, seeming to be a potential therapeutic target (Lee *et al*., 2017). Here, for the first time, we analyzed the *PIK3CA* mutational status in 15 gliomas recurrent cases and we detected the presence of these mutations in 2 of these cases. Importantly, *PIK3CA* mutations were present on the primary tumors and in the relapses, suggesting that *PIK3CA* mutations constitute early events in the development of gliomas, which are maintained during all the relapses and stages of the tumor development (Table 4.17).

According to our results, the importance of *PIK3CA* mutations during tumor progression seem to corroborate with the idea that these mutations could be a good therapeutic target. Despite its lower frequency, *PIK3CA* mutations could be more easily and efficiently target than other genetic alterations more common.

6. Conclusions

In our work, the reorganization of gliomas according to 2016 WHO classification had impact mainly in the astrocytomas and oligodendrogliomas subgroups, being the distribution of oligoastrocytomas the current point of distinction between studies. In addition, this reclassification allowed to conclude that glioma molecular subgroups constitute better independent prognostic factors of overall survival in comparison with histologic subgroups used until now.

The characterization of our series for other biomarkers showed *PTEN* deletions and *TERT* mutations are very frequent in GBM *IDH* wildtype. Additionally, *EGFR* amplification seems to be frequent in *IDH* wildtype subgroups, as well as, *MGMT* methylation is prevalent in *IDH* mutated subgroups.

In our Portuguese cohort, *PTEN* deletions were considered prognostic factors of poor outcomes in astrocytomas *IDH* wildtype, while seem to be factors of good prognosis in GBM *IDH* wildtype. On the other hand, *EGFR* amplification has no impact in the overall survival of patients with GBM and astrocytomas *IDH* wildtype, as well as *TERT* promoter mutations did not constitute prognostic factors in GBM *IDH* wildtype. The response to therapy analysis highlighted the effect of *EGFR* amplification in providing a better response to radiotherapy and *MGMT* methylated samples a better response to chemoradiotherapy. Overall these results emphasized the importance of having an overall picture to understand the groups of tumors that we are studying.

The main objective of this work was to study the impact of *PIK3CA* mutations on the molecular stratification, prognosis, diagnosis, and response to therapy of gliomas molecular subgroups. The results demonstrate that *PIK3CA* mutations constitute a rare mutation in gliomas, appearing in only 5% of the Portuguese cohort. Despite this, we verified that these mutations are predominant in the oligodendroglioma subgroup (10%). Likewise, *PIK3CA* mutations seem to be mainly associated with the GBM *IDH* mutated subgroup in comparison with the *IDH* wildtype subgroup (1/11). However, the prognosis value was not determined owing its reduced frequency.

We also verified that mutations in exon 20 of *PIK3CA* appear mainly in GBM *IDH* wildtype subgroup. It was also identified 4 unreported variants in *PIK3CA* gene, 3 of them estimated as pathogenic variants (c.3040C>T; c.3112 T>C; c.2988T>C) and c.3210A>G predicted as polymorphic.

In GBM *IDH* wildtype the most *PIK3CA* mutated cases had *PTEN* heterozygous deletion suggesting that *PIK3CA* mutations could be the second event to activate the PI3K/Akt signaling pathway. Our results indicate the simultaneous occurrence of at least two events in the PI3K/Akt signaling pathway, in the most aggressive gliomas, reinforcing the importance of this signaling pathway.

Furthermore, the analysis of the glioma recurrent group allowed to conclude that *PIK3CA* mutations are early events in the development of gliomas maintained during its progression and evolution. This corroborating with the idea that *PIK3CA* mutations are constitutive events in the tumor, which, despite its frequency, seem to be a good therapeutic target.

7. Future Perspectives

In the continuation of this work it would be interesting to compare the impact of *PIK3CA* mutations in exon 9 with the mutations in exon 20 to determine if the last ones are associated with poor prognosis or GBM aggressiveness. To perform this task, it would be important perform functional assays using GBM cell lines transduced with both mutations (in exon 9 and 20). In addition, it would also be important to analyze whether *EGFR* amplification, *PTEN* deletion and *PIK3CA* mutations have a synergistic effect on PI3K/Akt signaling pathway using functional assays. To test the putative role of these deregulatory events in gliomas aggressiveness and prognosis, since in our study we verified that at least 2 deregulatory events occurred in GBM and astrocytomas *IDH* wildtype (the gliomas groups with poor overall survival).

In the future, it would also be relevant analyze the impact of the unreported variants found in *PIK3CA*, mainly c.3112T>C and c.3040C>T, in order to validate their pathogenic nature *in vitro*. Furthermore, another important aspect is the overall molecular pattern involved in the recurrent gliomas, in order to identify potential players in the initiation and development of gliomas using a panel of Next generation sequencing.

The next step corresponds to the investigation of the effect caused by the in vitro pharmacological inhibition of *PIK3CA* in GBM cell lines, alone and in combination with temozolomide. Since we verified that a *PIK3CA* inhibitor could be a good alternative of treatment in GBM bearing *PIK3CA* mutations, mainly for GBM *IDH* wildtype patients, whose response to the chemotherapy is limited. Our hypothesis is that a *PIK3CA* inhibitor, could be more efficient that inhibitors targeting the *PI3K* enzymes, due to its specificity for the Class IA of PI3K, allowing the normal function of the other classes of PI3K enzymes which are important for the cell survival.

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