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Glutamine availability as a target for the control of Glutamine Synthetase-negative human cancers: the case of oligodendroglioma

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Abstract

The amino acid Glutamine (Gln) is a nutrient of fundamental importance for cell metabolism. It is involved in many metabolic pathways, such as the synthesis of non essential amino acids, nucleotides and hexosamines, the regulation of cell volume, the response to oxidative stress (through the maintenance of intracellular glutathione). Moreover, it refuels the Krebs cycle with carbon moieties (anaplerosis) and activates mTOR, possibly through the energization of leucine influx.

It has been known since many years that several types of normal and cancer cells depend upon Gln availability to maintain adequate proliferative activity. Moreover, certain cancer cells require large amounts of Gln and undergo severe metabolic stress and apoptosis upon Gln restriction, a condition called "Gln addiction". However, the molecular basis of Gln addiction and, more in general, the molecular and metabolic features that underlie the sensitivity to Gln depletion have not yet been defined.

The impossibility to identify Gln-dependent tumors has hampered, until now, the possibility to exploit sensitivity to Gln depletion for therapeutic purposes. However, a drug that produces Gln depletion in plasma has an established clinical use for many years. This drug, L-asparaginase (ASNase), is one of the first-line agents for the therapy of Acute Lymphoblastic Leukemia, since it produces a marked depletion of plasma asparagine, which ALL blasts cannot synthetize. However, ASNase also produces a partial depletion of plasma Gln.

This thesis aims at identifying determinants of Gln sensitivity in human cancer cells, using the sensitivity to ASNase as a device of potential translational interest. The cancer model adopted in this study is oligodendroglioma. Oligodendrogliomas are rare brain cancers, which are often characterized by lack of expression of Glutamine Synthetase (GS negativity).

The hypothesis of the study is, therefore, that low GS expression may render oligodendroglioma cells particularly dependent upon external Gln.

For verifying this hypothesis, the effects of the ASNase from *Erwinia chrysanthemi*, the most glutaminolytic variant in clinical use, were compared in two different human oligodendroglioma lines, HOG and Hs683, and in two glioblastoma cell lines, U87 and U373. Compared to glioblastoma cells, oligodendroglioma cells expressed far lower amount of the mRNA of *GLUL*, the gene which encodes for GS. Consistently, GS protein expression was readily detectable in U87 and in U373 cells, but not in HOG and Hs683 cells, remaining undetectable even after ASNase treatment, a condition known to increase GS abundance in

other cell models. Under the same condition, GS expression was instead clearly increased in glioblastoma lines.

Upon ASNase treatment, a marked depletion of cell Gln was detected in all the cell models. However, while HOG and Hs683 underwent an almost complete suppression of cell viability, glioblastoma lines were less sensitive. Moreover, the IC_{50} values for ASNase were lower in oligodendroglioma than in glioblastoma cells. The addition of the GS inhibitor methionine-L-sulfoximine (MSO) did not synergize ASNase effects in oligodendroglioma cells, while enhanced the effect of ASNase in U87 and U373 glioblastoma cells, confirming the absence of a functional GS in the former cell models. Moreover, HOG and Hs683 cells were more dependent than glioblastoma cells on the availability of extracellular Gln.

These results consistently point to a relationship between lack of GS and dependence on extracellular Gln. Nevertheless, transfection experiments with *GLUL* in HOG and Hs683 cells, while produced a marked GS expression in transfected oligodendroglioma cells, failed to yield a significant protection from Gln deprivation. This result may suggest that the severe consequences of Gln depletion in oligodendroglioma cells are not the result of the sole poor GS expression. Definite conclusions would require the measurement of Gln content along with the determination of GS activity in *GLUL*-transfected cells.

However, further support to the dependence of oligodendroglioma cells from extracellular Gln has come from studies on Gln transporters. Gln is transported into mammalian cells by several transport systems. Most of Gln influx is due to sodium-dependent transporters belonging to the SLC1 (ASCT transporters) and SLC38 (SNAT transporters) gene families. In particular, ASCT2, the product of the *SLC1A5* gene, appears overexpressed in several tumors and in many models of Gln-addicted cancer cells *in vitro*. A preliminary characterization of Gln-transporter expression in oligodendroglioma cells indicated that these cells express members of both SLC1 and SLC38 families and, in particular, Hs683 cells showed high expression of the SNAT1 transporter. In the attempt to hinder the activity of these transporters and, hence, Gln transport, oligodendroglioma and glioblastoma cells were incubated in the presence of high concentrations of specific inhibitors. The results indicated that transport inhibition had larger inhibitory effects on the viability of oligodendroglioma cells, compared with glioblastoma cells. In particular, both HOG and Hs683 cells were very sensitive to the specific inhibitor of SNAT transporters 2-methylaminoisobutyric acid (MeAIB). These results suggest that oligodendroglioma cells depend upon this transporter for Gln fuelling.

In the attempt to verify if the cytotoxic effects of Gln depletion were due to the inhibition of mTOR, the activity of the kinase was also studied in oligodendroglioma and glioblastoma

cells. While ASNase caused a severe inhibition of the kinase activity in HOG cells (as well as in glioblastoma cells), mTOR activity was spared in Hs683 cells. However, both HOG and Hs683 cells exhibited a very low mTOR activity when incubated in amino acid-free saline solution, indicating that sensitivity to essential amino acids, such as leucine, is maintained in both models. Gln restitution to cells pre-incubated in amino acid free saline solution restored mTOR activity in HOG but not in Hs683 cells. Hs683 cells also exhibited enhanced sensitivity to the mTORC1 inhibitor rapamycin, thus showing a mTOR-dependent phenotype. This behavior is probably to attribute to a *MTOR* mutation, previously described in these cells, that constitutively increases the phosphorylation of the mTOR substrate S6K1.

While these results exclude that mTOR inhibition plays a role in the cytotoxic effects of ASNase in oligodendroglioma cells, they indicate that Hs683 cells yield a model of Gln-independent mTOR and demonstrate that leucine and Gln have independent roles in the stimulation of mTOR.

In summary, the results recounted in this thesis indicate that GS-negative oligodendroglioma cells are markedly dependent on extracellular Gln and that mTOR inhibition is not involved in the effect. Lack of significant GS expression may, therefore, constitute a marker of sensitivity to therapeutic approaches based on the reduced availability of the amino acid. This hypothesis awaits conclusive confirmation *in vivo* with models of oligodendroglioma and, possibly, other GS-negative tumors.

1. Introduction

1.1. Cancer and metabolism

According to the revised list of cancer hallmarks proposed by Hanahan and Weinberg in 2011, cancer is considered also a metabolic disease (Hanahan et al. 2011). Indeed, cancer cells require alterations in their metabolism to incorporate continuously nutrients in their mass and to sustain their uncontrolled proliferation. Their metabolism is expected, therefore, to differ from that of the normal tissue from which cancer arises (Hu et al. 2013).

The first concept of metabolic alteration in cancer was proposed by Otto Warburg in 1924, and it is the so called "aerobic glycolysis" or Warburg Effect (Vander Heiden et al. 2009).

He observed that tumor cells, even in the presence of oxygen, ferment glucose to produce lactate, instead of oxidizing it and generating ATP through oxidative phosphorylation (Figure 1). At first he explained this behavior suggesting that it was a consequence of mitochondrial damage in cancer cells (Warburg 1956), hence forced to rely on glycolysis even in the presence of a normal tension of oxygen. Yet, this hypothesis wasn't validated because it was demonstrated that mitochondria are not defective in most cancer cells (Vander Heiden et al. 2009). Moreover the progress in viral carcinogenesis and molecular oncology overcame the concept of cancer as a metabolic disease, leading to its definition as a genetic pathology, the dominant thought until recently (Chiu et al. 2012).

Notwithstanding this, Warburg effect is now considered an hallmark of cancer and the dependence of most tumor cells on glucose has provided the basis for an important test in oncology, the ^{18}F-deoxyglucose Positron Emission Tomography (FDG-PET), that allows the measure of glucose uptake and the visualization of malignant lesions in patients tissues using a radioactive glucose analogue.

However, why tumors prefer upregulated glycolysis is still matter of arguing, and a lot of hypotheses have been proposed over the years.

The most accepted theory is that cancer cells do aerobic glycolysis to sustain the continuous fuelling of carbon intermediates (3-4-5 C) in TCA cycle. This concept, called "anaplerosis", would explain why tumor cells, while endowed with adequate energy supply, avoid the depletion of the intermediates necessary for the synthesis of macromolecules like nucleotides, amino acids and lipids ensuring the continuous availability of an intracellular pool of nutrients to sustain their high proliferation rate (Vander Heiden et al. 2009).

Another aspect of interest is that the alterations in some glycolytic enzymes present in glucose-dependent cancer cells are also involved in apoptosis regulation, like overexpression

of hexokinase II (HXK II), which negatively regulates apoptosis through its binding to the mitochondria. Studies have shown, indeed, that this binding may prevent mitochondrial dysfunction during cell stress and injury through its interaction with the voltage-dependent anion channel (VDAC); the binding of HXK II to the mitochondria is mediated by Akt activation that negatively regulates the activity of glycogen synthase kinase 3β (GSK3β), which phosphorylates VDAC and inactivates it (Pastorino et al. 2005). VDAC regulates the apoptotic process as Bax, Bak, and Bim (belonging to the Bcl-2 family proteins) can interact with VDAC and induce the formation of a novel large pore releasing, thus, cytochrome *c*., which in turn activates caspases, hence, apoptosis (Shimizu et al. 1999).

Moreover, the high cytosolic amount of ATP produced through glycolysis inhibits mitochondrial ATP synthase, induces a chemiosmotic backpressure and hyperpolarizes the mitochondrial membrane, fixing mitochondria in an anti-apoptotic state (Naviaux 2012).

Cancer cells dependent on glucose express, in addition, the low-activity pyruvate kinase type M2 (PKM2), an isoform of the enzyme pyruvate kinase (PK), which promotes proliferation and helps cancer cells to accumulate metabolic intermediates upstream of phosphoenolpyruvate (PEP), thus providing an ample supply of metabolic intermediates for the synthesis of precursor substances (Songfang et al. 2013).

Another feature linked to aerobic glycolysis is the lactate produced by glycolytic cancer cells as a waste product. Indeed, tumor cells tend to export lactate in the extracellular milieu implicating, thus, the acidification of the tumor environment (Wike-Hooley et al. 1984) and triggering the generation of a local inflammatory response. This activates innate immune cells, like macrophages, and their secretion of cytokines and growth factors, promoting tumor growth and metastasis (Yabu et al. 2011; Shime et al. 2008). Furthermore, lactate secretion facilitates tumor progression provoking a damage of adaptive immune response (Fischer et al. 2007).

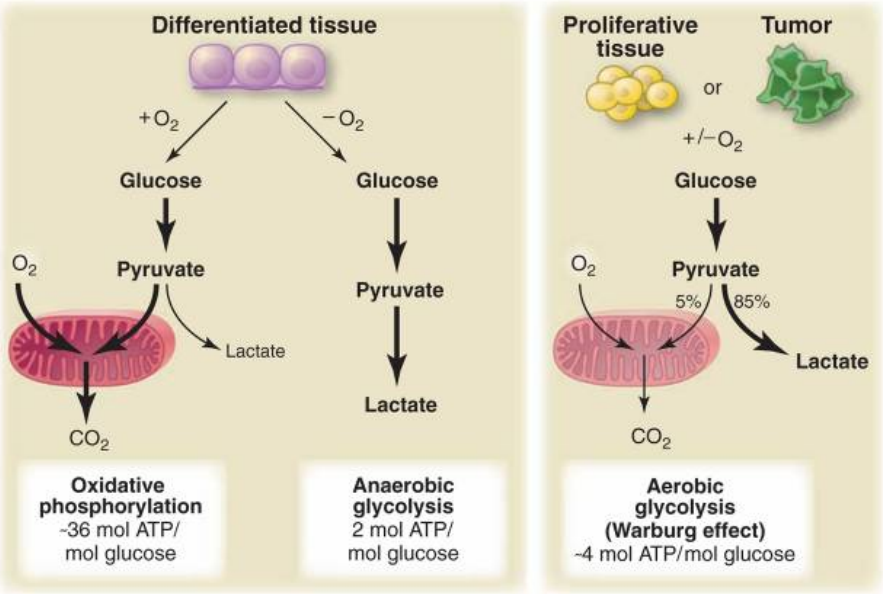


Figure 1. Comparison between anaerobic and aerobic glycolysis or Warburg effect (from Vander Heiden et al. 2009)

1.1.2. Mutations in cancer cells lead to altered metabolism

Altered metabolism in tumors is a consequence of several genetic mutations that affect cancer cells (Cairns et al. 2011, Shulze et al. 2012). The acquired phenotype is the result of a combination of alterations of both oncogenes and tumor suppressor genes, which promote increased consumption of nutrients to support cell growth and proliferation (Chiu et al. 2012). For instance, it is now known that *p53* controls a lot of aspects of cell metabolism regulating the availability of nutrients inside the cell. Wild type p53 checks, in fact, the glycolytic fueling lowering the expression of glucose transporters GLUT1 and GLUT4 (Schwartzenberg-Bar-Yoseph et al. 2004) and inactivates the pentose phosphate pathway (PPP) binding to glucose-6-phosphate dehydrogenase (G6PD); this is the first and rate-limiting enzyme of the PPP, and *p53* prevents the formation of the active dimer with $NADP^+$, thus lowering NADPH production and, hence, macromolecular biosynthesis (Jiang et al. 2011).

Moreover *p53* directly controls cell metabolism balancing inhibition of glycolysis and the promotion of oxidative phosphorylation. Indeed, it upregulates the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), an enzyme that decreases the levels of the glycolytic activator fructose-2-6-bisphosphate, resulting in the inhibition of glycolysis and an overall decrease in intracellular reactive oxygen species (ROS) levels protecting, thus, cells from ROS associated apoptosis (Bensaad et al. 2006); on the other hand, *p53* activates the expression of cytochrome c oxidase 2 (SCO2) which is required for the assembly of the

cytochrome c oxidase complex of the electron transport chain (Matoba et al. 2006). In addiction, *p53* may interact also with the transcription factor OCT1 that acts in an opposite manner to TIGAR activating the transcriptional genes that drive glycolysis and suppress oxidative phosphorylation. The transcriptional program initiated by OCT1 supports resistance to oxidative stress, which may be associated to the loss of *p53* during transformation (Shakya et al.2009).

PI3K signaling is another pathway that impacts on cell metabolism besides providing growth and survival signals to tumor cells (14). Its oncogenic properties may be due to mutations both in tumor suppressor genes, like *PTEN*, a phosphatase that degrades the phosphoinositide products of PI3K, or PI3K itself (Wong et al. 2010).

PI3K alterations affect mainly glycolysis through the activation of its downstream target AKT1 (also known as PKB) that enhances the glycolytic rate increasing the expression and membrane translocation of glucose transporters, like GLUT1, and phosphorylating key glycolytic enzymes, such as hexokinase (see before) and phosphofructokinase 2 (also known as PFKFB3), whose principal reaction product, fructose-2,6-bisphosphate (Fru-1,6-P_2) is the most potent known allosteric activator of PFK1 (Robey et al. 2009). Moreover, the increased AKT1 signalling, associated with transformation, inhibits Forkhead Box subfamily O (FOXO) transcription factors that, when active, can regulate circulating glucose homeostasis by promoting gluconeogenesis and repressing the expression of glycolytic enzymes in hepatocytes; thus their inactivation increases glycolytic capacity (Khatri et al. 2010).

In addition AKT1, and therefore PI3K pathway, stimulates signaling through the kinase mTOR, which is strongly dependent on nutrient availability (see below), phosphorylating and inhibiting its negative regulator tuberous sclerosis complex 2 (TSC2) (Robey et al. 2009) (Figure 2).

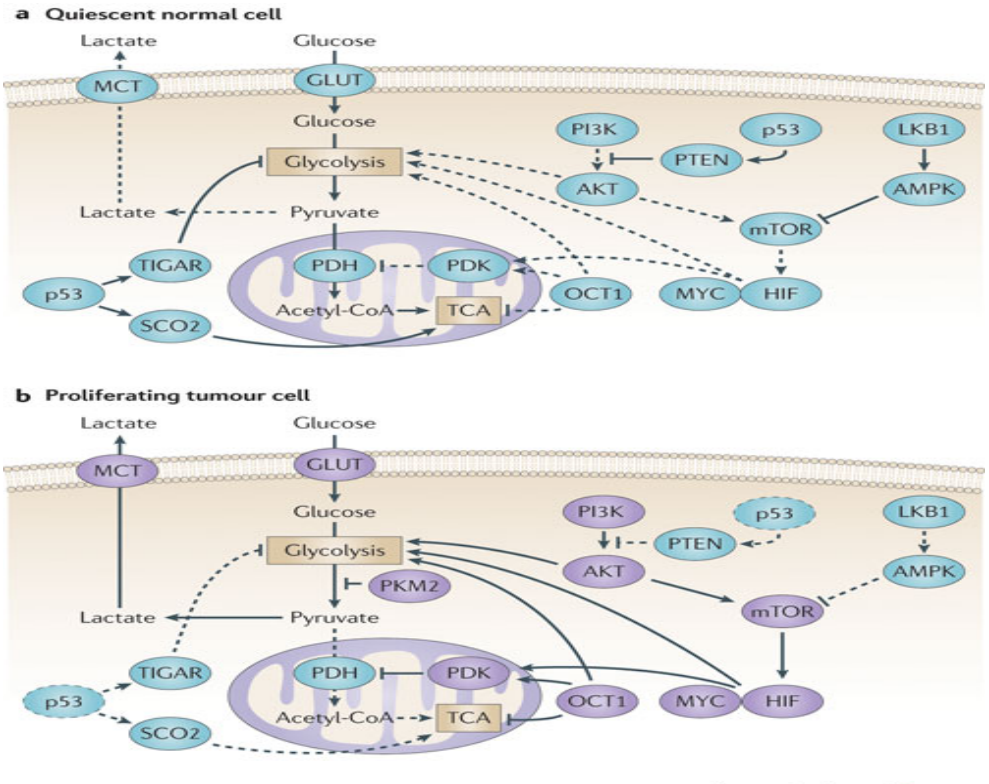


Figure 2. Molecular mechanism driving the Warburg effect (from Cairns et al. 2011)

Besides mutations in classical oncosuppressors and protooncogenes, such as *p53* and PI3K, but also *MYC*, also mutated enzymes belonging to metabolic pathways can promote cancerogenesis in selected cancer cell models.

For instance, germ-line inactivating mutations in genes encoding for Krebs Cycle enzymes, such as succinate dehydrogenase (SDH) and fumarate hydratase (FH), are mutated in rare human tumors, behaving as oncosuppressor genes. SDH drives the conversion from succinate to fumarate, and mutations of this enzyme have been identified in familial forms of the neuroendocrine tumors pheochromocytoma (PCC) and paraganglioma (PGL) (Mullen et al. 2012).

Fumarate hydratase, instead, catalyzes the hydration of fumarate to malate, and its mutations severely reduce enzyme activity, causing the accumulation of high levels of fumarate in tumors. These alterations are responsible for the familial syndrome of benign leiomyomas of the skin and uterus, and highly aggressive renal tumors termed Hereditary Leiomyomatosis Renal Cell Cancer (HLRCC) (Toro et al. 2003). Inactivation of FH in these cancers drives to aerobic glycolysis resulting in decreased levels of AMPK, reduced expression of p53, and activation of anabolic factors that stimulate tumor growth (Tong et al. 2011).

More in general, high levels of succinate and fumarate, generated by SDH and FH mutations, inhibit HIF-α prolyl hydroxylases (PHD) leading to the stabilization of hypoxia inducible

factor-1α (HIF-1α) under normal tension of oxygen (pseudohypoxia) (Selak et al. 2005; Isaacs et al. 2005). Physiologically, HIF promotes the adaptation of cells to low oxygen level by inducing glycolysis thanks to an increase of expression of glucose transporters (GLUT1 and GLUT3) (Semenza et al. 2010); its stabilization, moreover, upregulates other downstream targets, like VEGF, facilitating, thus, angiogenesis and metastasis and, in this way, tumor growth and diffusion (Pollard et al. 2005).

Another enzyme whose mutations can lead to HIF stabilization is isocitrate dehydrogenase (IDH). This enzyme is present in three isoforms; two catalyze the reversible conversion of isocitrate to α-ketoglutarate (α-KG) (IDH1 and IDH2, respectively in cytoplasm and mitochondria), whereas the other (IDH3) that promotes the irreversible decarboxylation of isocitrate to α-ketoglutarate in the Krebs cycle.

Somatic mutations in IDH1 and IDH2 occur in a lot of human cancers including low-grade gliomas, oligodendrogliomas and secondary glioblastomas (Yan et al. 2009), but they have been found also in chondrosarcoma, cholangiosarcoma and several subtypes acute myeloid leukemia (Ward et al. 2010).

IDH1 and IDH2 mutants, instead of producing α-KG, acquire the capability of using it producing to D-2-hydroxyglutarate (D-2HG), an "oncometabolite" that, normally, is present at very low levels in the cells (Yang et al. 2013). The production of D-2HG implicates, therefore, a decrease in the levels of α-KG that is, moreover, an obliged PHD substrate (it is used together with O_2 to hydroxylate HIF-1α). α-KG depletion causes, therefore, the inhibition of the enzyme and, hence, HIF stabilization (Bayley et al. 2010).

Moreover IDH1 and IDH2 mutants implicate changes in a lot of metabolic processes involved in glutamine, citrate and fatty acids metabolism (Borodovsky et al. 2012). In fact, it has been found that cells carrying these mutations exhibit decreased levels of citrate, coupled with increases in acetyl-CoA, triglyceride and phospholipid precursors; this phenomenon may indicate that cells shuttle citrate out of the TCA cycle to produce lipids necessary for cell growth. Also glutamine metabolism is upregulated in IDH1 and IDH2 mutated tumors because α-KG, derived from its degradation, is needed as the substrate for the production of D-2HG and as a source of oxaloacetate (OAA), which replenishes the mitochondrial pool of citrate exported for fatty acid synthesis (Wolf et al. 2010). As a consequence, the inhibition of glutaminase (the first enzyme of glutamine metabolism) with siRNA or the inhibitor bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide (BPTES) slowed growth of glioblastoma cells expressing mutant IDH1 (Seltzer et al. 2010).

Another enzyme that is focussing a great interest is phosphoglycerate dehydrogenase (PHGDH), which is involved in the synthesis of serine and glycine from glucose (Figure 3). It oxidizes the glycolytic intermediate 3-phosphoglycerate (3PG) to 3-phospho-hydroxypyruvate (3POHPyr), the first step in the synthesis of the two amino acids, and the amplification of this gene on chromosome 1p12 occurs in a significant percentage of melanomas and breast cancer (Mullen et al. 2012). Activating mutations in this enzyme may implicate advantages for tumor cells as serine is required as a precursor for lipids, proteins, nucleotides and amino acids biosynthesis, and, moreover, glycine can protect the cells from the oxidative stress, as it is a precursor for the major antioxidant glutathione (Shanware et al. 2011). High activity of PHGDH also enhances anaplerosis; in fact from the transfer of an amino group from glutamine-derived glutamate by phosphoserine aminotransferase-1 (PSAT1) to 3POHPyr, cells manage to obtain up to 50% of the α-KG supply, contributing thus to the replenishment of TCA cycle intermediates and also to ATP generation (Possemato et al. 2011).

Enzyme mutations linked to the production of oncometabolites are being discovered very fast. For instance, one of the last discoveries is glycine decarboxylase (GLDC), which was originally found highly expressed in Tumor Initiating Cells (TICs) of Non Small Cell Lung Cancer (NSCLC) and whose overexpression promotes cellular transformation and tumorigenesis inducing dramatic changes in glycolysis and glycine/serine metabolism and leading to changes in pyrimidine metabolism to regulate cancer cell proliferation (Zhang *b)* et al. 2012). In addiction, the attention has been recently focused also on prolyl oxidase (POX), which catalyzes the first step in the catabolism of proline. This enzyme, induced by p53, can regulate cell survival as well as programmed cell death and, at first, it was thought to behave as a tumor suppressor causing G_2 cell cycle arrest (Liu *b)* et al. 2009) but under hypoxic conditions it can work as a pro-survival factor (Liu *a)* et al. 2012).

The discovery of these characteristics is involving the scientific research to use them as potential targets for the cancer diagnosis or treatment.

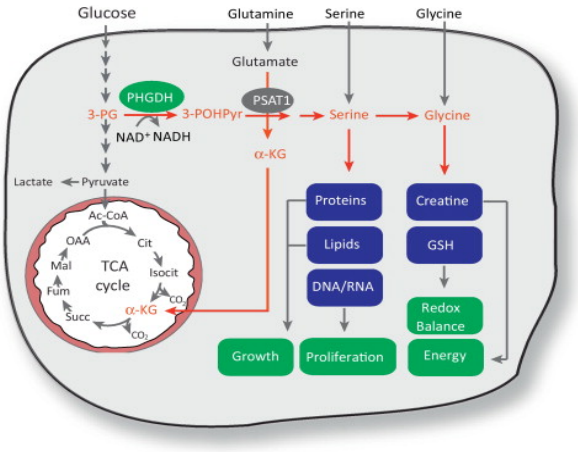


Figure 3. Role of Phosphoglycerate dehydrogenase (PHGDH) in cell metabolism (from Mullen et al. 2012)

1.2. Glutamine and its role in cancer metabolism

Although tumor cells mostly rely on aerobic glycolysis, glucose is not the only nutrient that they utilize to satisfy their continuous need of energy and intermediates for macromolecular synthesis.

In fact, many cancer cells require also the uptake of Glutamine (Gln or Q), whose accelerated metabolism is a major component of the metabolic phenotype of proliferating tumor cells (DeBerardinis, *b)* et al. 2010).

Gln is the most abundant amino acid in the bloodstream (its concentration is between 600 and 900 μM), as well the major source of nitrogen exchanged among body tissues. Mammals can synthesize it through the reaction between Glutamate (Glu) and ammonium, NH_4^+, catalyzed by the enzyme Glutamine Synthetase (GS) but during periods of rapid growth or illness the cell demand of Gln outstrips the capability of synthesis of the cells, becoming, thus, essential (hence its designation as a "conditionally" essential amino acid) (Lacey et al. 1990).

Gln is engaged in a lot a of functions inside the cells partecipating as a nitrogen donor in the synthesis of nucleotides, non essential amino acids, glucosamine, but also providing a major source of carbon for anaplerosis.

Although nitrogen donation to nucleotides accounts only for a small fraction of total Gln consumption, it is of pivotal importance in growing cells. In *de novo* purine and pyrimidine synthesis, the γ nitrogens of two Gln molecules are added to the growing purine ring and a third is used in the conversion of xanthine monophosphate to guanosine monophosphate; for the pyrimidine ring, instead, Gln contributes with one nitrogen together with one derived from aspartate; an additional nitrogen is added to uridine triphosphate to form cytidine triphosphate.

In cancer cells the consumption of the largest fraction of Gln is due for the production of Glu, through the action of Glutaminase (GLS), a mitochondrial enzyme highly expressed in tumors, which promotes Gln conversion to Glu and ammonium NH_4^+. Gln-derived Glu is the source of amino groups involved in the synthesis of non essential amino acids like alanine and aspartate through transamination reactions, but also serine, glycine and proline (Hensley et al. 2013). Importantly, it is also a precursor of glutathione (GSH), the major cellular antioxidant; GSH is a tripeptide formed by glutamate, cysteine and glycine and its formation is highly dependent on glutamine, as glutamate not only takes part in GSH formation, but it is also necessary for cells to acquire cysteine through the X^-_c exchanger, an antiporter that exports glutamate and imports cystine (the dimer of cysteine (Cys)). Cystine can then be converted to cysteine inside the cell and used in GSH synthesis.

High levels of GSH protect tumor cells from the oxidative stress associated with enhanced metabolism, DNA damaging agents, exposure to several drugs or ionizing radiations (Estrela et al. 2006).

In glutamine-consuming cells, Glu is mostly converted to α-ketoglutarate, obtained by oxidative deamination, through the action of glutamate dehydrogenase (GDH) in the mitochondrion, or by transamination to produce nonessential amino acids either in the cytosol or in the mitochondrion. Under conditions of glucose abundance, the transamination pathway predominates, but when glucose is scarce, GDH becomes a major pathway to supply glutamine carbon to the TCA cycle and is required for cell survival. α-KG is an intermediate of Krebs cycle; its metabolism generates reducing equivalents for the electron transport chain (ETC) and oxidative phosphorylation; moreover it is an important anaplerotic nutrient sustaining a net production of oxaloacetate (OAA) to counteract export of intermediates from the cycle (cataplerosis) (Figure 4). For instance, OAA together with the acetylCoA (AcCoA) derived by glucose metabolism, is the precursor of citrate, needed not only for the Krebs cycle but also for the lipid synthesis when it is exported to the cytosol.

The importance of Gln metabolism is evident in a lot of cancer cell types: for example estrogen stimulation induces glutaminolysis in breast cancer cells and the use of Nuclear Magnetic Resonance (NMR) spectroscopy with ^{13}C-labeled substrates has revealed the use of Gln as the major anaplerotic precursor in proliferating glioma cells in both rats and humans (DeBerardinis, *a)* et al. 2007). However, not all cancer cells need an exogenous supply of glutamine, but some tumors, like some forms of breast cancer (Kung et al. 2011), hepatocellular carcinoma (Tardito, *b)* et al. 2011) or glioma (Lieberman et al.) show an high dependence on external Gln and, thus, they are called “Glutamine addicted”.

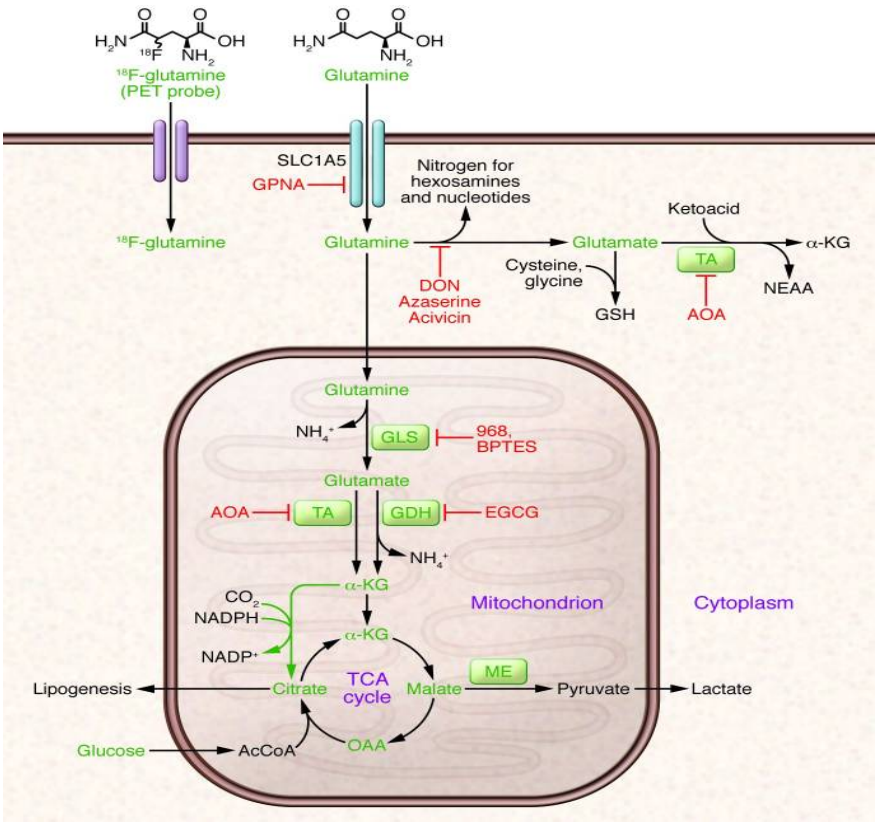


Fig. 4 Glutamine implications in cell metabolism (from Hensley et al. 2013)

In addition to these basic metabolic reactions, Gln modulates other pathways in the cell. For instance, it contributes to energy supply also promoting glycolysis through the suppression of the negative regulator of glucose uptake thioredoxin-interacting protein (Kaadige et al. 2009) and sustains proliferating signaling favoring the activation of the serine/threonine kinase mTOR (see below).

Gln addition is also associated with enhanced activation of Myc; in fact it promotes the expression of *SLC1A5* (it codes by for the Na^+- dependent amino acid transporter ASCT2) and GLS genes driving, thus, Gln uptake and catabolism counteracting Gln deprivation (Gao et al. 2009; Wise et al. 2008).

Given the important role of Gln in cell metabolism, it is not surprising that several cells types undergo severe consequences when starved of the amino acid. Most cells exhibit a cell cycle arrest, while cell death processes may be activated.

1.3. L-Asparaginase: history and function

L-Asparaginase (ASNase) is a drug used against acute lymphoblastic leukemia (ALL) since the late 1960s. The original observation leading to ASNase use came in the 1950s, when Kidd administered serum of guinea pig into mice and rats and observed a regression of transplanted lymphomas. Subsequently, in 1960 Broome discovered that the anti-lymphoma activity of the guinea pig serum was due to ASNase (Agrawal *b)*. et al. 2013).

Enzyme preparations from several organisms were evaluated, and in 1964 Mashburn and Wriston demonstrated that the EC-2 enzyme obtained from *Escherichia coli* had optimal characteristics.

The first clinical studies implicating the use of ASNase were performed in 1967 by Old and Hill that established benefit from the enzyme in some patients with childhood acute lymphoblastic leukemia. The benefit that was brought in patients by the enzyme was due to the ability to deplete asparagine in tumor cells, auxotrophs for this amino acid, because of their low asparagine synthetase (AS) expression.

High intracellular concentrations of asparagine has been observed in plants, so that this enzyme has been found in a lot of tissues of herbivores, but not carnivores (Clementi 1922); also bacteria possess two types of ASNases which are genetically and biochemically distinct: the cytoplasmic (type I) and the periplasmic (type II). Type I are required for the growth of bacteria, while the second ones are expressed under anaerobic conditions and are widely used as anti-leukemic drug for the ALL treatment; moreover type II enzyme shows higher affinity for the substrate than type I with a *K*m in the range of 1μM versus 3.5 mM of type II (Emadi et al. 2014).

ASNases catalyze the hydrolysis of asparagine to aspartic acid and ammonium and produce a sustained reduction in serum asparagine, which is required for rapid proliferation of leukemic cells.

Over the years it has been found that L-ASNases have also a glutaminolytic activity (the products of this reaction are glutamate and ammonium) that causes a reduction of Gln levels in the serum. This is a very important aspect of the mechanism of action of the enzyme because the only asparaginolytic activity seems not to be sufficient for effective cytotoxic activity against leukemia cell lines. Infact the decrease of Gln levels in the serum can prevent cells to use it to resynthesize asparagine via a transamidation reaction where aspartate + glutamine + ATP give asparagine + glutamate + AMP + PPi; moreover, its depletion results very limiting for cell survival (see above).

1.3.1. Pharmacology and clinical use

E. coli ASNase often causes allergic reactions and has a short serum half-life. These complications leads to the characterization of other two forms of the enzyme approved for clinical use: one derived from *Erwinia chrysantemi* (Erwinase) and the other consisting of a pegylated *E. coli* form (PEG Asparaginase: polyethylene glycol-L-Asparaginase), both of which improve pharmacokinetics (Agrawal *b)* et al. 2013). In particular, pegylation delays the elimination of the enzyme by the reticuloendothelial system, which prolongs the half-life of the drug (Emadi et al. 2014).

ASNases are administrated either intramuscularly or intravenously and are thought to remain in the vascular space even if they have been detected in the pleural fluids and ascites, but not in cerebrospinal fluid.

The *in vivo* half-life of the *E. coli*-derived enzyme is longer when it is administered intramuscularly (46 h) rather than intravenously (7-28 h) and it is longer also than that of *E. chrysantemi* (16 h).

Although they have high therapeutic efficacy, there are still some problems that exist in the therapeutic field, such as immunological response and side effects (i.e. fever, skin rashes, allergic reactions, even anaphylactic shocks but also thrombosis, pancreatitis and neurologic dysfunctions). Thus, trying to avoid toxicities phenomena and immune responses toward ASNase, new ways were searched to avoid the activation of the immune system and to improve the therapeutic index.

Two options are actually taken into consideration, nanoparticle or RBC encapsulation ASNAse. The first nanosized ASNase drug delivery system was developed in 2008 and consisted in a carboxymethyl konjac glucomannan-chitosan (CSGM-CS) system; it was demonstrated that the CSGM-CS entrapped ASNase had a longer half-life retaining 77% of its activity after 90 minutes in opposition to only 60% for unbound enzyme and, moreover, under acidic pH of 6, the enzyme was found to have 25.8% activity relative to only 17.9% for unbound enzyme (Wang *b)* et al. 2008).

The other nanosized system was developed in 2009 and utilized a hydrogel-magnetic nanoparticle entrapped ASNase, which ensured an activity retention of 61.05% (Teodor et al. 2009).

As far as the utilization of RBC as delivery system is concerned, the first approach of this method was attempted in 1979 when the first studies to load therapeutic agents into red blood cells (“carrier erythrocytes”) were made. RBC provides an ideal drug delivery mechanism not only due to their biodegradability and lack of toxic product formation, but also lack of

immunogenicity, a side effect shared, although with different probability, by *E. coli, E. chrysantemi,* and PEG ASNase. Moreover, this delivery system improved pharmacokinetics with protection from circulating proteolytic enzymes and avoidance of early liver or renal clearance. Studies have demonstrated that doses of 30-200 U/Kg of human RBCs, loaded with ASNase administered systemically to cancer patients, had an half-life of 28 days, and at 200 U/kg, plasma asparagine was eliminated for 50 days with no observation of allergic reactions, even if anti-ASNase antibodies were developed and side effects like thrombocytopenia and anemia were similar to naked ASNase (Agrawal *b)*. et al. 2013).

1.3.2. Mechanisms of resistance to ASNase

ASNase instability or proteolysis, production of anti-ASNase antibodies and stromal production of asparagine are some of the mechanisms of clinical resistance described during the use of ASNase (Agrawal *b)* et al. 2013).

Several proteases, like cathepsin B and asparaginyl endopeptidase (AEP), degrade ASNase *in vivo.* The latter is present in large quantities in high-risk acute lymphoblastic leukemia blasts and may contribute to reduced ASNase half-life and efficacy, also contributing to allergic reactions too. Studies have demonstrated that the N24 is one of the cleavage sites of ASNase (together with D124 and N143) and modifications at this site rendered the enzyme resistant to AEP action providing, thus, a more effective drug (Patel et al. 2009).

Production of anti-ASNase antibodies causes premature clearance as well higher asparagine levels, poorer remission rates and allergic reactions. The immune response has been seen both with the use of *E. coli*, PEG-AA and *E. chrysantemi* ASNase (Armstrong et al. 2007; Dinndorf et al. 2007; Wang *a)* et al. 2003). Cross-reactivity has been observed between pegylated and native *E.coli* ASNase.

Moreover, ASNase efficacy is hampered by the stromal and tumor cell asparagine synthesis; bone marrow derived mesenchymal cell, with higher levels of asparagine synthetase than ALL cells (up to twenty times), form the microenvironment for the leukemia cells and can protect ALL cells from the action of the drug (Iwamoto et al. 2007).

1.3.3. Studies of ASNase in non-ALL neoplastic cell lines

Although majority of tumors sensitive to ASNase was of lymphoid origin and most of solid tumors were generally found to be resistant to the drug, the sensitivity to ASNase treatment was demonstrated, in vitro, also for other forms of cancer like soft tissue sarcoma (Tardito a), et al. 2007), ovarian cancer (Lorenzi et al. 2008), β-catenin mutated hepatocellular carcinoma (Tardito b), et al. 2011), hepatocellular carcinoma with low expression of asparagine synthetase (Zhang a) et al. 2013) and gastric adenocarcinoma (Scotti et al. 2010).

Moreover the enzyme has been tested, for clinical use, also in other hematologic malignancies including acute myeloid leukemia (AML) and myelosarcoma (Takahashi et al. 2012), non-Hodgkin's lymphoma (NHL) (Wróbel et al. 2000), multiple myeloma (Agrawal a) et al. 2003) and, more recently, also for extranodal natural killer (NK)/T-cell lymphoma (Ishida et al. 2010).

1.4. mTOR: a key regulator in cell growth and metabolism

Cell growth depends on the availability of nutrients and energy by which cells synthetize new cellular components and manage to proliferate. On the other hand they also rely on a complex set of programs to cope with situations of nutrient starvation and low energy availability. In this context the highly conserved serine/threonine kinase mammalian target of rapamycin (mTOR) plays a pivotal role (Zoncu et al. 2011).

mTOR belongs to the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family and assembles into two complexes with distinct inputs and downstream effects: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Yang et al. 2013). The former contains the rapamycin-sensitive regulatory associated protein of mTOR (Raptor), whereas the latter contains the rapamycin-insensitive companion of mTOR (Rictor) (Murugan A. K. et al 2013); these are the distinctive elements of the two complexes and function as scaffolds for their assembling and for binding substrates as well as regulators (Zoncu et al. 2011).

Further unique components exist in both complexes: indeed, mTORC1 comprises a negative regulator, PRAS40, whereas mTORC2 contains Protor and mSin1, which likely help complex assembly and localization, respectively (Frias et al. 2006). Moreover mTORC1 and mTORC2 share mLST8 and the recently identified Deptor, which function as positive and negative regulators, respectively (Peterson et al. 2009).

In response to growth factors and nutrients (in particular insulin and amino acids), mTORC1 regulates cell growth and proliferation through the phosphorylation and regulation of downstream effector substrates, among which the most important and best characterized is the ribosomal protein S6 kinase 1 (S6K1; also known as p70S6K) (Hay N., 2004).

In particular, at the base of mTORC1 activation there is an integration of multiple signals: growth factors induce GTP loading of the G protein Rheb, enabling it to physically interact with mTORC1, while amino acids cause the RAG GTPase-mediated shuttling of mTORC1 to the lysosomal surface, where Rheb resides (Figure 5) (Zoncu et al. 2011).

mTORC1 translocation to lysosomal surfaces, besides Rag GTPases, involves also the Ragulator complex and the vacuolar ATPase (v-ATPase). Through a poorly understood mechanism requiring the v-ATPase, luminal amino acids activate the guanine nucleotide exchange factor (GEF) activity of Ragulator towards RAG GTPase (exist as obligate heterodimers of RagA or RagB, which are highly homologous, with either RagC or RagD, which are also very similar to each other) that, when GTP-loaded, recruits mTORC1 to the lysosomal surface. Upon amino acid withdrawal RagA/B become GDP-bound by unknown

mechanisms and mTORC1 leaves the lysosomal surface and maintains an inactive status (Betz et al. 2013).

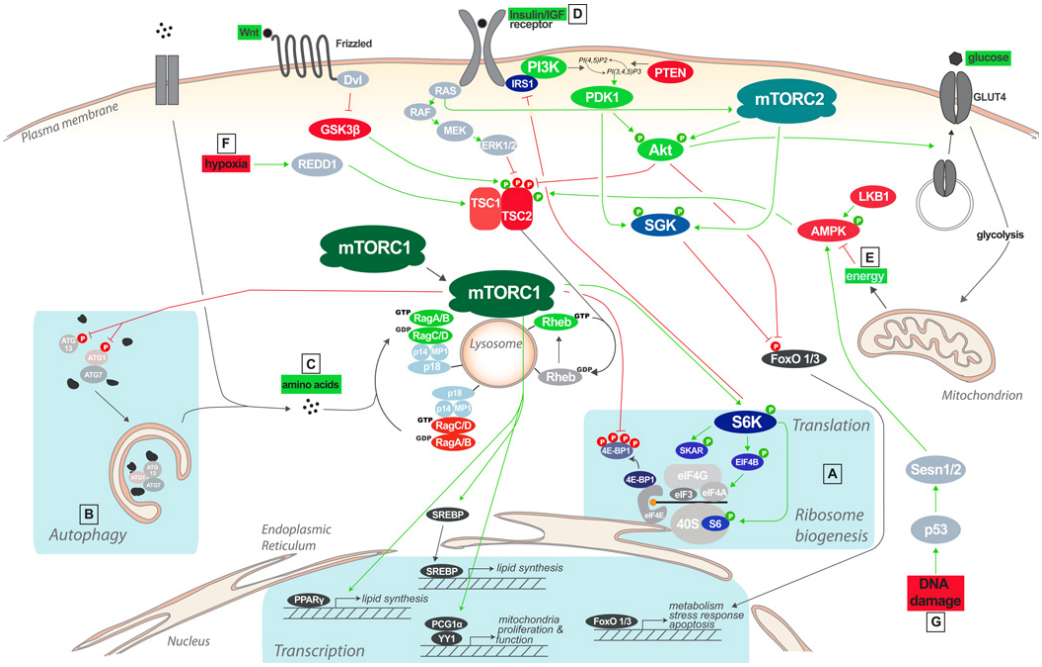


Figure 5. mTORC1 pathway (from Zoncu et al. 2011)

As far as mTORC2 complex is concerned, its function is less well understood, but it is involved in the modulation of the PI3K/Akt pathway and cytoskeletal physiology (Murugan et al. 2013).

1.4.1. Amino acids sensing to mTOR

It is known that amino acid availability, in particular that of leucine, arginine and glutamine, is essential for mTORC1 activation.

It has been demonstrated that amino acids participate to mTORC1 activation through a mechanism that involves RAG GTPases (see above).More recently, the enzyme leucyl-tRNA synthetase (LRS) has been specifically involved in the kinase activation interacting with RAG GTPase in an amino acid-dependent manner and functioning as a GTPase-activating protein (GAP) for Rag GTPase (Han et al. 2012).

Although the regulation of RAG proteins is not yet well known, recently it has been identified a complex, called GATOR, which interacts with the RAG proteins behaving as a critical regulator of amino acids signaling to mTORC1. It is composed of two subcomplexes, GATOR 1 and GATOR 2.

GATOR 1 (formed by DEPDC5, Nprl2, and Nprl3) has GTPase activating protein (GAP) activity for RagA and RagB, thus participating to the shuttle of mTORC1 to the lysosomal surface, while GATOR 2 (formed by Mios, WDR24, WDR59, Seh1L, Sec13), is an upstream inhibitor of GATOR 1.

In cancer cells presenting a loss of GATOR 1 function, mTORC1 is hyperactivated and insensitive to amino acid starvation. Moreover, tumors carrying this alteration show an hypersensitivity to the treatment with the inhibitor rapamycin (Bar-Peled et al. 2013).

It is not well defined how amino acids enter the cell to regulate the kinase activation (Nicklin et al. 2009), but their uptake is typically coupled with fluxes of ions such as Na^+ (also K^+, or Cl^-). Na^+ and K^+ transporters are referred to as being secondary active transporters since they are dependent on the plasma membrane Na+/K+ ATPase that maintains the ion gradients across the membrane.

A lot of transporters that provide amino acid supply have been discovered over the years. Two families of carriers involved in the regulation of mTORC1 signalling are the system L and the system A transporters. The system L type transporters are amino acid exchangers that primarily function to import the branched chain amino acids in exchange for other intracellular amino acids, while System A is a family of Na^+- dependent, unidirectional concentrative transporters which allowthe uptake of neutral amino acids, in particular of glutamine (Mackenzie et al. 2004).

In particular the transporters LAT1 for system L and SNAT2 for system A are coupled together through a tertiary active transport mechanism, in which SNAT2 maintains intracellular glutamine levels in order to drive the LAT-1 exchanger and promote leucine uptake, which is believed to be the main responsible of mTORC1 activation (Dodd et al. 2012) (Figure 6).

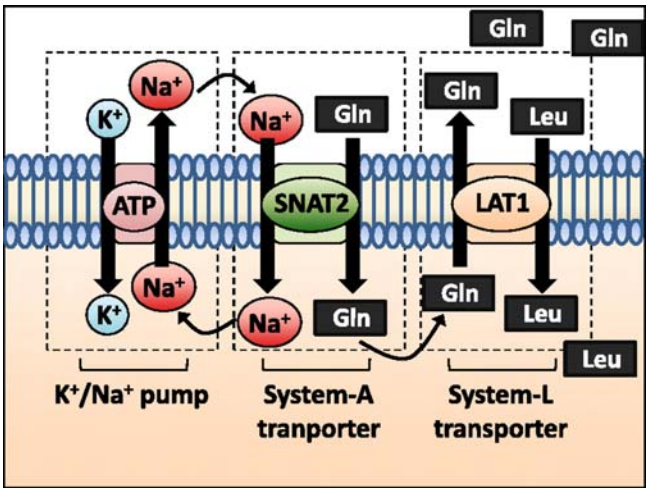


Figure 6. Amino acids transporters influencing mTORC1 signalling (from Dodd et al. 2012)

A variant of this mechanism was proposed in 2009 by Nicklin, who described a model in which glutamine is necessary for mTORC1 activation. Even in this case the uptake of glutamine through the high-affinity neutral amino acid transporter ASCT2 was required for leucine-induced mTORC1 activity in conjunction with the LAT amino acid exchanger (Nicklin et al. 2009).

Conversely, a study from our laboratory demonstrated that in HepG2 and HeLa cells glutamine-free incubation lowered mTORC1 activity, although cell leucine was not decreased (Chiu et al 2012). Thus glutamine and leucine stimulate mTORC1 independently, suggesting the existence of two distinct stimulatory signals.

1.4.2. Tumorigenicity of mutated MTOR gene

Aberrant activation of mTOR signaling, particularly through the mTORC1 complex, occurs commonly in human cancers (Guertin et al. 2007). A lot of loss-of-function mutations have been identified along the mTOR pathway for many years, mostly at level of tumor suppressors, such as phosphatase and tensin homolog (*PTEN*) and tuberous sclerosis 1/2 (*TSC1/2*), but also at level of *KRAS*, *PIK3CA*, or *AKT* (Zoncu et al. 2011). More recently inactivating mutations have been demonstrated also at the level of the subcomplex GATOR 1, upstream of mTORC1: these mutations lead to the overstimulation of the kinase and make it insensitive to amino acid starvation (see above) (Bar-Peled et al. 2013).

Notwithstanding few mutations of the *MTOR* gene rarely have been found in human cancers (Murugan et al. 2012), recent studies have demonstrated that point mutation in mTOR structure are associated with cancer conferring an hyperactivation of the kinase (Grabiner et

al. 2014). These alterations occur especially at level of the FAT, FRB and Kinase domains of mTOR structure and are present in multiple cancer subtypes, with the highest number in colorectal, endometrial, and lung cancers, although these cancer subtypes are also considered to have the highest mutation rates (Figure 7).

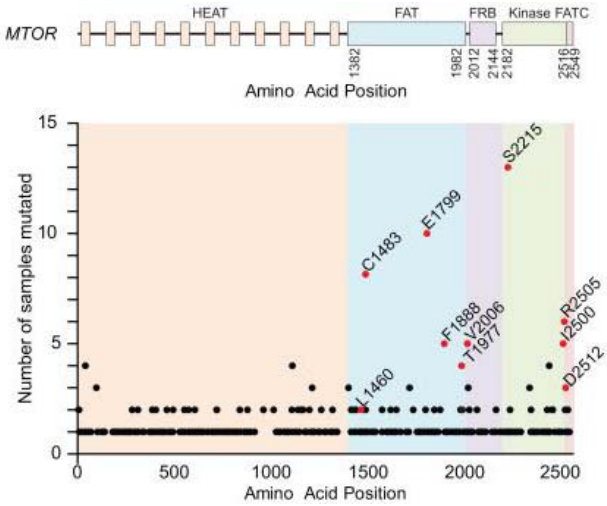


Figure 7. Cancer associated *MTOR* mutations (from Grabiner et al. 2014)

Probably *MTOR* mutations might increase S6K1/Akt1 phosphorylation by altering mTORC1 or mTORC2 assembly. In fact it has been demonstrated that the *MTOR* mutations may diminish the binding of mTOR to its endogenous inhibitor Deptor, which, in normal conditions, binds to the FAT domain of the protein (Peterson et al. 2009). Thus, this behavior implicates an overstimulation of the kinase which increases its activity also when cells are undergoing nutrient starvation (Murugan et al. 2012).

Interestingly, it has been noted both *in vitro* and *in vivo* that cell lines carrying activating *MTOR* mutations are hypersensitive to rapamycin treatment, thus indicating a mTOR-dependent phenotype (Grabiner et al. 2014).

In conclusion, the presence of hyperactivating *MTOR* mutations in cancer cells may serve as a marker to identify tumors that are likely to respond to mTOR inhibitors.

2. Aim of the thesis

Gln is a nutrient of fundamental importance for the metabolism of normal and cancer cell and is implied in many metabolic pathways as well as in anaplerosis and in mTOR activation. Although it has been known for several years that certain cancers cells are strongly dependent on Gln availability and undergo severe consequences upon Gln deprivation, the molecular bases underlying this phenotype are not precisely known.

Aim of this thesis is to ascertain if lack of expression of Glutamine Synthetase (GS), the enzyme which synthetize Gln from Glu and ammonium, is a determinant of Gln dependence.

Two cell lines derived from human oligodendroglioma, a rare brain tumor in which GS expression is usually low or absent, were chosen as the experimental model to verify the hypothesis.

3. Materials and Methods

3.1. Cell lines and culture methods

The HOG cell line (provided by Dr. Glyn Dawson, University of Chicago), derived from a human oligodendroglioma, and the U87 cell line (provided by Dr. Daniela Parolaro, University of Insubria), derived from a human glioblastoma, were grown in a high-glucose (4.5 g/l) DMEM (Dulbecco's modified Eagle's Medium) with 3.7 g/l of bicarbonate, supplemented with 10% of fetal bovine serum (FBS), 2 mM of Glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The human oligodendroglioma Hs683 cell line (provided by Dr. Robert Kiss, University of Bruxelles) and the human glioblastoma U373 cell line (provided from Dr. Jacopo Meldolesi, University Vita Salute, Milan) were grown in a low-glucose (1 g/l) DMEM supplemented with 10% of FBS, 2 mM of Glutamine and antibiotics. Bicarbonate concentration was 2.2 g/l for Hs683 cells and 3.7 g/l for U373 cells. The different bicarbonate concentrations fixed pH value at 7.4 for HOG, U87 and U373 and 7.2 for Hs683 at 5% CO_2.

All the cell lines were grown at 37°C and 5% of CO_2 and after thawing they were not used for more than 10-12 passages.

For amino acid depletion, cells were incubated in EBSS (Earle Balanced Salt Solution): $NaHCO_3$ (2.2 g/l), glucose (1 g/l), 10% of a EBSS-x10 solution (NaCl 68 g/l; KCl 4g/l, NaH_2PO_4 1.249 g/l; $MgSO_4$ 2 g/l; $CaCl_2$ 0.993 M), Phenol Red (NaOH) (2 ml/l). FBS?

3.2. Viability Assay

The viability of oligodendroglioma and glioblastoma cells in response to experimental treatments (glutamine depletion, ASNase, ASNase + MSO, rapamycin, 2-methylaminoisobutyric acid (MeAIB) and threonine (Thr)) was assessed with resazurin assay. Resazurin is a blue and non fluorescent die that is reduced to resorufin, pink and highly fluorescent, when the cells are metabolically active (increase of $NADH/NAD^+$) (O'Brien J. et al., 2000). Cells were seeded in 24 well plates (25 x 10^3/well/500 µl for HOG and Hs683 cells and 30 x 10^3/well/500 µl for U87 and U373 cells for experiments with glutamine depletion, ASNase, ASNase + MSO, rapamycin; 5 x 10^3/well/500 µl for HOG, Hs683 and for U373 for experiments with 2-methylaminoisobutyric acid (MeAIB) and threonine (Thr) in complete growth medium. After 24 hours, they were incubated with fresh medium containing different concentrations of glutamine (ranging from 0 to 2 mM) or ASNase (ranging from 0 to 1 U/ml),

ASNase + MSO (1mM), rapamycin (ranging from 0 to 10 nM) for 48 hours or with MeAIB e/o Thr for 72 hours according to the experimental schemes reported for each experiment.
At the end of the treatments cells were incubated in medium supplemented with resazurin (11 μg/ml) without FBS and Gln. After 3 h of incubation at 37°, fluorescence was measured at 572 nm with a fluorimeter (Multimode Plate reader Enspire, Perkin Elmer).
Afterwards tThe effect of the treatments was also evaluated counting the cells with a Coulter Z1 particle counter.
The effect of ASNase (1 U/ml) and ASNase (1U/ml) + MSO (1mM) was evaluated also in a time-dependent manner (72 hours), keeping the same cellular density and assessing the viability every 24 hours from the beginning of the treatments. Viability was assessed as described before.

3.3. Western Blot

After the experimental treatments, medium was collected and cells were lysated in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton, 2,5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 2 mM imidazole, and a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche). Lysates were transferred in Eppendorf tubes, sonycated for 5 s and centrifuged at 12000 g for 10 min at 4° C. The protein concentration (μg/μl) was assessed with the Bradford method and the equivalent of 30 μg of proteins were mixed with Laemmli buffer 4x (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.4 M DTT), warmed at 95° C for 5 min and loaded on a 10 % polyacrylammide gel for electrophoresis (SDS-PAGE). After the electrophoretic running, the gel was transferred to a polyvinylidenedifluoride membrane (Millipore-Immobilion-P), the non-specific binding sites of proteins were blocked in a 5 % milk (Sigma-Aldrich) solution or in a 10%-blocking solution (Roche Diagnostic SpA, Milan, Italy) for 1 hour at room temperature.
Then the membranes were then hybridized with the specific antibodies diluted in a 5% bovine serum albumin TBS Tween solution at 4° C overnight. The primary antibodies used for the blots were: anti-Glutamine Synthetase (GS) (mouse monoclonal, 1:2000, BD Transduction Laboratories), anti-total p70S6K, anti-phospho-p70S6K (T389) (both rabbit polyclonal, 1:1000, Cell Signaling Technology), anti-β-tubulin (mouse monoclonal 1:2000, Santa Cruz Biotechnology), anti-GAPDH (rabbit,1:5000; Chemicon, CA, USA). After two washes, the blots were incubated for 1 hour at room temperature to HRP-conjugated anti-mouse or anti-rabbit secondary antibody (ExactaCrutz, Santa Cruz Biotechnology), diluted 1:20000 (mouse) or 1:10000 (rabbit) in blocking solution. After washing, the membranes were exposed to

Immobilion Western Chemiluminescent HRP Substrate (a solution of H_2O_2 and luminol) (Millipore), and the signal was impressed on photographic paper.
Every expression level was normalized to the housekeeping protein used as loading control, specified in each experiment.

3.4. RNA extraction, Reverse Transcription and Semi-Quantitative PCR

To assess gene expression in HOG, Hs683, U87 and U373 lines, cells were seeded in 6-well plates (4 x 10^5/well/2 ml) with complete growth medium, and, after 24 hours, total RNA was extracted (kit Gen Elute™ Mammalian Total RNA Miniprep (Sigma Aldrich)).
Then, 1 μg of total RNA was reverse transcribed to cDNA using (ImProm-II™ Reverse Transcription System, Promega). For real time PCR, cDNA was amplified with GoTaq® qPCR Master Mix (Promega) using the primers reported in Table 1.
PCR was performed in a 36-well Rotor Gene 3000 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). Each cycle consisted of a denaturation step at 95° C for 15 s, an annealing step (30 s, 55-57°) and an extension step (30 s, 72° C). Fluorescence was monitored at the end of each extension step. A no-template, no-reverse transcriptase control (NTC) was included in each experiment. At the end of the amplification cycles a melting curve analysis was added. The analysis of the data was made according to the Relative Curve Standard (Bustin S.A., 2000). Every gene expression values were normalized to *RPL-15* (Rybosomal Protein L-15) values used as housekeeping gene.

GENE	PRIMERS	TEMPERATURES
GLUL *(Glutamine Synthetase)*	For 5'-TCATCTTGCATCGTGTGTGTG-3' Rev 5'-CTTCAGACCATTCTCCTCCGG-3'	57° C
RPL-15 *(Rybosomal Protein L-15)*	For 5'-GCAGCCATCAGGTAAGCCAAG-3' Rev 5'-CGGACCCTCAGAAGAAAGC-3'	55° C

SLC38A1, SNAT1 (Solute carrier family 38 member 1)	For 5'-CACCACAGGGAAGTTCGTAATC-3' Rev5'-CGTACCAGGCTGAAAATGTCTC-3'	57° C
SLC38A2, SNAT2 (Solute carrier family 38 member 2)	For 5'-ATGAAGAAGGCCGAAATGGGA-3' Rev 5'-TGCTTGGTGGGGTAGGAGTAG-3'	57° C
SLC38A4, SNAT4 (Solute carrier family 38 member 4)	For 5'-GAAATTCCAAATACCCTGCCCT-3' Rev 5'-GCGGTGGGTGTAATCCATCA-3'	57° C
SLC1A5, ASCT2 (Solute carrier family 1 member 5)	For 5'-TGGTCTCCTGGATCATGTGG-3' Rev 5'-TTTGCGGGTGAAGAGGAAGT-3'	57° C
$ATB0^+$ (Solute carrier family 6 member 14)	For 5-GCTGCTTGGTTTTGTTTCTCCTTGGTC-3' Rev 5'-GCAATTAAATGCCCCATCCAGCAC-3'	60° C

3.5. Amino Acid analysis

After the treatments, cells were rinsed twice with cold PBS (Phosphate Buffered Saline) (Na_2HPO_4 1,126 g/l; KH_2PO_4 0,2 g/l; KCl 0,2 g/l; NaCl 8 g/l), and amino acid content was extracted with a solution of ethanol-acetic acid (150 ml absolute ethanol + 1 ml acetic acid 1M). After extracts were collected, they were transferred in Eppendorf tubes, centrifuged at 12000*g* for 10 min at 4° C and lyophilized (VR-I Hetovac). Every sample was then resuspended in LiOH buffer, pH 2.2, filtrated, and analyzed with a Biochrom 20 Amino Acid Analyzer (Biochrom, Cambridge, UK) (Gazzola G.C. et al. 1981) Data were normalized to the protein content of cell population obtained, after the extraction, with the Lowry (Lowry O.H., 1951). Results were expressed as nmol/mg protein.

3.6. Glutamate Assay

Glutamate Assay Kit, Fluorometric (Abcam). was used to assess Glu and Gln content in oligodendroglioma and glioblastoma cells. The assay consists of two coupled reactions between L-Glutamic acid and NADP+ to generate NADPH, which is then recognized by a NADPH sensor and recycled back to NADP+. During the reaction a red fluorescence product is produced and the signal is read at Ex/Em = 530-570 nm / 590-600 nm with a fluorimeter ((Multimode Plate reader Enspire, Perkin Elmer).

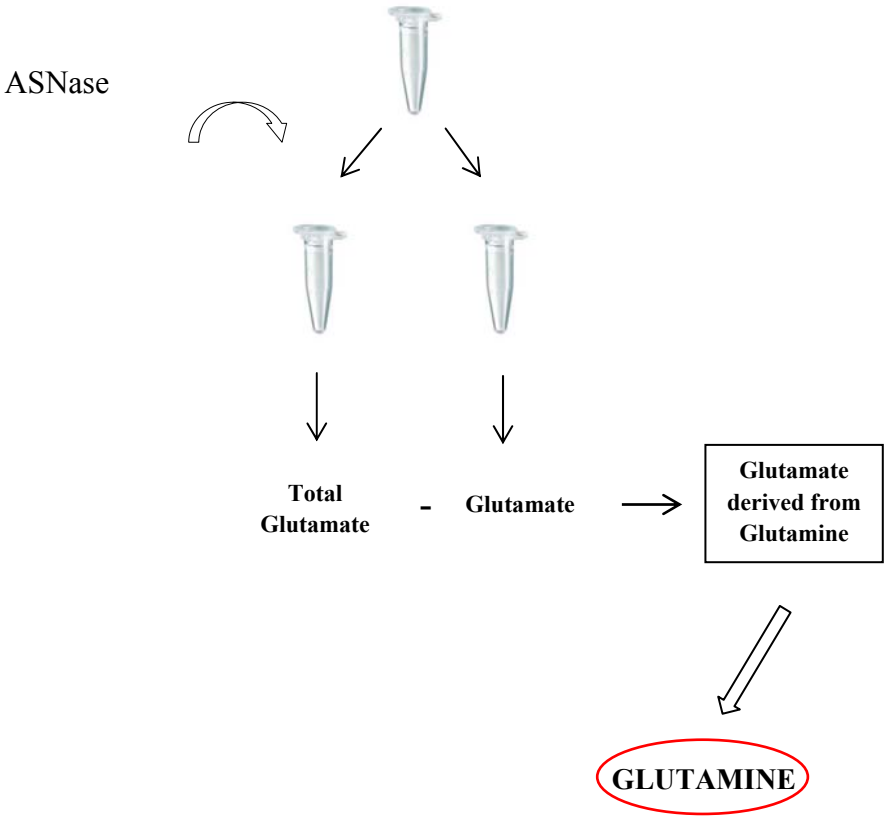
The Amino acids were extracted as described previously. After lyophilization, samples were solved in Dilution Buffer and divided into two Eppendorf tubes to measure Glu and Gln, respectively. To assess Gln, ASNase (15 U/ml) was added to the sample that was incubated for 2 hours at 37°C. According to the protocol kit, after ASNase reaction, 50 µl of sample were plated in wells of black microplates and 50 µl of glutamic acid assay mixture were added to start the reaction at room temperature. After 30 and 45 minutes, fluorescence at 572 nm was detected with a Multimode Plate reader Enspire, Perkin Elmer.

Since ASNase catalyzes Gln hydrolysis into Glu and ammonium, samples treated with ASNase yielded Glu + Gln ("total" Glu), while the samples incubated without ASNase yielded Glu.

The subtraction between "total" Glu and Glu gives Glu stoichiometrycally derived from Gln, yielding Gln content of the sample calculated from a Standard Curve built in parallel. Data were normalized to the protein content of the cells obtained with the Lowry method and results were expressed as nmol/mg prot.

ASNase

$$GLN \longrightarrow GLU + NH_4^+$$



3.7. Gene transfection

The *GLUL* gene, encoding for Glutamine Synthetase, was transiently transfected in HOG and Hs683 cells.

3.7.1. Plasmid amplification and purification

pCMV-*GLUL* and pCMV, used as negative control (OriGene Products, Rockville, Maryland, USA), were used for transfection experiments.

An high plasmid concentration was obtained recombining an *E.coli* culture with the vectors and purifying it with GenElute Plasmid Miniprep kit (Sigma Aldrich). Briefly, according to the protocol kit, recombinant *E.coli* culture, grown in LB (Luria broth), was pelleted at 12000 g for 1 minute and well resuspended in 200 µl of the Resuspension Solution (containing RNAase). After, the resuspended cells were lysed adding 200 µl of the Lysis Solution and mixed by inversion for no more of 5 minutes. Then 350 µl of Neutralization/Binding Solution were addicted and after all the cell debris, proteins, lipids and chromosomal DNA were precipitated by centrifuging at 12000 g for 10 minutes. The cleared lysate was then transferred in a GenEluteMiniprep Binding Column (previously prepared adding 500 µl of the Column Preparation Solution and centrifuging at 12000 g for 1 minute) and centrifugated at 12000 g for 1 minute. The column was then washed at first with the Optional Wash Solution (500 µl and centrifugation at 12000 g for 1 minute) and after with the Wash Solution (750 µl and centrifugation at 12000 g for 1 minute). Finally the column was transferred to a fresh collection tube and eluted adding 100 µl of Elution Solution and centrifugated at 12000 g for 1 minute to obtain the purified plasmid DNA.

The DNA obtained was then precipitated and resuspended in TE (Tris-HCl, EDTA : Tris-HCl 100 mM, EDTA 10mM).

3.7.2. Transfection

For transfection experiments, cells were seeded in 24-well plates (10 x 10^3/well/500 µl for HOG cells and 13 x 10^3/well/500 µl for Hs683 cells) in complete growth medium, and, after 24 hours, they were transfected with pCMV-*GLUL* or pCMV using Fugene® 6 Transfection Reagent (Promega), following the manufacturer's instructions. The transfection reagent is a lipofectamine that creates an intussusception in the cell membrane and allows the entrance of the plasmids into the cells. The final transfection volume was 500 µl/well. According to the kit protocol, the growth medium was removed and substituted with 400 µl of fresh medium

without FBS and antibiotics. Then, a transfection mixture was prepared for all the wells adding transfection reagent (1µl/well) to the medium (99 µl/well); after an incubation of 5 min at RT, the mixture was divided into two aliquots. The plasmid vectors (600 ng/ml for both pCMV-*GLUL* and pCMV) were added to each aliquot and incubated for 15 min at RT. The mixture was then distributed into the wells (100 µl/well).
transfection, medium was removed and substituted with complete fresh medium with FBS and antibiotics. The experimental treatments started after 72 hours from transfection to allow the expression of the transfected gene.
When we wanted to evaluate the sensitivity of transfected cells to Gln depletion, cells were maintained at 5% FBS to reduce Gln concentration in the medium.

3.8. Inhibition of Glutamine transporters

2-methylaminoisobutyric acid (MeAIB) and threonine (Thr) were used to inhibit Gln transporters of System A and System ASC transporters respectively.
For these experiments HOG, Hs683 and U373 cells were seeded in 24-well plates (5 x 10^3/well/500 µl) in complete growth medium. After 24 hours they were incubated with fresh medium containing MeAIB (20 mM), Thr (10 mM) or MeAIB + Thr at the same concentrations (20 mM + 10 mM) for 72 h. Since MeAIB and Thr were used at high concentrations, difference of osmolarity was balanced using sucrose 30 mM in control condition, 10 mM in MeAIB condition and 20 mM in Thr condition. Viability was assessed as described before.

3.9. Materials

ASNase (Erwinase ®, Eusa Pharma,) was provided from EUSA Pharma, while serum was obtained from Euro Clone.
Whenever not specified otherwise, Sigma-Aldrich was the source of chemicals and reagents.

4. Results and Discussion

4.1. Oligodendroglioma cell lines are "GS-negative"

Oligodendrogliomas (OD) are rare brain cancers; among other markers, they show a lack of Glutamine Synthetase (GS) expression in immunohistochemistry so that they are considered "GS negative" (Pilkington et al. 1982). This marker differentiates them from astrocytomas, facilitating differential diagnosis (Zhuang et al. 2011).

The expression of GS was assessed in two human oligodendroglioma cell lines, HOG and Hs683, through both RT-PCR (determining the expression of the mRNA of *GLUL*, the gene that encodes GS) and Western Blot analysis of GS protein; results were compared with GS expression in U87 and U373 human glioblastoma lines, used as positive controls (Figure 1*a* and 1*b*).

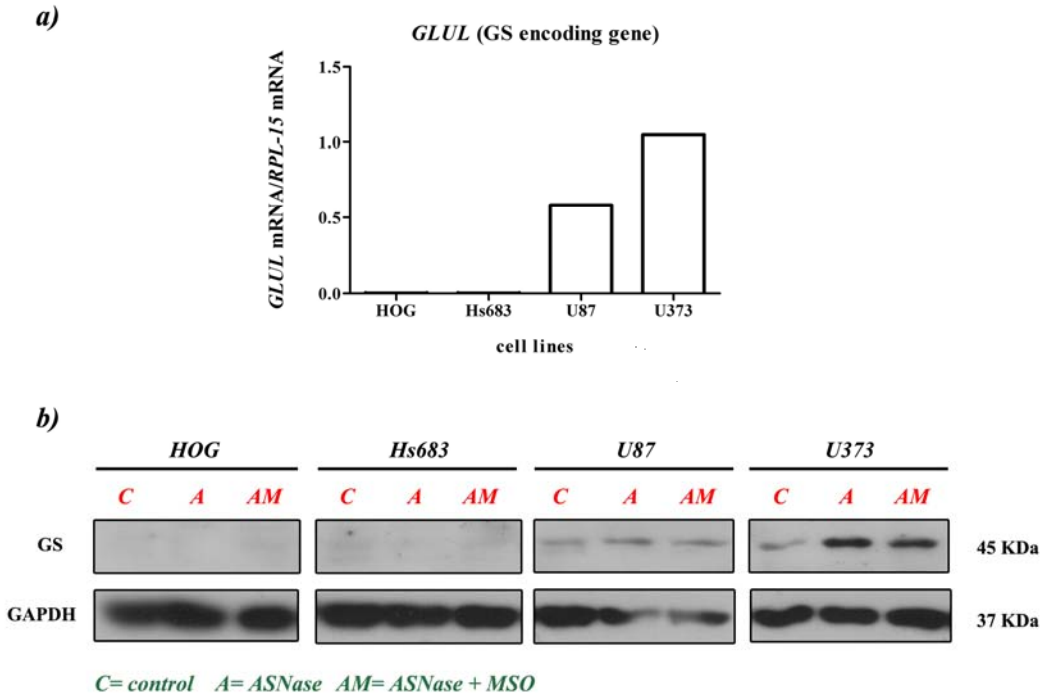


Figure 1. *GLUL* mRNA *a)* and GS protein *b)* expression in oligodendroglioma and glioblastoma cell lines.
a) Cells (4 x 10^5/well/2 ml) were seeded in 6-well plates. After 24 h total RNA was extracted, reverse transcribed to cDNA, and amplified through RT-PCR (see Materials and Methods). Data are means of two determinations and *GLUL* expression is expressed relatively to the expression of the *RPL-15* housekeeping gene. U87 and U373 cells were used as positive controls. Values of *GLUL* mRNA expression were 0.001346 and 0.003788 for, respectively, HOG and Hs683 cells.
b) Cells (7 x 10^5/well/2 ml) were seeded in 6-well plates. After 48 h cells were lysed, proteins were extracted, and GS expression was evaluated in the lysates through Western Blot analysis (see Materials and Methods), using GAPDH as loading control. U87 and U373 cells were used as positive controls. The experiment was performed twice with comparable results.

HOG and Hs683 cells show a much lower expression of *GLUL* gene than U87 and U373, while at protein level GS expression was not detectable at all in Western Blot in both oligodendroglioma cell lines, but clearly visible in glioblastoma cells.

The treatment with asparaginase (ASNase) and ASNase + MSO (methionine-L-sulfoximine, an irreversible GS inhibitor), while increased GS expression in U87 and U373 cells, did not enhance GS abundance in HOG and Hs683. Increased GS expression in glioblastoma cells, although never described before, is expected. Indeed, ASNase and ASNase + MSO cause a severe intracellular depletion of Gln (see below and Chiu et al., 2014). Since the shelf life of GS protein is inversely dependent on the intracellular levels of Gln (Tardito *c)* et al., 2012), Gln depletion causes an increase in GS protein shelf life and, hence, an apparent increased abundance of the enzyme. The fact that, even under these extreme conditions, oligodendroglioma cells do not exhibit any detectable expression of GS protein is consistent with the exceedingly low levels of expression of the *GLUL* gene detected in these cells, which, therefore, can be considered “GS negative”.

4.2. Oligodendroglioma cell lines are sensitive to Glutamine starvation and ASNase

The effects of the incubation of the four cell lines under Gln-free conditions are presented in Figure 2. Since the experiment was performed in the presence of 5% Fetal Bovine Serum ([Gln] about 500-600 µM), the actual extracellular Gln concentration under nominally Gln-free conditions is 25-30 µM, corresponding to a 95% decrease in the physiological plasma Gln.

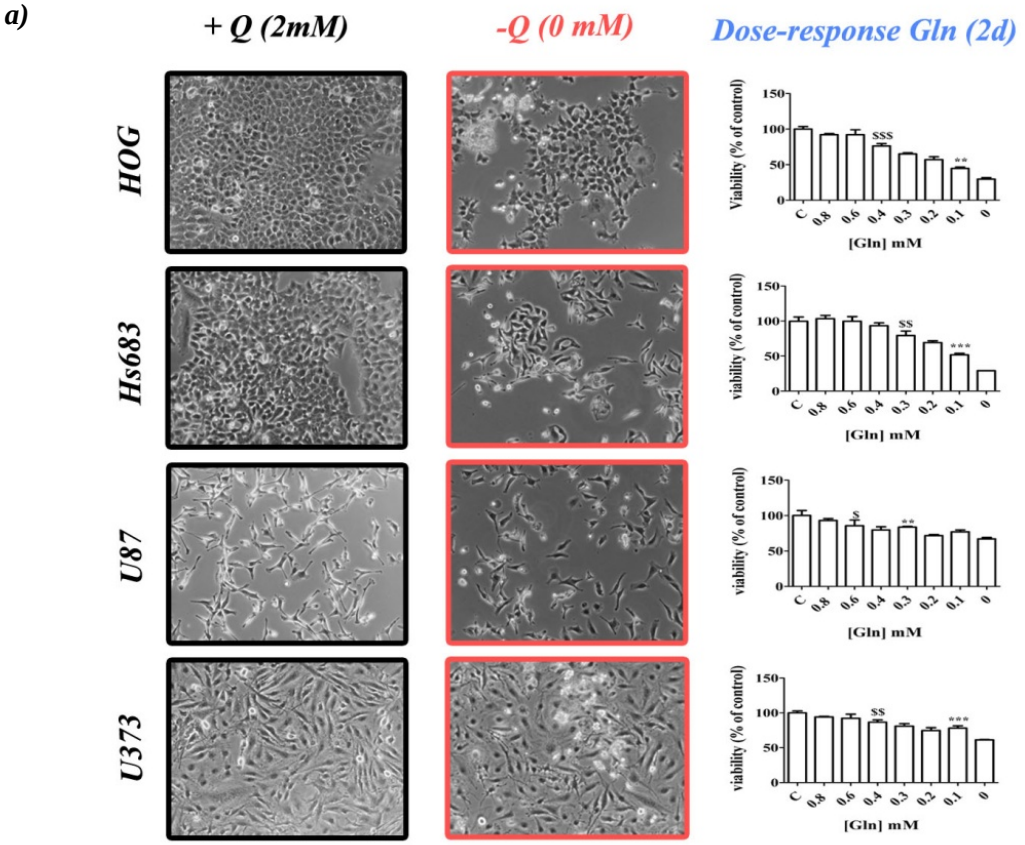


Figure 2. Sensitivity of oligodendroglioma and glioblastoma cells to Gln deprivation

a) Cells (2.5 x 10^4 /well/500 µl for HOG and Hs683 and 3 x 10^4 /well/500 µl for U87 and U373) were seeded in 24-well plates. After 24 h they were incubated with fresh medium (at 5% of FBS) containing different Gln concentrations (from 0 to 2 mM, see Materials and Methods) for 48 h. At the end of the treatments, cultures incubated at 0 and 2 mM Gln were observed (left, x100) and cell viability was evaluated with resazurin test (right, see Materials and Methods). Data are means ± S.D. of three independent determinations in an experiment performed twice with comparable results. Statistical analysis was performed with ANOVA followed by the Bonferroni post hoc test;* $p<0.05$ ** $p< 0.01$ *** $p< 0.001$ versus the lowest Gln concentration; $ $p<0.05$ $$ $p< 0.01$ $$$ $p< 0.001$ versus control cells ([Gln] 2 mM).

HOG and Hs683 cells showed high sensitivity to Gln deprivation. In particular, the maximal effect, in terms of decrease in cell viability upon a 48h-incubation in Gln-free medium, was 70% for both the oligodendroglioma cell lines, but only 30% for U87 and 40 % for U373 glioblastoma cells. This difference was also evident at the microscopic observation.

Data analysis indicated that the extracellular Gln concentration sufficient to allow a half maximal growth is between 0.1 and 0.2 mM for HOG cells, around 0.1 mM for Hs683 cells, and < 0.03 mM (the Gln concentration under nominally Gln-free conditions) for both the glioblastoma cell lines. Consistently, the slight increase of extracellular Gln from nominally free conditions to 0.1 mM has significant effect on cell growth in oligodendroglioma cells but not in glioblastoma cells. It is apparent that lack of GS expression is associated with higher sensitivity to incubation under Gln-deprived conditions.

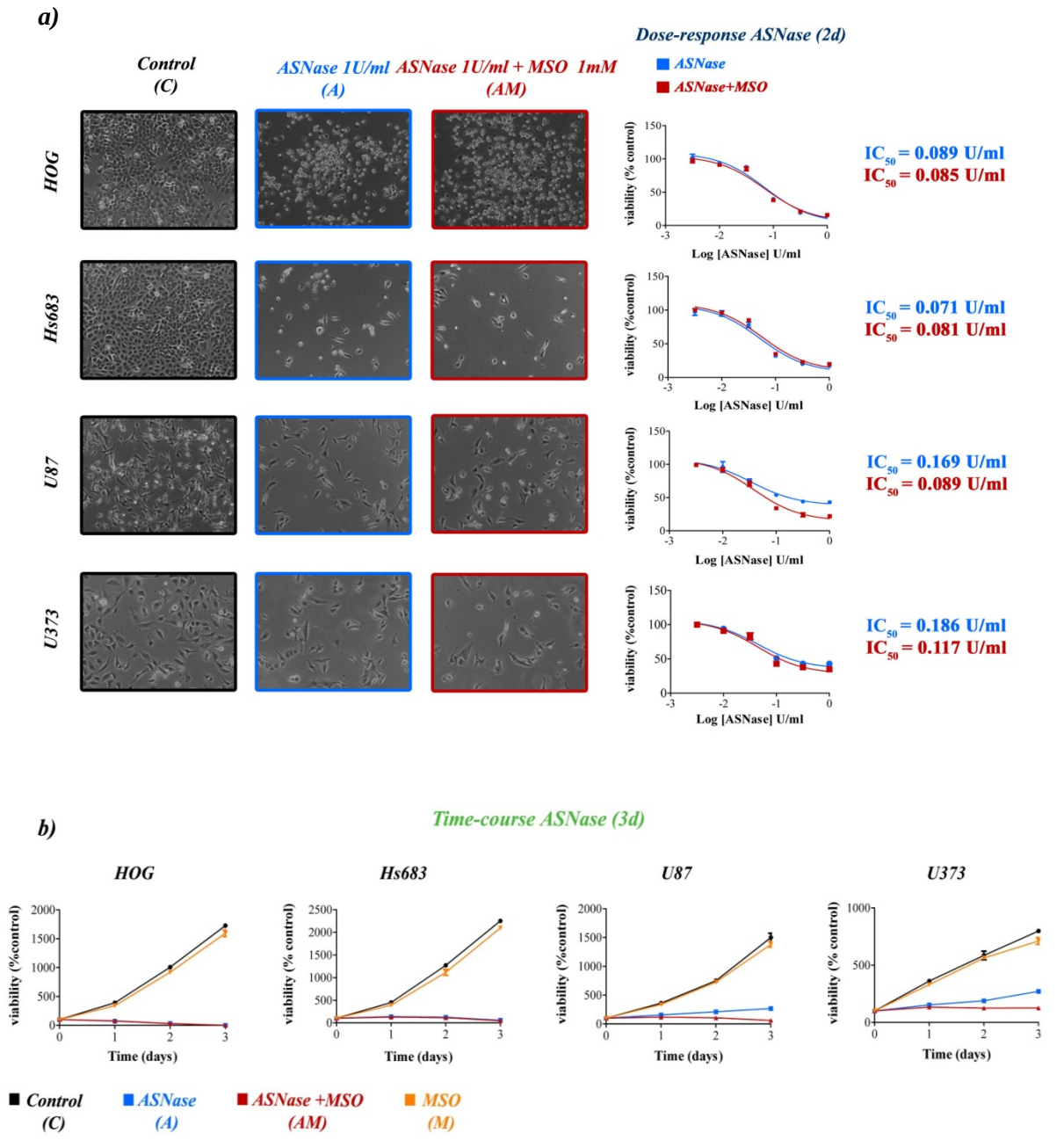


Figure 3. Sensitivity of oligodendroglioma and glioblastoma cells to ASNase

a) Cells (2.5 x 10^4 /well/500 µl for HOG and Hs683 and 3 x 10^4 /well/500 µl for U87 and U373) were seeded in 24-well plates. After 24h they were incubated for 48 h with fresh medium containing different ASNase concentrations (from 0.003 to 1 u/ml, see Materials and Methods) in the presence or in the absence of the GS inhibitor MSO (1mM). At the end of the treatments cultures were observed at the microscope (upper left) and viability was evaluated with resazurin test (upper right, see Materials and Methods). Data are means ± S.D. of three independent determinations in an experiment performed twice with comparable results. For each dose-response the half-maximal inhibitory concentration (IC_{50}).was calculated with the GraphPad Prism5™ software.
b) Cells (2.5 x 10^4 /well/500 µl for HOG and Hs683 and 3 x 10^4 /well/500 µl for U87 and U373) were seeded in 24-well plates. After 24 h cells were incubated with fresh medium containing ASNase (1U/ml) or ASNase (1U/ml) +MSO (1mM) for 72 h. Viability was assessed every 24 hours with resazurin test (see Materials and Methods).

Oligodendroglioma cells were also very sensitive to ASNase treatment (Figure 3). The IC_{50} values, calculated in dose-response experiments, were < 0.1 U/ml (0.089 U/ml and 0.071 U/ml for HOG and Hs683 cells, respectively). Since an IC_{50} = 0.1 U/ml is usually adopted as a threshold value for sensitivity to ASNase in leukemia cells, these values allow to define the two oligodendroglioma cell lines as "sensitive" to ASNase.

The addition of the GS inhibitor MSO to ASNase-treated cells had no effect on cell viability of the two oligodendroglioma cell lines and the IC_{50} values obtained in the absence and in the presence of the inhibitor were comparable. This result is consistent with the very low GS expression detected in oligodendroglioma cells and demonstrates that GS is functionally silent in these cells.

In contrast, glioblastoma cells exhibited an IC_{50}> 0.1 U/ml for ASNase and, therefore, can be defined poorly sensitive to the enzyme. In these cultures MSO decreased markedly the IC_{50} for ASNase, which was lowered below 0.1 U/ml in U87 cells. Moreover, the microscopic observation of the cultures suggested that massive cell death was triggered in oligodendroglioma but not in glioblastoma cells.

Figure 3, Panel *b*, shows the time course of ASNase effect. In both the oligodendroglioma cell lines ASNase caused a progressive and complete loss of cell viability which was completely suppressed after an incubation of 72 hours with the enzyme. MSO did not significantly affect cell viability both in the absence and in the presence of ASNase. In U87 and U373 lines ASNase caused a rapid inhibition of cell growth compared to the untreated control, already detectable after a 24h-treatment. However, both U87 and, more evidently, U373 cells showed a partial recovery of cell growth at later times of incubation. This result is important, since it indicates that glioblastoma cells are able to adapt to the antitumor enzyme. In glioblastoma cells, MSO did not significantly affect cell viability in the absence of ASNase but significantly lowered cell viability in the presence of ASNase. More importantly, no sign of

growth recovery is observed if ASNase treatment is carried on in the presence of the GS inhibitor, indicating that the adaptation to ASNase is likely due to GS activity.

4.3. ASNase depletes intracellular Gln in both oligodendroglioma and glioblastoma cells

The effects of ASNase on intracellular Glu and Gln content of HOG, Hs683 and U87 cells are reported in Figure 4.

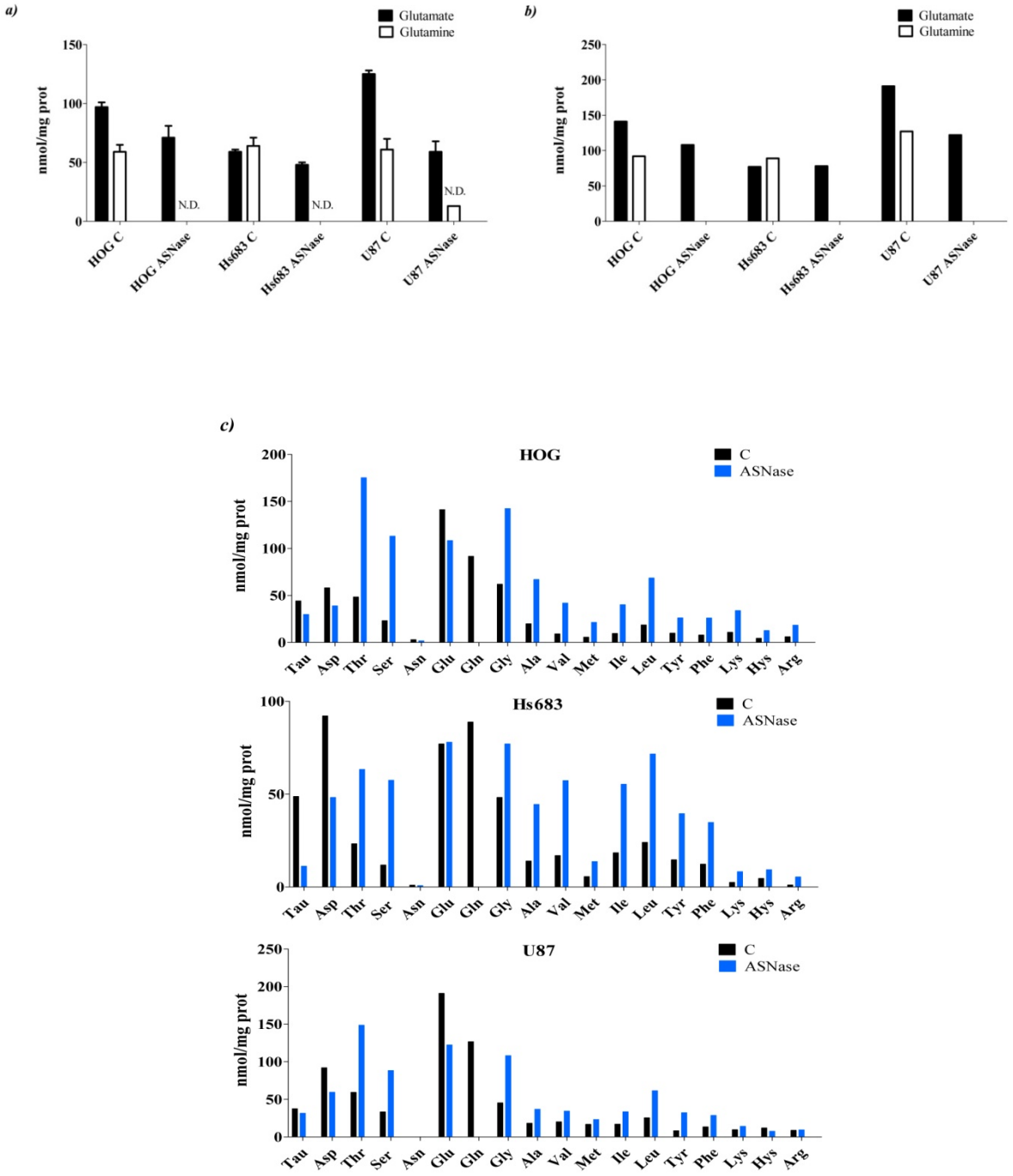


Figure 4. Glutamine and Glutamate content in oligodendroglioma and glioblastoma cells

Cells ($1x10^5$/well/1ml for HOG and U87 and $1.2\ x10^5$/well/1ml for Hs683) were seeded in 24 well plates. After 24 h cells were treated with ASNase (1 U/ml) for 18 h. At the end of the treatment cells were rinsed twice with cold PBS and amino acids were extracted with 500 µl of a solution of ethanol-acetic acid. Extracts were collected, transferred in Eppendorf tubes, centrifuged and lyophilized. Glu and Gln contents were assessed with Glutamate Assay Kit (*a*) or HPLC (*b*) (see Materials and Methods). In both cases data were normalized to the protein content of the cell population obtained, after the extraction, with the Lowry method. *c)* Complete amino acidic profiles of HOG, Hs683 and U87 cell lines measured as in *b).* Data, expressed as nmol/mg protein, are means ± S.D. of three independent determinations in an experiment performed twice with comparable results (*a*) or the results of a representative experiment (*b, c*) .

The cell content of amino acids was determined in parallel cultures with two independent methods. The first (Glutamate Assay Kit) consists in an indirect estimation of Glutamate from the NADH produced by the activity of Glutamate Dehydrogenase (GDH). To estimate Glutamine, the cell extract is treated, before the GDH reaction, with ASNase in order to hydrolyze all the Gln into Glu. Gln content is then calculated as the difference between the fluorescence recorded in the sample treated with ASNase and that recorded in the untreated sample. The results obtained with the Glutamate Assay Kit indicated that under control conditions the three cell lines had comparable contents of Gln although Hs683 showed an higher content of Gln than Glu, while the opposite result was obtained for the other lines. In all the three models ASNase caused a dramatic decrease of Gln content. Gln depletion was complete in both oligodendroglioma cell lines while U87 cells ASNase caused a drop of Gln content (from 61 ± 9 to 13 ± 15 nmol/mg protein). This result was expected since ASNase hydrolyzes extracellular Gln and the "GS-negative" oligodendroglioma cells cannot synthetize it. Interestingly, in both U87 and (much less evidently) in oligodendroglioma cells, also cell glutamate decreased in ASNase-treated cells. In U87 cells this decrease is attributable to the enhanced activity of GS (see Figure 1) due to Gln depletion. However, this explanation obviously cannot be applied to the two oligodendroglioma cells. Instead, in these models it is likely that the decrease in cell Glu is due to the anaplerotic use of the amino acid (e.g. the synthesis of oxoglutarate). If this is true, HOG cells, which exhibit a larger decrease than Hs683, would use Gln for anaplerosis faster than the other cell line under normal conditions (ASNase absent) and would obtain much glutamate and oxoglutarate from Gln. However, after ASNase addition, no Gln is available and HOG cells are forced to use Glutamate pool to sustain anaplerosis. At the light of these consideration, it is possible that the marked decrease in cell glutamate observed in U87 is, indeed, the results of both GS activity and the anaplerotic use of glutamate.

Results of the fluorimetric kit were confirmed with HPLC, which is instead based on the direct determination of the various amino acid species after chemical derivatization. The analysis with HPLC gave roughly comparable results, although the absolute values obtained were higher, possibly for an higher sensitivity of the method. HPLC did not detect a residual Gln in U87 treated with ASNase but it should be noted that, if one considers the rough HPLC data (see Supplementary Results, Figure S1-S6), the peak of Glu in U87 treated with ASNase exhibits a small shoulder that may correspond to a small, residual intracellular pool of Gln.

In conclusion, these data demonstrate definitely that the treatment with ASNase massively depletes the cell content of Gln. The possible residual Gln detected in U87 possibly suggests that the depletion is slower in these cells than in oligodendroglioma cells, a likely effect of GS expression.

HPLC analysis allowed also to determine the complete amino acidic profile of all the three cell lines (*c*). Besides Glu and Gln discussed above, ASNase treatment caused an increase of almost all the amino acids, except for taurine (Tau) and aspartate (Asp). The drop of the latter is evident, since it is used by the cells to synthesize asparagine (Asn), the first target of ASNase. Of note it was also the increment of the essential amino acid content, in particular of leucine (Leu). A possible explanation of this behavior is that cells transport Gln through multiple transport systems, some of which are also used by essential amino acids. Thus, when extracellular Gln is depleted by ASNase the essential amino acids are transported much more easily into the intracellular compartment.

4.4. Glutamine deprivation has a different effect on mTOR activity in oligodendroglioma cell lines

The highly conserved protein kinase mammalian target of rapamycin (mTOR) controls cell growth in response to nutrient availability and growth factors and is frequently deregulated in cancer (Yang H. et al. 2013). It is known that the availability of amino acids, in particular leucine (Leu) and Gln, is needed for mTOR activation, although the precise details of the mechanisms involved are not yet defined. Moreover, it is not known if the various amino acids use the same transduction mechanism to regulate mTOR activity.

Figure 5 reports the effects of ASNase and ASNase + MSO on mTOR activity in oligodendroglioma and glioblastoma cells. To monitor mTOR activity during the experimental treatments, we have evaluated the phosphorylation of its downstream target S6K1 in Thr 389, a mTOR-specific site.

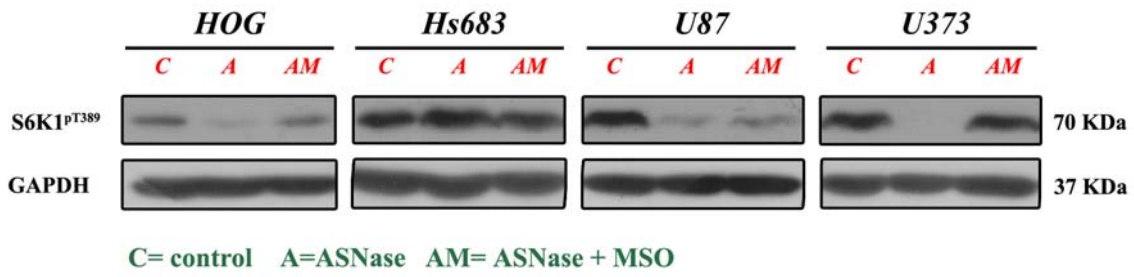


Figure 5. Effect of ASNase and ASNase + MSO on mTOR activity in oligodendroglioma and glioblastoma cell lines. Cells (7 x 10^5/ well/ 2 ml) were seeded in 6-well plates. After 24 h cells were treated with ASNase (1 U/ml) and ASNase + MSO (1mM) for 18 h. At the end of the treatment cells were lysed. Proteins were extracted and mTOR activity was evaluated through the expression of the phosphorylated form of the substrate S6K1 with Western Blot analysis (see Materials and Methods) using GAPDH as loading control. The experiment was performed twice with comparable results.

The treatment with ASNase, while lowered mTOR activity in HOG, U87 and U373 cells, did not turn off the activity of the kinase in Hs683 cells.

The lowering of S6K1 phosphorylation observed in HOG, U87 and U373 cells was expected since, in several other cell models, mTOR is affected by the Gln depletion caused by ASNase.

The addiction of MSO to ASNase during the treatment increased S6K1 phosphorylation in HOG, U373 and, less evidently, in U87 cells, while did not cause significant changes in Hs683 cells.

This effect was already observed in other cell models, where it was demonstrated that MSO is an mTOR activator, possibly mimicking the effects of the natural activators leucine and Gln

(Tardito *c)* et al., 2012). The behavior observed confirms that MSO is able to activate mTOR in Gln-depleted cells, thus mimicking the role of Gln.

Interestingly, the increase of Leu content observed in ASNase-treated cells cell lines (see above) was not sufficient to maintain mTOR activity upon ASNase treatment. This result clearly contradicts the model proposed by Nicklin et al. (Nicklin et al. 2009) (see the Introduction). Indeed, if the mechanism used by Gln to stimulate mTOR activity only consisted in promoting leucine entry, the increased intracellular concentration of leucine detected in ASNase-treated cells would cause a stimulation of mTOR activity rather than an inhibition. Conversely, the results obtained indicate that the activity of the kinase in the cells in which it shows Gln-sensitivity just depends on the intracellular concentration of Gln, not from that of leucine.

Conversely, the behavior observed in Hs683 cells was unexpected and not immediately explainable. As shown before, at 18h of treatment with ASNase, intracellular Gln levels in Hs683 cells were very low and comparable, or even lower, compared to the levels observed in other cell lines. It is, therefore, unlikely that the different behavior observed is attributable to different outcomes of ASNase treatment in terms of intracellular depletion of Gln. The peculiarity of amino acid influence on mTOR activity is also highlighted by MSO effects. In contrast with the other three cell lines, the GS inhibitor does not enhance kinase activity but, rather, seems to slightly inhibit it.

The experiment shown in Figure 5 was finalized to assess if intracellular Gln is needed for mTOR activity. Given the results obtained, we decided to assess the stimulatory effect of amino acids on the kinase. To this purpose, cells were incubated in amino acid-free EBSS (Earle's Balanced Salt Solution), a condition that causes a rapid and severe depletion of the whole intracellular pool of amino acids and, in particular, of Gln and essential aminoacids. Upon EBSS incubation, mTOR activity is thus lowered to a basal level and the stimulatory effects of amino acid restoration can be assessed (Figure 6).

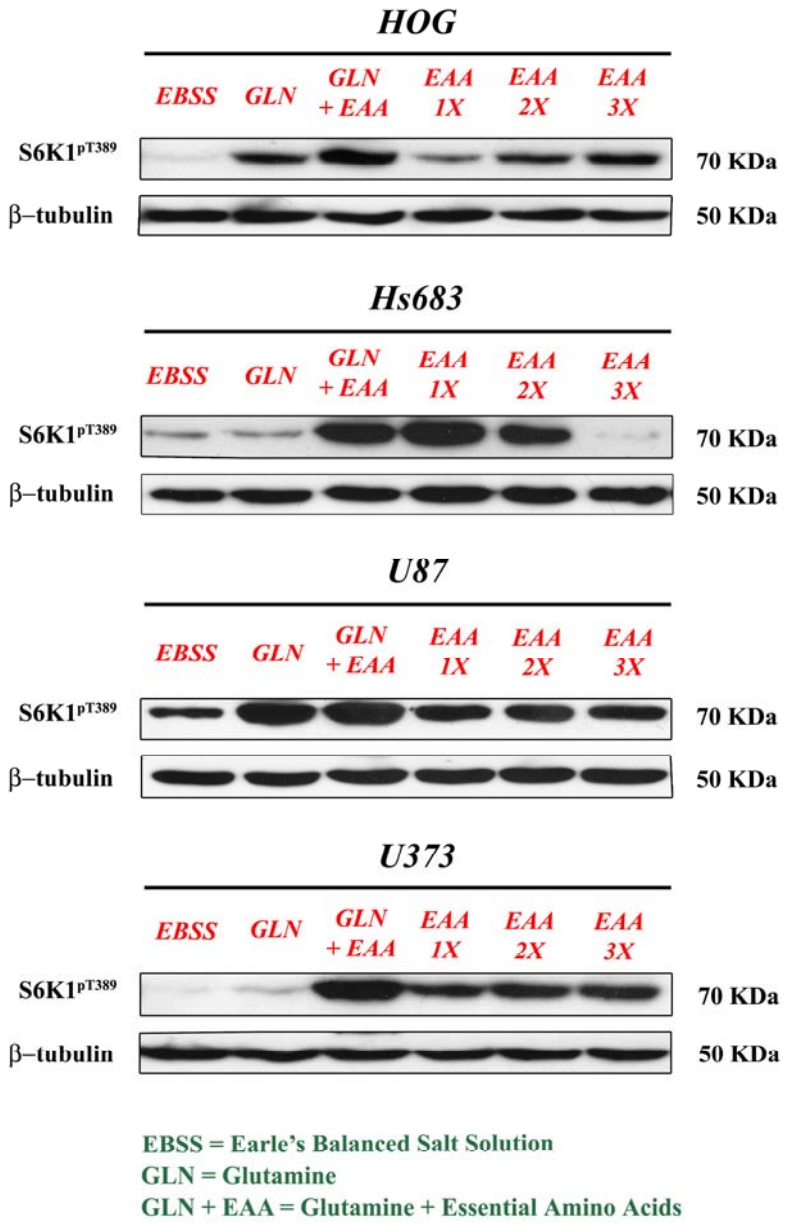


Figure 6. Cells (7 x 10^5/ well/ 2 ml) were seeded in 6-well plates. After 48h cells were incubated in EBSS (Earle's Balanced Salt Solution) for 3 hours and then incubated for 1h with Gln (2 mM), Gln (2 mM) + EAA (Essential Amino Acids, at the concentration present in the culture medium), EAA at the concentration present in the culture medium (EAA 1X), or at double (EAA 2X) or tripled concentration (EAA 3X). At the end of the treatments, proteins were extracted and mTOR activity was evaluated through the expression of the phosphorylated form of the substrate S6K1 with Western Blot analysis (see Materials and Methods) using β-tubulin for loading control. The experiment was performed twice with comparable results.

In all the four cell lines the restitution of Gln and Essential Amino Acids (EAA, including 1 mM leucine) to the EBSS dramatically increased mTOR activity compared to the levels observed in unsupplemented EBSS.

Conversely, the supplementation of Gln or EAA (used at the same concentration present in the growth medium or at double or triple concentration) highlighted a markedly different behavior of the cells.

HOG cells showed a marked recovery of kinase activity upon Gln and, to a lesser extent, with EAA 1X. Moreover the S6 phosphorylation increased with the increase of EAA concentration.

The maximal stimulation of mTOR was observed when both Gln and EAA were restored although, under this condition, the intracellular concentration of leucine is expected to be lower than that achieved with 3X EAA (see Tardito *c)* et al., 2012). The conclusion suggested is clear: Gln and EAA (leucine) provide a synergistic stimulation of mTOR activity in HOG cells.

The results are quite different for Hs683 cells. In this model, Gln addition had no effect on the recovery of the kinase activity, confirming that mTOR is Gln-insensitive in Hs683. Conversely the kinase turned maximally on when EAA were added to the incubation medium at physiological concentrations (EAA 1X). Interestingly, at variance with what observed for the other cell lines, the maximal rescue of mTOR activity was observed with EAA 1X addition. Gln addition to EAA did not further increase mTOR activity. Thus, in order to stimulate mTOR in Hs683 cells, leucine does not require Gln (another argument that contradicts the Nicklin model). Surprisingly, mTOR activity progressively decreased at EAA 2X and, even further, at EAA 3X. A possible explanation for the paradoxical decrease of $S6K1^{pT389}$ expression with the increase of EAA extracellular concentration could be the triggering of feed-back mechanisms, aimed at avoiding an overstimulation of mTOR. However, it is clear that the regulation of mTOR activity in Hs683 exhibits peculiar features.

The two glioblastoma cell lines exhibited a behavior similar to what observed with HOG cells, although with some difference. In both lines, Gln restores (partially in U373 cells, very markedly in the U87 cell line) mTOR activity. In U87 cells, in fact, the sole addition of Gln was sufficient to rescue mTOR activity at levels substantially comparable to those observed with Gln + EAA, while EAA markedly restored kinase activity but at clearly submaximal levels. Moreover, in these cells there was no evident dose-dependency in the EAA effect. In U373, instead, S6 phosphorylation was more evident upon the addition of EAA rather than Gln, even if the maximal, synergistic effect was detected with the combined treatment of Gln + EAA. Gln per se had only a minor stimulatory effect.

In conclusion, each cell model exhibits different modes of amino acid dependent stimulation of mTOR activity, suggesting the existence of multiple pathways that connect nutrient

availability with the kinase function and, hence, the regulation of protein synthesis. While Gln is able to stimulate mTOR activity in three of four cell lines, EAA are mTOR activators in all the cell models. Thus, these data are consistent with the hypothesis that Gln and EAA stimulate mTOR through different and distinct pathways. Hs683 behavior appears unique and deserves, therefore, further considerations.

4.5. Hs683 cells are more sensitive to rapamycin than HOG cells and glioblastoma cell lines

The unexpected maintenance of mTOR activity upon ASNase treatment, observed in Hs683 cells, indicates that kinase activity is not sensitive to Gln depletion and that, in these cells, it is constitutively activated. Bar-Peled et al. (Bar Peled et al., 2013, see the Introduction) have demonstrated the presence of two complexes upstream of mTOR, named GATOR 1 and GATOR 2, which interact with the G-proteins RAG and activate mTOR in presence of amino acids. Thus, cancer cells with mutations at the level of GATOR 1 complex show a constitutive mTOR activation, even during amino acid starvation, and are hypersensitive to the mTORC1 inhibitor rapamycin (IC_{50} between 0.1 and 0.4 nM).

To ascertain if Hs683 cells would behave as GATOR 1 mutated cells, the sensitivity to rapamycin of these cells was compared to HOG cells and the glioblastoma cell lines U87 and U373 (Figure 7).

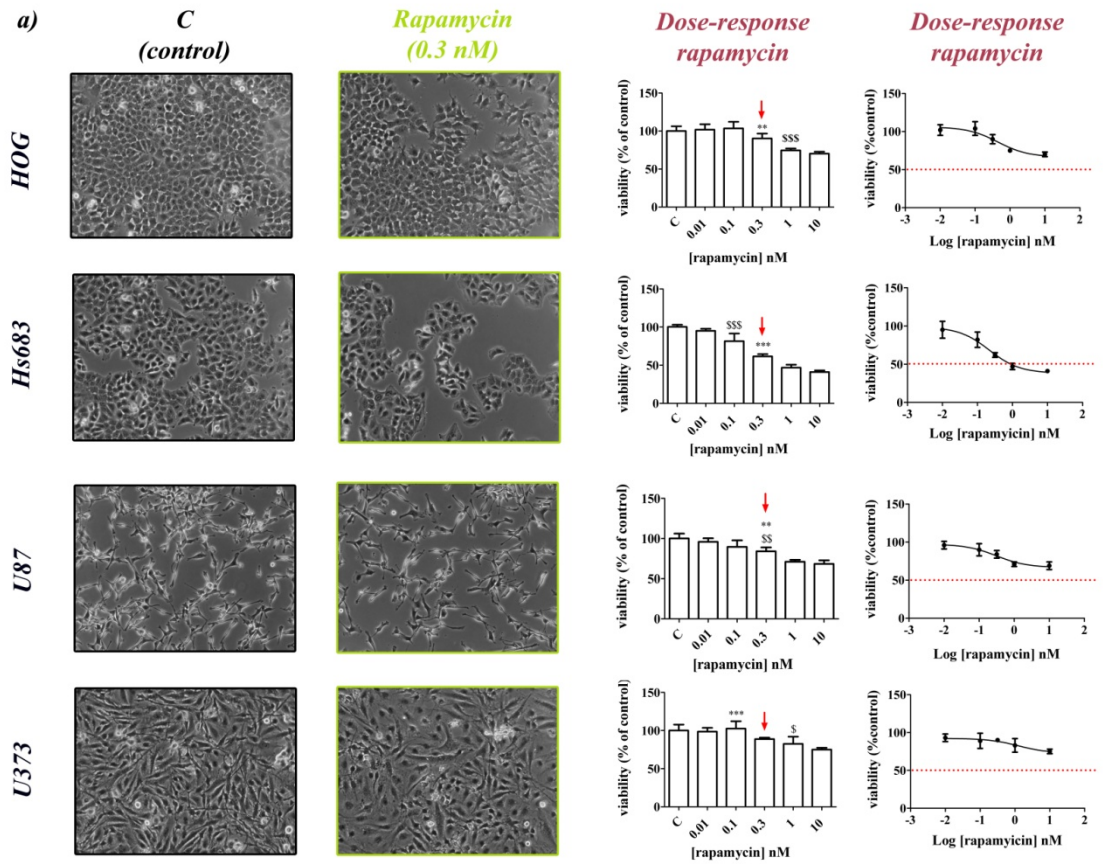


Figure 7. Sensitivity to rapamycin of oligodendroglioma and glioblastoma cells. Cells (2 x 10^4 /well/500 µl for HOG, 2.3 x 10^4 /well/500 µl for Hs683 and U87 and 2.5 x 10^4 well/500 µl for U373) were seeded in 24-well plates. After 24h, cells were incubated for 48 h with fresh medium containing different rapamycin concentrations (from 0.01 nM to 10 nM, see Materials and Methods). Since rapamycin is diluted in Dimethyl Sulfoxide (DMSO), this compound was also added in control cell incubation medium at the maximal concentration. At the end of the treatments, control cultures and cultures incubated with 0.3 nM rapamycin were observed at the microscope (left), while viability was.evaluated with the resazurin test throughout the whole range of rapamycin doses tested (right, see Materials and Methods). On dose-response graphics the red arrows indicate the cell viability change at 0.3 nM rapamycin in all the four cell lines. Data are means ± S.D. of four independent determinations in an experiment performed twice with comparable results.
Statistical analysis was performed with ANOVA followed by the Bonferroni post hoc test; * $p<0.05$ ** $p< 0.01$ *** $p< 0.001$ versus the highest rapamycin concentration; $ $p<0.05$ $$ $p< 0.01$ $$$ $p< 0.001$ versus control cells ([rapamycin] 0 mM). For each dose-response the half-maximal inhibitory concentration (IC_{50}).was calculated with the GraphPad Prism5™ software.

Hs683 showed an higher sensitivity to rapamycin than HOG cells and glioblastoma lines. This is evident both at the highest rapamycin concentration (10 nM), when cell viability was lowered to 41% of control for Hs683 cells, (70 % for HOG cells, 69% for U87 and 75 % for U373 cells) and also at 0.3 nM, when Hs683 viability was 62 % of control, compared to 90% of HOG cells, 84 % of U87 and 90% of U373. This effect was also evident at the microscopic observation. It was possible to calculate IC_{50} (0.91 nM) only for Hs683 cells, while in the

other three cell lines 50% inhibitory effect on cell viability was not reached at the maximal concentration used (IC_{50} > 10 nM).

Although rapamycin at 0.1 nM had only a limited effect on cell viability in Hs683 cells (82 % of control), statistical analysis showed that the difference in viability between cells treated with this concentration and control cells (no rapamycin) was highly significant ($p < 0.001$), while it was not significant for the other cell lines that needed much higher rapamycin doses to exhibit a significant viability decrease. A significant decrease of viability was obtained at 1 nM rapamycin for HOG cells, 0.3 nM for U87 cells and 1 nM for U373 cells. These data indicate that Hs683 cells have a mTOR-dependent phenotype. Rapamycin sensitivity was also tested in all the four cell lines evaluating mTOR activity in response to the treatment with the inhibitor at 0.3 nM and 10 nM (Figure 8).

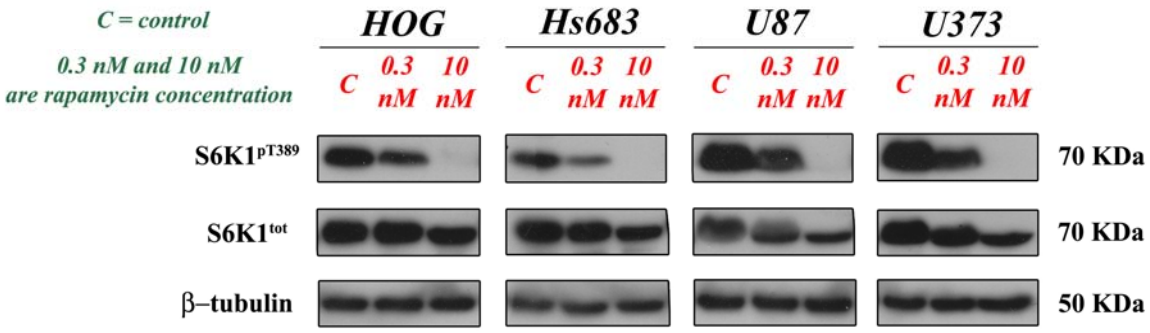


Figure 8. Effect of rapamycin on mTOR activity in oligodendroglioma and glioblastoma cell lines.
Cells (3.5 x 10^5/well/1.6 ml for HOG, 3.7 x 10^5/well/1.6 ml for Hs683 and U87 and 4 x 10^5/well/1.6 ml for U373) were seeded in 6-well plates. After 48 h cells were treated with rapamycin (0.3 nM or 10 nM) for 6 hours. Vehicle (DMSO) was added to control medium at the maximal concentration used to dilute rapamycin stock solution. At the end of the treatments, proteins were extracted and mTOR activity was evaluated through the phosphorylated form of the substrate S6K1 with Western Blot analysis (see Materials and Methods) using β-tubulin for loading control. The experiment was performed twice with comparable results.

In all the four lines mTOR kinase activity was completely suppressed at the highest concentration of rapamycin used (10 nM, Figure 8). Also the limiting concentration used in cell viability experiment (0.3 nM, see above) caused a decrease of the phosphorylation of S6K1 in all the four cell lines. To compare the effective inhibition of mTOR activity by 0.3 nM rapamycin among the four cell lines, it is necessary to take into account the variability observed in the expression of total S6K1 and, even more evidently, in the abundance of phosphorylated S6K1 detected under control conditions.

The expression of the total form of the kinase was comparable in HOG, Hs683 and U373 cells and lower in U87 cells. Moreover, S6K1 levels were dose-dependently lowered upon the treatment with rapamycin (U87 >> U373 > Hs683 > HOG).

The aliquot of S6K1 kinase phosphorylated by mTOR can be estimated from the ratio between pS6K1 and tot S6K1. This ratio (Fig. 9a) is more than 4 for U87 cells and less than 1 for Hs683 cells, with U373 and HOG cells showing intermediate levels. Given the different primary antibodies used for the two forms, it is obviously not possible to reach an absolute quantification of phosphorylated S6K1. However, this ratio is useful to calculate the extent of inhibition at 0.3 nM rapamycin. Under this condition, the ratio pS6K1/totS6K1 becomes 0.5, .3, 2, 0.5 for, respectively HOG, Hs683, U87 and U373 cells. This allows the calculation of the percentage inhibition of mTOR activity at 0.3 nM rapamycin reported in Figure 9b corresponding to 49% for HOG, 64% for Hs683, 59% for U87 and 53% for U373 cells.

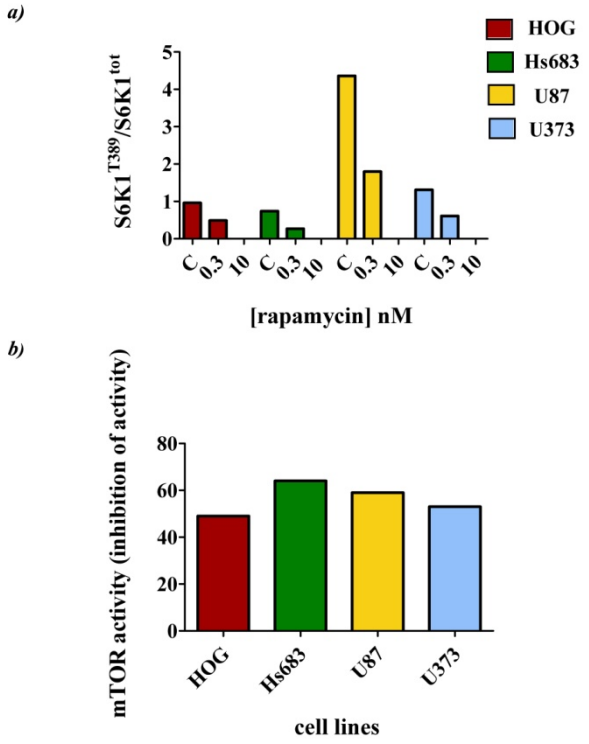


Figure 9. Densitometric analysis of expression of S6K1^{pT389}/S6K1^{tot} and 0.3 nM/C in oligodendroglioma and glioblastoma cells. The Figure reports the results of the densitometric analysis of the Western Blot reported in Figure 8. See text for explanation.

These results support the hypothesis that Hs683 cells express a mTOR variant more sensitive to rapamycin than the other cell lines. Moreover, they allow a further interpretation of the data presented in Figure 8. The very significant difference between 10 nM rapamycin (a dose at which mTOR is completely inhibited) and 0.3 nM rapamycin, detected in Hs683, HOG and U87 cells, indicated that at 0.3 nM rapamycin the residual mTOR activity is still able to give a significant stimulus to proliferation in these lines. Given the stronger inhibition at 0.3 nM observed for Hs683 compared to other cells and the highly significant difference, it is possible to hypothesize that these cells are indeed the most dependent on mTOR activity for their proliferation. Conversely, the difference in viability between 0.3 and 10 nM rapamycin is not significant for U373, indicating that the residual activity is not able to exert an appreciable proliferative stimulus, making these cells the least dependent on mTOR for their proliferation. Interestingly, it is known that Hs683 cells have a mutation in the *MTOR* gene which results in the substitution of a residue of Glu1799 with a residue of Lysine (Lys) (COSMIC; Catalogue of somatic mutations in cancer), belonging to the FAT domain of the protein. Analyzing some mutants of mTOR it has been demonstrated that this mutation increases S6K1 phosphorylation diminishing the binding of mTOR to its endogenous inhibitor Deptor (which

binds the FAT domain) confering, thus, an hyperactivation of the protein (Grabiner et al. 2014). Moreover other cell lines carrying this mutation (like the endometrial cancer cells HEC59 and the kidney cancer cells SNU349) show hypersensitivity to rapamycin both *in vitro* and *in vivo* (this result observed only for the HEC59 line). These assumptions could be a good explanation for the behavior observed in Hs683 cells, which, thus, conform to a mTOR dependent phenotype.

Moreover, the results obtained in response to rapamycin treatment and to glutamine depletion demonstrate that oligodendroglioma cell lines, HOG and Hs683, show a different regulation of mTOR activity, although they have both a very high sensitivity to Gln starvation in terms of decrease in cell viability. Indeed, in Hs683 cells, Gln is not implicated in the regulation of the kinase function, while its presence or absence causes the "turning on" or the "turning off" in HOG cells. Thus, in conclusion, Hs683 and HOG cells show a different regulation of the mTOR pathway and possible repression of mTOR activity should be excluded as a mechanism underlying the effects of Gln depletion on oligodendroglioma cell viability.

4.6. GLUL transfection does not protect oligodendroglioma cells from Gln depletion

It is tempting to attribute the peculiar Gln-dependence of oligodendroglioma cells to their very low expression of Glutamine Synthetase (see above).

To obtain a definite demonstration of this hypothesis, a transient transfection experiment with *GLUL* gene has been performed to test if GS expression may protect oligodendroglioma cells from the effects of Gln starvation.

The effects of the transient transfection of the *GLUL* gene on GS expression are reported in Figure 10. In this t experiment the negative control was represented by the unrelated vector pRL-TK (Renilla Luciferase).

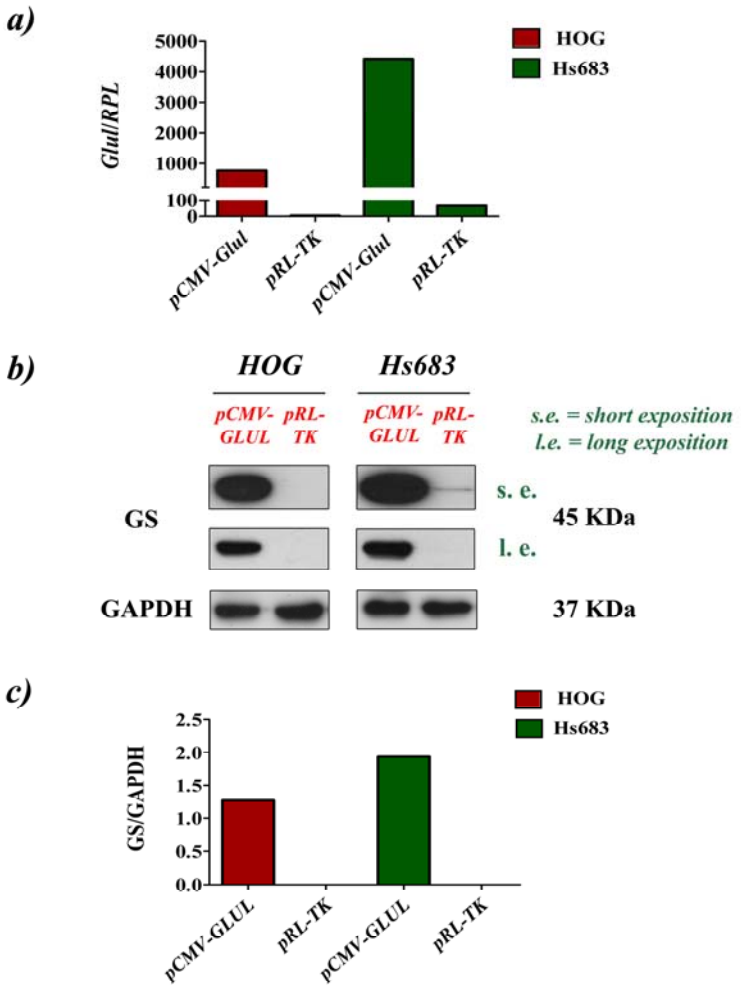


Figure 10. GS expression in transiently transfected oligodendroglioma cell lines. *a)* Cells (1.5 x 10^4/well/500 µl for HOG and 1.8 x 10^4/well/500 µl for Hs683) were seeded in 24-well plates. After 24 h cells were transfected in medium without FBS (Fetal Bovine Serum) and antibiotics (see Materials and Methods) supplemented with 600 ng/ml of pCMV-*GLUL* or of pRL-TK (Renilla Luciferase) as a negative control. After 24h, the transfection medium was replaced with fresh complete medium. After further 24h, total RNA was

extracted, reverse transcribed to cDNA, and amplified through RT-PCR (see Materials and Methods). *GLUL* expression is expressed relatively to the expression of the *RPL-15* housekeeping gene.
b) Cells (1.5 x 10^4/well/500 µl for HOG and 1.8 x 10^4/well/500 µl for Hs683) were seeded and transfected as in *a)*. After 72 h from transfection cells were lysed. Proteins were extracted, and GS expression was evaluated with Western Blot analysis (see Materials and Methods) using GAPDH as a loading control. s.e., short exposure (30 s); l.e., long exposure (10 min).
c) Densitometric analysis of the blot presented in b) long exposure

The transient transfection with pCMV-*GLUL* induced a marked expression of the *GLUL* gene in both HOG and Hs683 cell lines. In particular, Hs683 showed a *GLUL* mRNA expression four times higher than HOG cells (4413 and 759 Arbitrary Units, respectively); a small expression was also appreciable in their negative control (transfection with the unrelated gene pRL-TK Renilla Luciferase). The same trend was observed also for GS protein expression (Figure 10 *b*). Indeed, GS was markedly expressed in both the transfected cell lines and, even in this case, Hs683 cells showed an higher GS expression than HOG cells, although with a smaller difference with respect to *GLUL* mRNA expression. Also in this case, a barely detectable GS expression was appreciable in Hs683 cells transfected with the empty vector.
To quantify the difference between GS expression in HOG and Hs683 cell lines a densitometric analysis was effectuated relating GS with the housekeeping protein GAPDH (Figure 10 *c*). The ratio between GS and GAPDH was higher in Hs683 than in HOG cells (1.94 and 1.27 respectively).
The effect of Gln starvation on the transfected cell lines is shown in Figure 11.

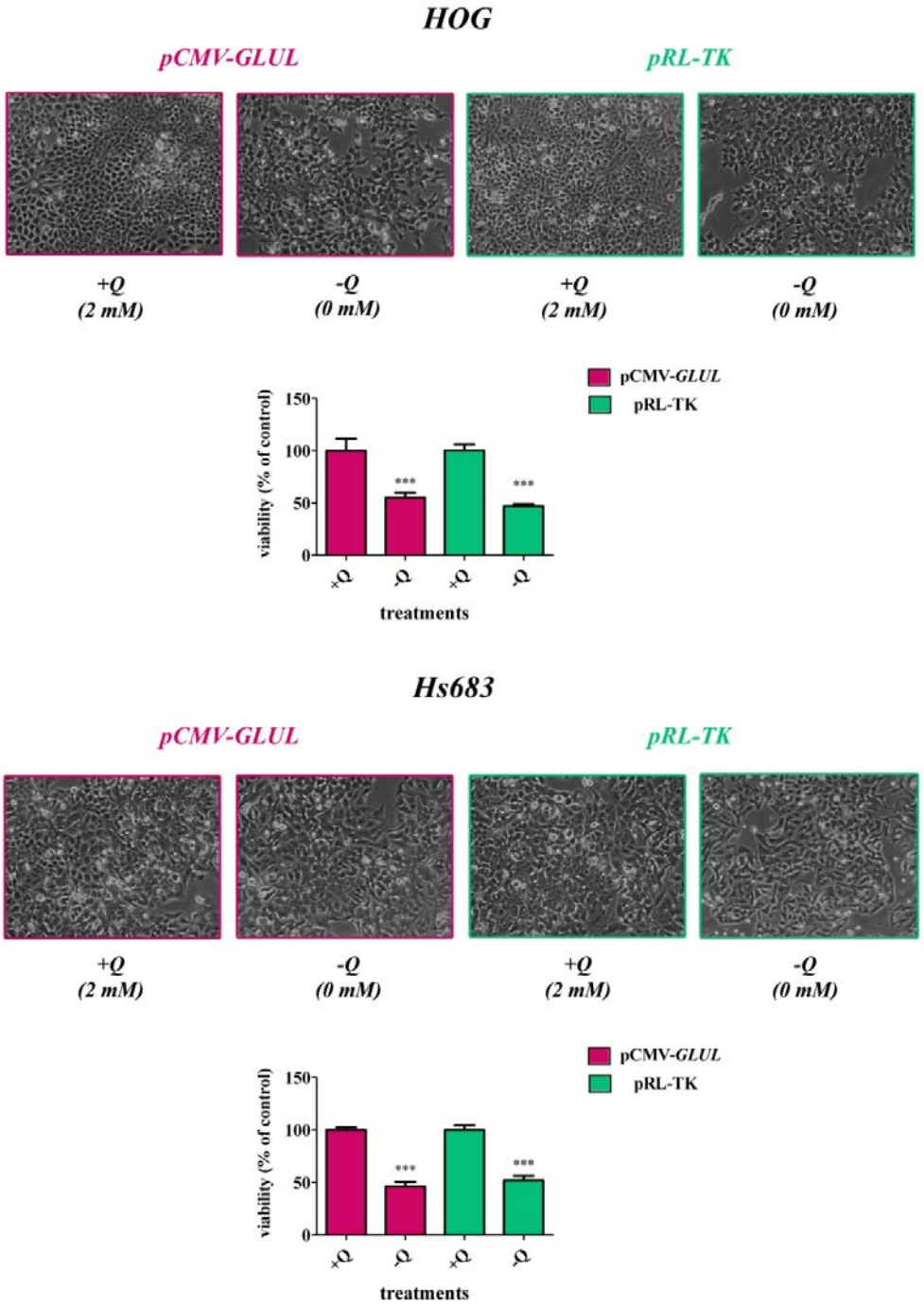


Figure 11. Effect of Gln starvation in *GLUL* transfected oligodendroglioma cell lines. *a)* Cells (1.5 x 10^4/well/500 µl for HOG and 1.8 x 10^4/well/500 µl for Hs683) were seeded in 24-well plates. After 24h, cells were transfected with pCMV-*GLUL* or pRL-TK (Renilla Luciferase) as a negative control (600 ng/ml) in medium without FBS (Fetal Bovine Serum) and antibiotics (see Materials and Methods). After 24h, transfection medium was replaced by fresh complete medium. After 48h, cells were incubated with fresh medium (at 5% of FBS) at "nominally" Gln 0 mM or 2 mM (see Materials and Methods) for 48 h. At the end of the treatments, cultures were observed (x100) and cell viability was evaluated with the resazurin test (see Materials and Methods). Data are means ± S.D. of three independent determinations. Statistical analysis was performed with ANOVA followed by the Bonferroni post hoc test (*** $p < 0.001$).

GLUL transfection did not significantly mitigate the sensitivity to Gln depletion in both HOG and Hs683 cells. In particular, in HOG cells the treatment at 0 mM Gln caused a viability reduction of 44% for cells transfected with pCMV-*GLUL* and of 53% for the respective negative control (cells transfected with pRL-TK), while in Hs683 cells a viability decreases of 54% and 48% (respectively for pCMV-*GLUL* and pRL-TK transfected cells) were observed. This was evident also at the microscopic observation, where, moreover, a change in the morphology of HOG cells was also noted, probably due to the transfection process. These results suggest that the abundant GS expression obtained through *GLUL* transfection does not protect the cells from Gln depletion.

Trying to find an explanation to this behavior, another transfection experiment was performed in both cell lines (Figures 13 and 14). In these experiments, Glu (2mM) was added to the medium during Gln depletion so as to increase the availability of substrate for glutamine synthesis through GS. Moreover, in these experiments the same vector used for *GLUL* transfection was used in negative controls. Figure 12 reports GS expression in cells transfected with either pCMV-*GLUL* or pCMV.

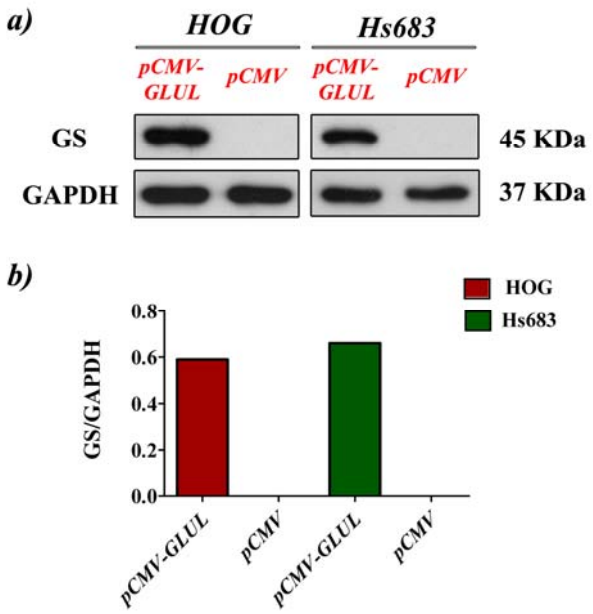


Figure 12. GS expression in transiently transfected oligodendroglioma cell lines. *a)* Cells (1 x 10^4/well/500 µl for HOG and 1.3 x 10^4/well/500 µl for Hs683) were seeded in 24-well plates. After 24h, cells were transfected with pCMV-*GLUL* or pCMV as a negative control (200 ng/ml) in medium without serum and antibiotics (see Materials and Methods). After 24 h transfection medium was changed with fresh complete medium. After 72h from transfection, cells were lysed. Proteins were extracted and GS expression was evaluated with Western Blot analysis (see Materials and Methods) using GAPDH as loading control. *b)* Densitometric analysis of expression of GS/GAPDH in transiently transfected oligodendroglioma cells.

Also in this experiment, although a lower amount of GLUL vector was used, a marked GS expression was observed in both pCMV-*GLUL* transfected oligodendroglioma cell lines, with a slightly higher protein abundance in Hs683 than in HOG cells (densitometric analysis, Figure 12 *b*). GS protein expression was not detectable in the negative controls transfected with pCMV.

Viability and GS expression observed in transfected cells treated with Gln 0 mM or 2 mM and/ or Glu 0 mM or 2 mM are reported in Figure 13 and Figure 14.

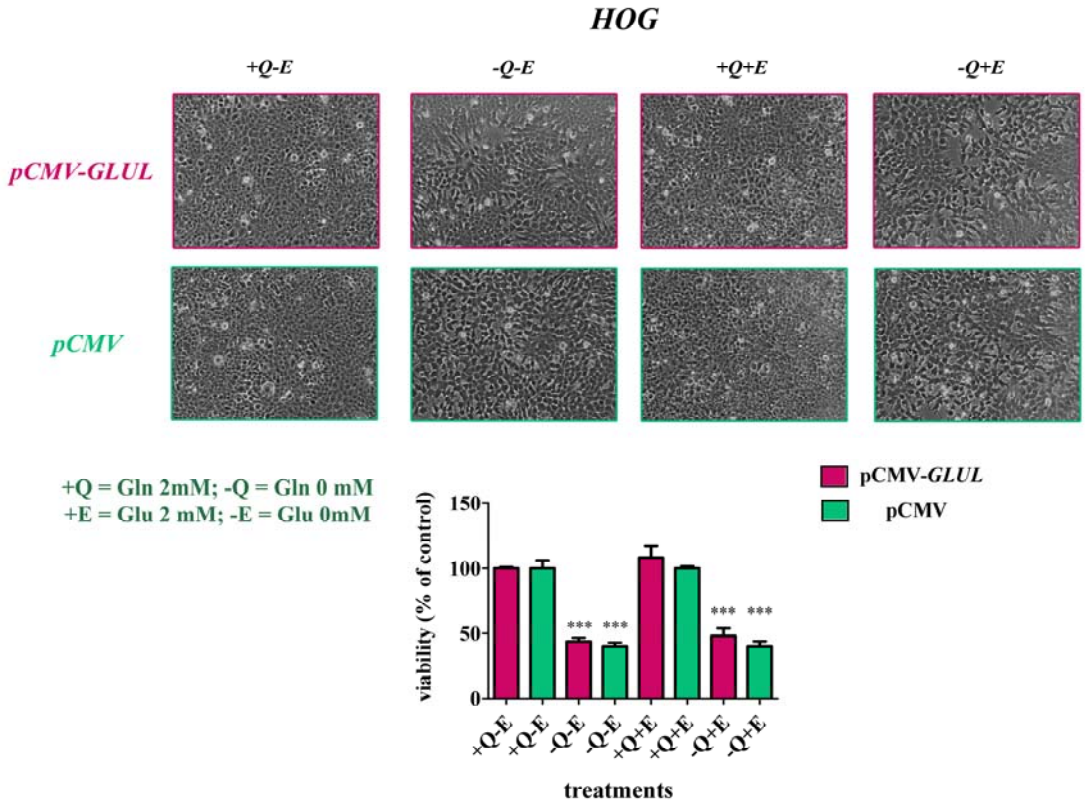


Figure 13. Effect of Gln starvation and Glu supplementation in transiently *GLUL*-transfected HOG cells. *a)* Cells (1.3 x 10^4/well/500 µl) were seeded in 24-well plates. After 24h, cells were transfected with pCMV-*GLUL* or pCMV as a negative control (200 ng/ml) in medium without FBS (Fetal Bovine Serum) and antibiotics (see Materials and Methods). After 24h, transfection medium was replaced by fresh complete medium. After 48h, cells were incubated with fresh medium (at 5% of FBS) at "nominally" 0 mM or 2 mM Gln with or without Glu (2mM) for 48 h (see Materials and Methods). At the end of the treatments, cultures were observed at contrast phase microscope (x100) and cell viability was evaluated with the resazurin test (see Materials and Methods). Data are means ± S.D. of three independent determinations. Statistical analysis was performed with ANOVA followed by the Bonferroni post hoc test (*** $p < 0.001$).

Even in these experiments, *GLUL* transient transfection did not protect HOG cells from Gln starvation. Indeed, the difference between the sensitivity of pCMV-*GLUL* transfected cells,

incubated in Gln free conditions, and their negative control (pCMV) was not significative with a drop of viability of 48% and 55% respectively. Moreover, also the addition of the GS substrate (Glu 2mM; E) was not able to increase cell viability upon Gln depletion (49% and 52% of viability for pCMV-GLUL and pCMV respectively). This was evident also at the microscopic observation where a change in the morphology of the cells was noted again.

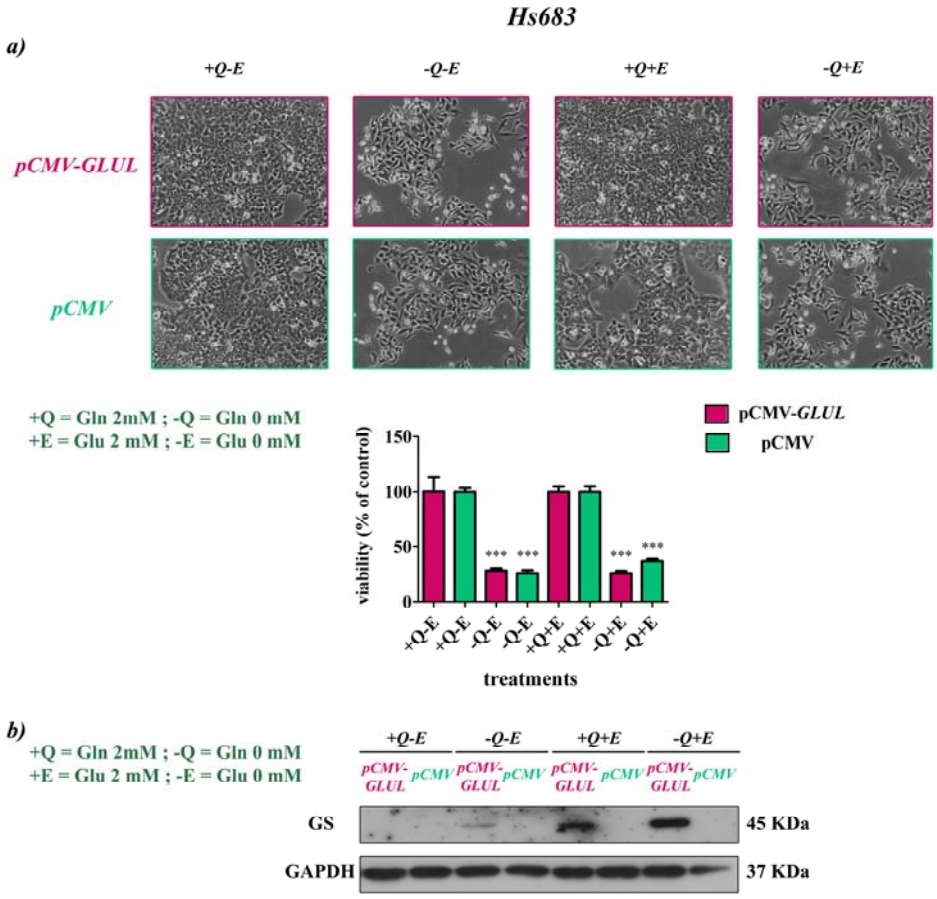


Figure 14. Effect of Gln starvation and Glu supplementation in transiently *GLUL*-transfected Hs683 cells. *a)* Cells (1.3 x 10^4/well/500 µl) were seeded in 24-well plates. After 24h, cells were transfected with pCMV-*GLUL* or pCMV as a negative control (200 ng/ml) in medium without FBS (Fetal Bovine Serum) and antibiotics (see Materials and Methods). After 24h, transfection medium was replaced by fresh complete medium. After 48h, cells were incubated with fresh medium (at 5% of FBS) at "nominally" 0 mM or 2 mM Gln with or without Glu (2mM) for 48 h (see Materials and Methods). At the end of the treatments, cultures were observed at contrast phase microscope (x100) and cell viability was evaluated with the resazurin test (see Materials and Methods). Data are means ± S.D. of three independent determinations. Statistical analysis was performed with ANOVA followed by the Bonferroni post hoc test (*** $p < 0.001$).

b) Cells (1.3 x 10^4/well/500 µl) were seeded, transfected and treated as in *a)*. At the end of the treatments (96h from transfection) cells were lysed, proteins were extracted and GS expression was evaluated with Western Blot

analysis (see Materials and Methods) using GAPDH as loading control. The experiment was performed twice with comparable results.

Viability data, consistently with the morphological analysis, confirmed that there was no significant difference between the drop of viability observed in pCMV-*GLUL* transfected cells compared to their negative control (72% and 74% of viability loss, respectively) upon incubation in Gln-free conditions in the absence of Glu supplementation. This outcome was not substantially changed by Glu supplementation. Indeed, the addition of Glu was not able to rescue cell viability from the effects of Gln depletion with a viability decreased by 74% and 63% for pCMV-*GLUL* and pCMV transfected cells, respectively.

At the end of the experiment (96h from the transfection), we also checked GS expression in Western Blot analysis. GS protein was not appreciable under control conditions while it was detectable in cells incubated in the absence of Gln. This behavior can be explained considering that, prolonging the time of incubation, while the expression of the transfected gene decreases, the shelf life of the protein is sensitive to Gln levels, which obviously are higher in Gln-fed than in Gln-depleted cells. Interestingly, the addition of Glu 2mM markedly increased GS expression both in control (+Q+E) and in cells incubated at Gln 0 mM (-Q+E), which, however, still exhibited the highest levels. In conclusion, also in transfected cells GS protein expression is sensitive to Gln, thus indicating that the transduced protein is regulated similarly to the native protein. Moreover, these data would point to stabilizing effect of Glu (the substrate of GS) on the enzyme, a finding thus far not described.

Overall, the preliminary results obtained from transfection experiments indicate for both HOG and Hs683 cells that GS expression is not able to protect these lines from Gln depletion. This may mean that the lack of an appreciable enzyme activity is not the only reason of their high sensitivity to the amino acid deprivation. However, before reaching this conclusion, it would be necessary to perform at least two types of control:

1) the analysis of the intracellular pool of amino acids of the transfected cells, to verify if the GS expressed in pCMV-*GLUL* transfected cells produces a sizable intracellular glutamine pool;
2) The determination of GS activity in the cell extracts of transfected cultures, so as to ascertain that the transfected protein is functional.

4.7. The inhibition of Gln transporters decreases viability of oligodendroglioma cells.

The results presented above suggest that HOG and Hs683 oligodendroglioma cell lines must take up Gln from the extracellular environment to offset the lack of Glutamine Synthetase activity.

Over the years a lot of Gln transporters have been characterized, many of which belonging to the System A and System N SLC38 solute carrier families. These families are the major regulated Na^+ - dependent amino acid transport systems and include five transporters: SNAT1, SNAT2 and SNAT4, coded for by *SLC38A1*, *SLC38A2* and *SLC38A4* for System A transporters, and SNAT3 and SNAT5, coded for by *SLC38A3* and *SLC38A5* for System N transporters. SNAT is the acronym for Sodium-coupled Neutral Amino acid Transporters, which work exploiting the transmembrane gradient of Na^+ electrochemical potential formed by the Na^+/K^+ pump to take up Gln and other neutral amino acids like alanine, asparagine and histidine.(Mackenzie et al. 2004).

Other transporters that mediate Gln uptake are the Na^+- dependent ASCT2 transporter (coded for by *SLC1A5*), belonging to the ASC system, and ATB^{0+} (coded for by *SLC6A14*), belonging to the family of Na,Cl-dependent transporters. The first one is an obligatory neutral amino acid exchanger with Gln as the preferential substrate; the second one has a very wide range of substrates including 18 of the 20 proteinogenic amino acids and was initially identified in early embryonic models (Ganapathy, 2009).

The expression of *SLC38A1*, *SLC38A2*, *SLC38A4, SLC1A5* and *SLC6A14* mRNAs has been evaluated in oligodendroglioma and glioblastoma cells, using the hepatocellular carcinoma/hepatoblastoma HepG2 cells (a model in which several Gln transporters are operative) as a positive control (Figure 15).

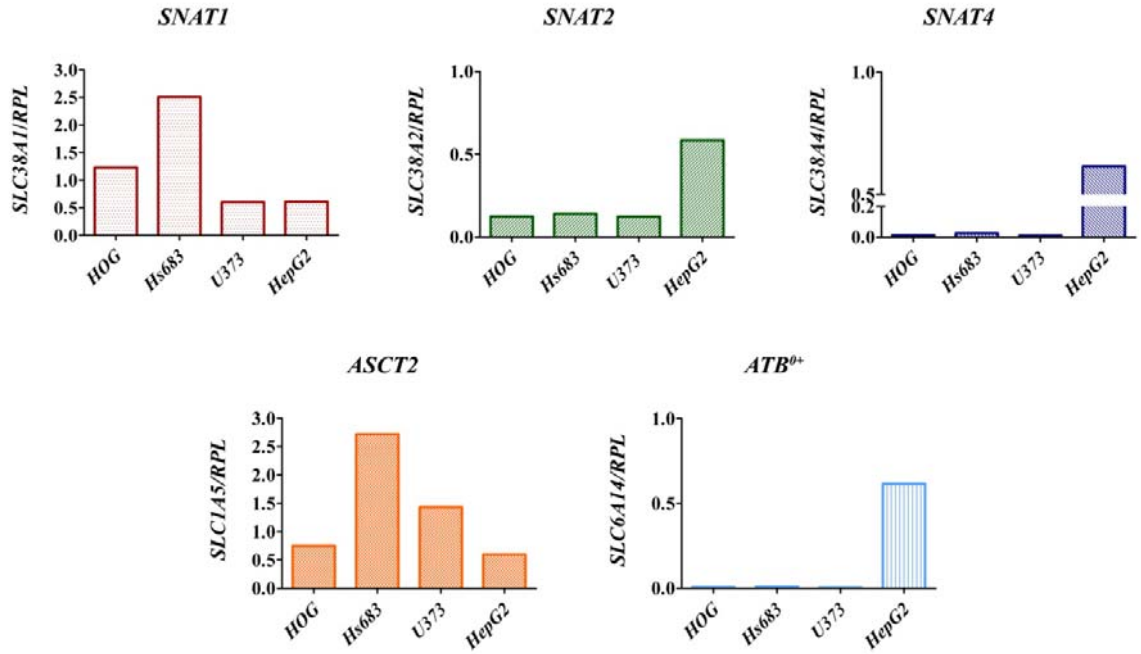


Figure 15. Expression of Gln transporters in oligodendroglioma and glioblastoma cell.

Cells (4 x 10^5/well/2 ml) were seeded in 6-well plates. After 24h total RNA was extracted, reverse transcribed to cDNA and amplified through RT-PCR (see Materials and Methods). Data are means of two determinations and gene expression is expressed relatively to the expression of the *RPL-15* housekeeping gene. HepG2 cells were used as a positive control.

Results obtained from RT-PCR demonstrated that oligodendroglioma cells HOG and Hs683 have a different profile expression of Gln transporters mRNAs, indicating that there is not an oligodendroglioma-specific amino acid transporter signature.

Interestingly, Hs683 showed the highest expression of *SLC38A1* and *SLC1A5* mRNAs, suggesting that these transporters are likely the major actors of Gln transport in this cell line. On the contrary, *SLC38A2*, *SLC38A4* and *SLC6A14* mRNAs exhibited a low expression, suggesting that they are not implied in Gln transport in this cell model.

Similarly, also HOG cells showed a comparably low expression of *SLC38A4, SLC38A2* and *SLC6A14*. However, their expression of *SLC38A1* was only second to that detected in Hs683 cells, while *SLC1A5* expression was lower than that recorded in Hs683 and U373 cells.

In conclusion, the transporter genes more expressed in oligodendroglioma than in the other cell models are those encoding for SNAT1 and (only for Hs683) ASCT2.

Actually, some of these results are not unexpected. Indeed, SNAT4 is the "hepatic" isoform of the SNAT transporter family (hence, its relative tissue-specific expression in liver-derived cells). Conversely, the expression of the SNAT1 isoform was originally described in brain tissue, while the SNAT2 carrier expression is substantially ubiquitous.

The hypothetic role of SNAT1 and ASCT2 as major Gln transporter in oligodendroglioma cells, has prompted us to verify their functional relevance in HOG and Hs683 cells.

For both transporters roughly specific inhibitors are available. Indeed, it has been known for many years that all the System A transporters are inhibited by 2-methylaminoisobutyric acid (MeAIB) that is considered a paradigm substrate for these carriers (Mackenzie et al. 2004).

It is relatively less widely acknowledged that the natural amino acid threonine (Thr) is a preferential substrate of system ASC transporters (Bussolati et al., 1991; Fuchs et al. 2005). Thus, high doses of Thr can be effectively used to reduce Gln uptake through ASCT2.

Thus, to try a functional assessment of SNAT1 and ASCT2 relevance for oligodebdroglioma cell growth, both transporters were blocked with high doses of MeAIB and Thr so as to lower Gln fuelling. In these experiments, medium Gln concentration was set at 0.6 mM (the physiological concentration). The results obtained are reported in Figure 16.

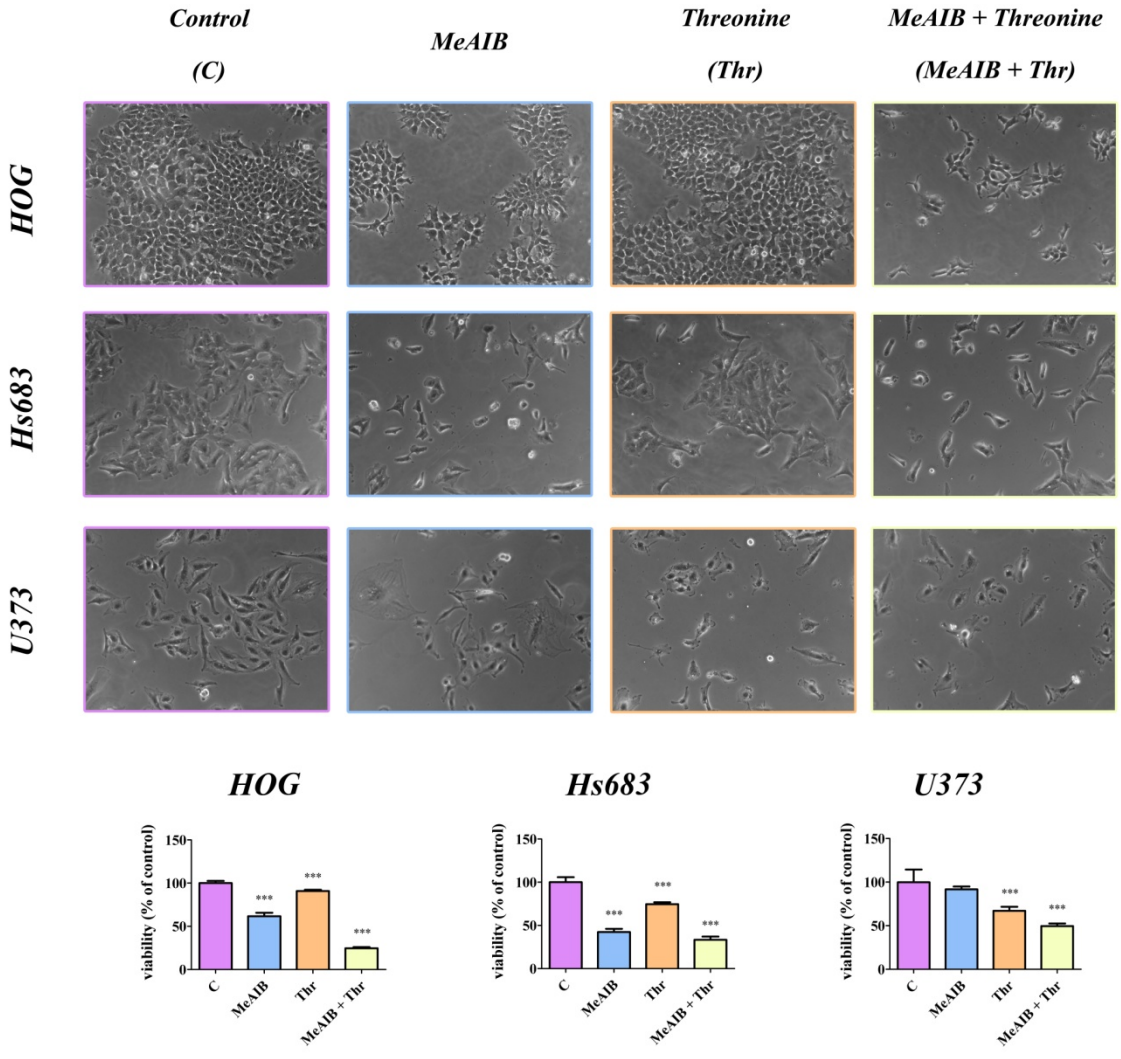


Figure 16. Effect of MeAIB and Thr supplementation on oligodedroglioma and glioblastoma cells.
Cells (5 x 10^3/well/ 500 µl) were seeded in 24-well plates. After 24h cells were incubated for 72h with fresh medium containing MeAIB (20 mM), Thr (10 mM) or MeAIB + Thr at the same concentrations (20 mM + 10 mM). During the treatments Gln was used at 0.6 mM. Sucrose was used to balance the difference of osmolarity. (see Materials and Methods). At the end of the treatments, cultures were observed at the microscope and viability was evaluated with the resazurin test (see Materials and Methods). Data are means ± S.D. of six independent determinations of an experiment performed twice with comparable results.

In all the three cells the addition of MeAIB and Thr had marked effects on cell viability. When both the inhibitors were added, cell viability was inhibited by 75% in HOG cells, 67% in Hs683 cells and by 50% in U373 cells.

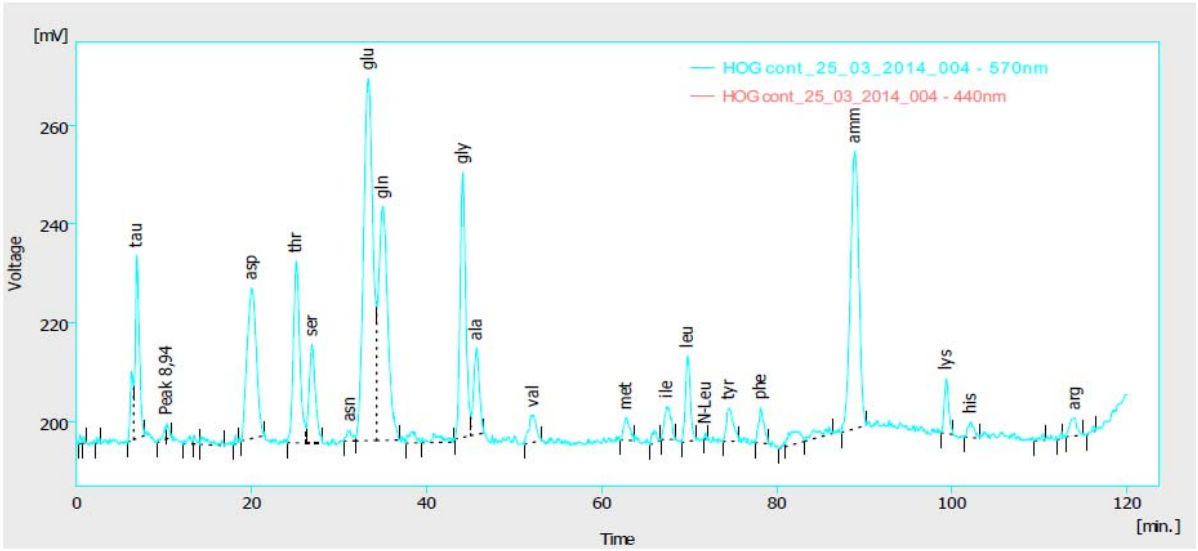
The contribution of MeAIB and Thr to these effects was markedly different in the three cell models. In particular MeAIB treatment significantly lowered viability of HOG cells (39%) and Hs683 cells (58%) cells, but did not significantly affect U373 viability (-9%). The effect of Thr was much smaller in HOG (- 9%) and Hs683 cells (- 25%), but larger in U373 cells (- 33%).

Thus, it is possible to conclude that oligodendroglioma cells are more sensitive to MeAIB (SNAT1 substrate) than to Thr (ASCT2 substrate), while the opposite is true for U373 cells. However, all the three lines are markedly affected by the combined treatment, although U373 are less sensitive than the other cell lines. This behavior is likely dependent on the GS expression exhibited by the latter cell model.

Interestingly, the behavior observed in all the three cell lines with the two single treatments roughly reflected the rank of mRNA expression profiles of the transporters noted in RT-PCR; in fact the major expression of SNAT1 in oligodendroglioma cell lines, and in particular in Hs683, was in line with the greater effect of MeAIB in these cells.

Supplementary Results

S1



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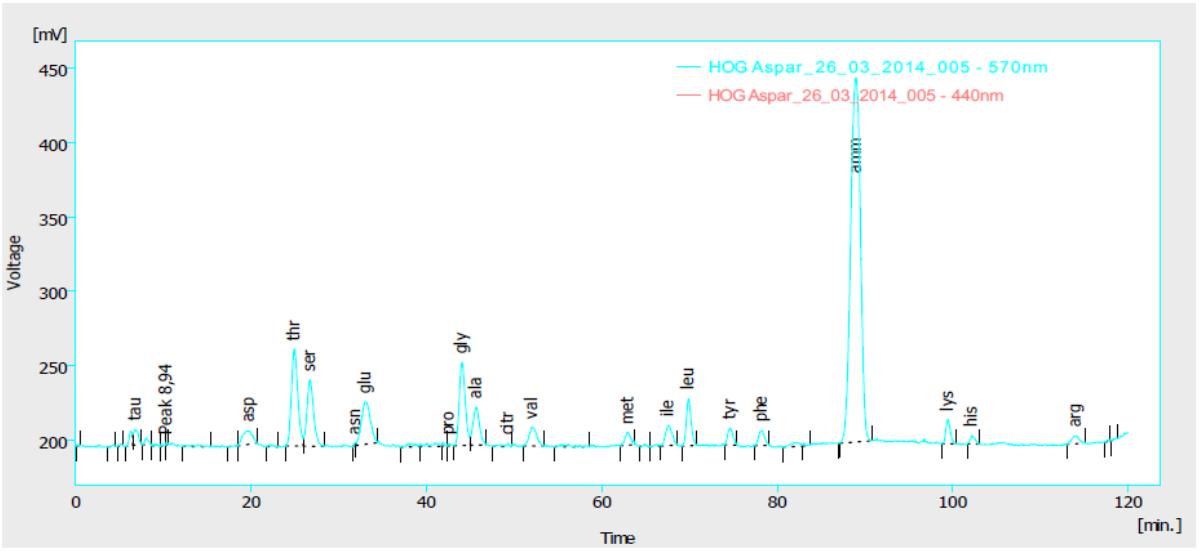

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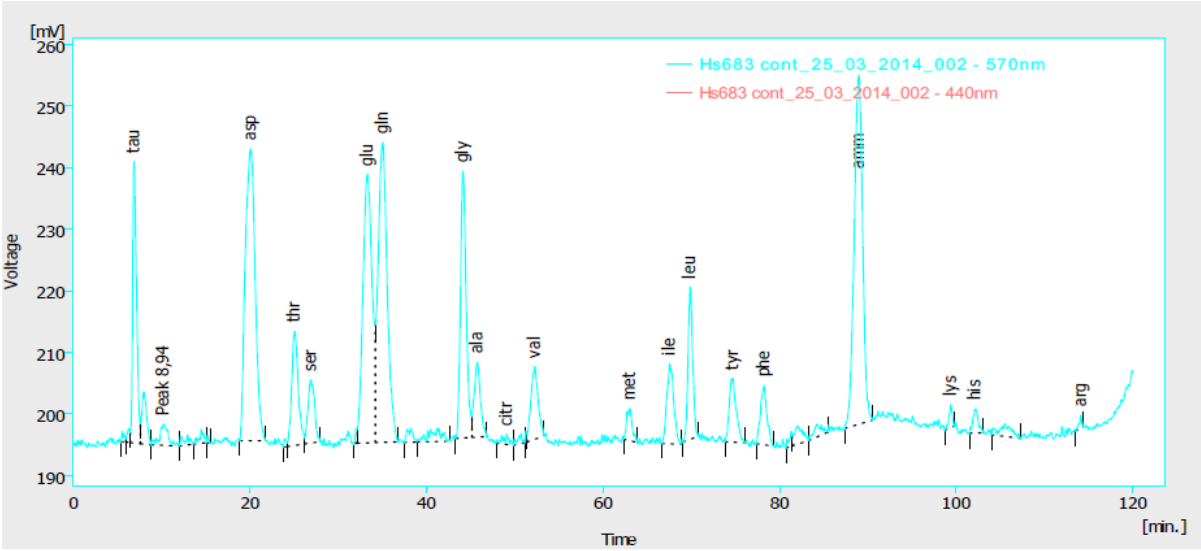
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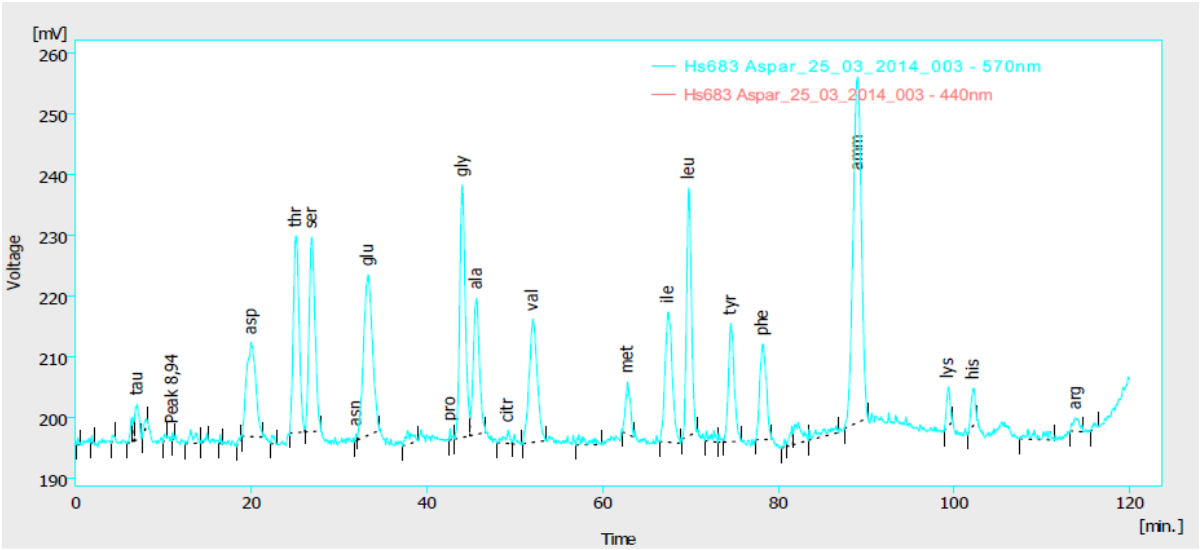
S4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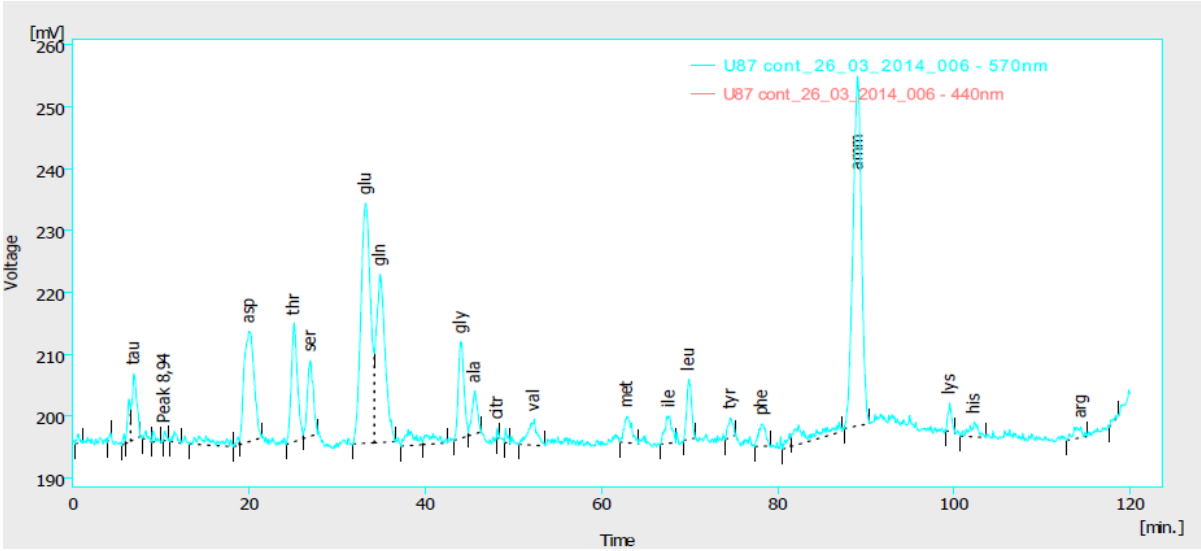
S5



S6

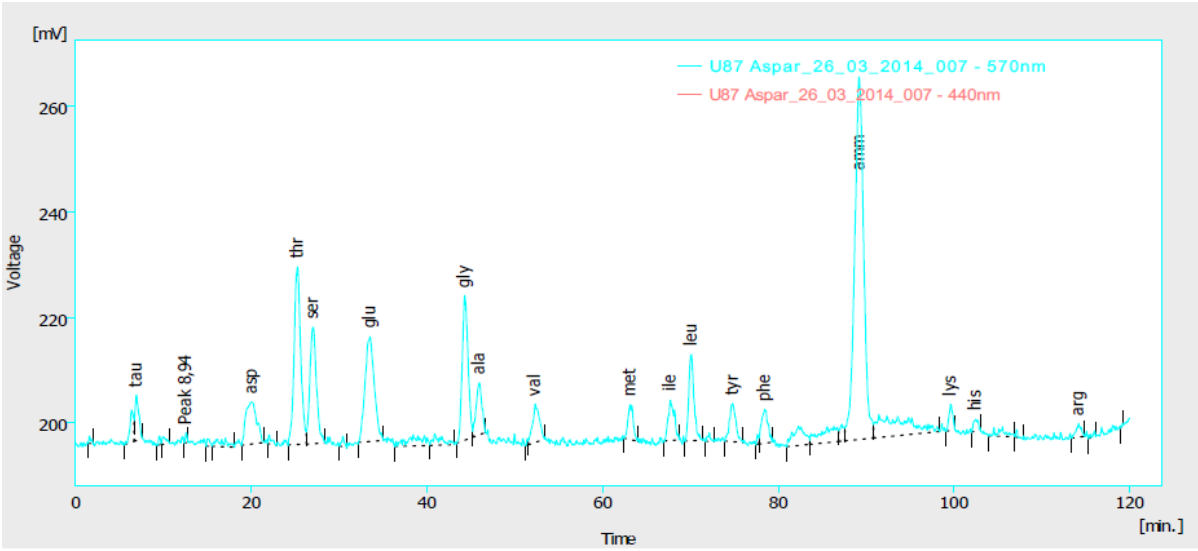


Figure S1-S6. Chromatograms of amino acid profiles of oligodendroglioma and glioblastoma cell lines.

5. Conclusions

In this study the effects of extracellular Gln deprivation were tested in two human "GS-negative" oligodendroglioma cell lines, HOG and Hs683 compared to two "GS-positive" glioblastoma cell lines, U87 and U373.

The results obtained indicate that HOG and Hs683 cells are extremely sensitive to Gln depletion, both when incubated under Gln-free conditions and when treated with the asparaginolytic and glutaminolytic drug asparaginase (ASNase). In particular ASNase treatment affects the viability of the cells in a dose and time-dependent manner eventually leading to the complete loss of cell viability. Consistently with the lack of GS expression, the addition of the GS inhibitor methionine-L-sulfoximine (MSO) does not synergize ASNase effects.

Conversely, the incubation in Gln depleted medium of the two GS-positive glioblastoma cell lines, U87 and U373, causes a less marked decrease of cell viability, evident also with ASNase treatment. Moreover, these cells adapt to the antitumor enzyme since a recovery of the cell growth is observed at later times of incubation. Growth recovery is not observed when MSO is added to the incubation medium during ASNase treatment, thus confirming the active role of GS in the adaptation to the nutritional stress imposed by ASNase.

However, preliminary data from transfection experiments indicate that the lack of GS is not probably the only reason of the high sensitivity to Gln deprivation in HOG and Hs683 cells. Indeed, although the expression of GS in *GLUL* transfected oligodendroglioma cells was readily detected, there is no significant protection on cell viability after incubation in Gln-free conditions. However, the regulation of the protein transduced is very similar to the native protein with a stabilization of its shelf-life inversely dependent on Gln cell levels.

Moreover GS seems to be stabilized by the presence of its substrate glutamate (Glu) that increases protein abundance.

Further studies are necessary to confirm these data. For example, it would be useful 1) to measure the intracellular pool of amino acids of *GLUL* transfected cells to verify if a sizable amount of intracellular glutamine is synthetized and 2) to determine GS activity in the extracts of transfected cells, to assess the functionality of the transfected enzyme.

Because of the lack of GS activity, both HOG and Hs683 cells are extremely dependent on extracellular Gln availability with a clear cut sensitivity to 2-methylaminoisobutyric acid (MeAIB), the substrate of the active Gln transporters of the SNAT family.
One of these transporters, SNAT1 (the product of *SLC38A1*), is expressed at higher levels in the two oligodendroglioma cells (especially in Hs683 cells) than in glioma cells.
Consistently, MeAIB has negligible effects on the viability of glioma cells. These data indicate that the phenotype of oligodendroglioma cells is deviated to a strict dependence on extracellular Gln that is efficiently taken up by SNAT1.

Another possible mechanism underlying the cytotoxic effects of Gln depletion consists in the inhibition of mTOR, which has been found sensitive to Gln availability in many other cell models. Although there is a marked drop of intracellular Gln levels upon ASNase treatment in all the cell models observed, the treatment with the drug has a different effect on mTOR activity in the two oligodendroglioma cell lines, causing the "turning off" of the kinase in HOG cells, but not in Hs683 cells. This excludes a role of mTOR inhibition in the cytotoxic effects of ASNase in oligodendroglioma cells.

Anyway, these results also indicate that the two cell lines have a different regulation of mTOR activity (Gln-dependent in HOG cells, but Gln-independent in Hs683 cells). Consistently, Gln is able to restore mTOR activity in HOG cells but not in Hs683 cells, pre-incubated in amino acid-free Earle's Balanced Salt Solution (EBSS) to suppress the kinase function. Similarly to HOG cells, also in the two glioblastoma cell lines, U87 and U373, mTOR activity is blocked by ASNase, indicating its dependence from Gln.
Thus, Hs683 represent a cell model in which mTOR is Gln-independent. Moreover, since mTOR is still sensitive to essential amino acids in the same cell model, these results strongly suggest that Gln and essential amino acids control mTOR activity through independent pathways. The recovery of the kinase activity in HOG, U87 and U373, when MSO is added to ASNase-treated cells confirms that, as in other cell models, the GS inhibitor is involved in mTOR regulation and suggests that MSO and Gln share a common pathway to activate mTOR.
It is possible that the different Gln-dependence of mTOR activity in the two oligodendroglioma cell lines HOG and Hs683 cells is to attribute to a *MTOR* mutation in Hs683 (substitution of a residue of Glu1799 with a residue of Lysine, already described in this model) that constitutively increases the phosphorylation of S6K1 (one of the downstream

targets of the kinase). This behavior renders Hs683 cells a mTOR dependent phenotype, as confirmed by their enhanced sensitivity to rapamycin and confirms a different regulation of the kinase activity in the two oligodendroglioma lines.

In conclusion, the results obtained in oligodendroglioma cell lines indicate that the impairment of Gln availability could be a good target for the control of these tumors. Moreover, these findings suggest a novel paradigm for sensitivity to the anti-tumor effects of ASNase, consisting in the lack of functional expression of GS. If this is true, "GS-negative" human cancers would be candidates for assessing the feasibility of a nutritional approach to therapy. Moreover, the results obtained on mTOR regulation in Hs683 cells point to this model as an interesting experimental tool to define the mechanism of mTOR dependence upon amino acids.

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Scientific publications and posters

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"Towards a metabolic therapy of cancer?"
Acta Biomed. 2012 Dec; 83(3): 168-176. (review)

- Martina Chiu, Saverio Tardito, Renata Franchi-Gazzola, Massimiliano G. Bianchi, Laura Ottaviani, Jacopo Uggeri, Annarosa Arcangeli, Serena Pillozzi, Ovidio Bussolati
"β-Catenin-Mutated Human Hepatocellular Carcinoma (HCC) Cells Show Features of Glutamine Addiction"
XXXI Meeting of the Italian Society of Pathology and Translational Medicine (SIPMeT), J. Pathol. XX (Suppl. XX), XX, Udine 12-15 September 2012 (poster)

- Laura Ottaviani**,** Martina Chiu, Davide Schiroli, Annarosa Arcangeli, Ovidio Bussolati
"Human oligodendroglioma cells are sensitive to glutamine deprivation induced by L-Asparaginase"
ABCD Congress; Ravenna 12-14 September 2013 (poster)

- M. Chiu, D. Bardelli, G. D'Amico, L. Ottaviani, F. Dell'Acqua, C. Rizzari, O. Bussolati
"Human Bone Marrow Mesenchymal Stromal Cells Adapt to L-asparaginase through Autophagy and Glutamine Synthetase Induction"

2nd Joint Meeting of Pathology and Laboratory Diagnostics - XXXII Congress of the Società Italiana di Patologia e Medicina Traslazionale and LXIV National Congress of the Associazione Italiana di Patologia Clinica e Medicina Molecolare, Palermo 17-20 September 2014 (poster)

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