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Glutamine deprivation and glutaminase 1 inhibitory effect in colorectal cancer cells: a metabolomic study

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Ph.D. Student: ***Martina Spada***

Supervisor ***Prof. Luigi Atzori***

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Abstract

Cancer cells reprogram their metabolism to fulfil the rising bioenergetic demand due to the high proliferation rate. Some nutrients become essential for cancer growth and development and their lack determines an arrest of cell proliferation or a metabolic adaptation. Glutamine is considered a "conditionally essential" amino acid and plays a pivotal role in cancer cell metabolism. Indeed, glutamine addiction is an emergent hallmark of several types of cancer, including colorectal cancer. Identifying tumours sensitive to glutamine deprivation represents a promising approach movable to the clinic to improve targeted therapeutic strategies and personalised medicine. In this regard, metabolomics offers the opportunity to ameliorate the knowledge of pathophysiological aspects in cancer metabolism and to identify potential biomarkers.

This study aimed to investigate glutamine addiction in colorectal cancer and to clarify the role of glutamine in cancer development by using an *in vitro* model consisting of four different cell lines: Caco-2, HCT116, HT29 and SW480. We investigated the effect of glutamine starvation or the pharmacological inhibition of its metabolism with CB839, a non-competitive allosteric inhibitor of glutaminase 1 (GLS1). The ultimate goal is to recognize and exploit tumoural metabolic dependencies to identify new therapeutic targets and to improve cancer therapy.

Our experiments demonstrated that glutamine deprivation reduces the growth rate and proliferative capacity of all studied cancer cell lines. The absence of glutamine caused alterations in the levels of glutathione, one of the most important intracellular antioxidants, and its oxidized form, highlighting modifications of the redox homeostasis. Furthermore, glutamine-deprived cells exhibited greater glucose uptake and higher expression of the GLUT1 transporter probably to exploit the glucose carbons for biosynthetic purposes or to replenish the Pentose Phosphate Pathway and restore the redox balance. Glutamine starvation induced profound changes at the metabolic level: energetic processes, such as glycolysis and Krebs cycle, and amino acid pathways were deeply affected by the starvation. Additionally, glutamine deprivation sensitized cancer cells to chemotherapeutic drugs, such as camptothecin, while it is not a winning strategy with the antimetabolite 5-FU. Recently, studies on the glutamine dependency of cell lines in vitro motivated the testing of glutamine metabolism inhibitors as cancer therapeutics. CB839, a non

competitive glutaminase 1 (GLS1) inhibitor, exerted antiproliferative effects and limited cell survival and proliferation of the colorectal tumour cell lines. Both glutamine deprivation and enzymatic inhibition of its metabolism similarly affected the energetic and amino acid pathways (alanine, aspartate and glutamate metabolism; glycine, serine and threonine metabolism; phenylalanine, tyrosine, and tryptophan metabolism; glutathione metabolism and glyoxylate and dicarboxylate metabolism).

This work represents an intriguingly starting point to clarify the role of glutamine in colorectal cancer metabolism and to identify potential strategies useful to improve targeted therapies.

List of abbreviations

APC = adenomatous polyposis coli

AFAP = attenuated familial adenomatous polyposis

BPTES = bis-2- (5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulphide

CIMP = CpG island methylator phenotype

CIN = chromosomal instability

CPT = camptothecin

CRC = colorectal cancer

DAPI = 4′,6-diamidino-2-phenylindole

DMEM = Dulbecco's Modified Eagle's Medium

DMSO = dimethyl sulfoxide

FA = fatty acid

FACS = fluorescence-activated cell sorting

FAP = familial adenomatous polyposis

FASN = fatty acid synthase

FBS = fetal bovine seru

FH = fumarate hydratase

FTICR = Fourier transform cyclotron ion resonance analyzer

GC = gas chromatography

GC-MS = gas chromatography -mass spectrometry

GLN = glutamine

GLS = glutaminase

GLS1 = glutaminase 1

GLUD = glutamate dehydrogenase

GLUT1 = glucose transporter 1

GOT = glutamic-oxalacetic transaminases

GPT = glutamic-pyruvic transaminases

GSH = glutathione

GSSG = glutathione disulfide

G6PD = glucose-6- phosphate dehydrogenase

HNPCC = hereditary non-polyposis colon cancer

HIF1α = hypoxia-inducible factor 1 α

HKII = hexokinase 2

HPLC = high-performance liquid chromatography

IDH = isocitrate dehydrogenase

JPS = juvenile polyposis syndrome

LDH1 = lactate dehydrogenase 1

LOH = loss of heterozygosity

MAP = MUTYH-Associated Polyposis

MMR = mismatch rapair

MS = mass spectrometry

MSI = microsatellite instability

MSTFA = N-Methyl-N-trimethylsilyl)-trifluoroacetamide

mTORC = mammalian target of rapamycin complex

mTOR = mammalian target of rapamycin

MTT = ((3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) tetrazolium

NADPH = nicotinamide adenine dinoucleotide phosphate

NMR = nuclear magnetic resonance techniques

Nrf2 = nuclear factor erythroid 2–related factor 2

OD = optical density

PC = principal components

PCA = principal component analysis

PDK1 = pyruvate dehydrogenase kinase 1

PI = propidium Iodide

PJS = Peutz–Jeghers syndrome

PPP = pentose phosphate pathway

PTEN = phosphatase and tensin homologue

ROS = reactive oxygen species

QC = quality control

SD = standard deviation

SDH = succinate dehydrogenase

SSLs = sessile serrated lesions

TSAs = traditional serrated adenomas

TCA = tricarboxylic acid

TOF = time-of-flight

2-HG = 2-hydroxyglutarate

2-NBDG = fluorescent 2- deoxy-2-[(7-nitro-2,1,3- benzoxadiazol-4-yl)amino]-D-glucose

2-OGDD = 2-oxoglurate-dependent dioxygenase

5-FU = 5-fluorouracil

αKG = alpha-ketoglutarate

I. Introduction

Colorectal cancer

Epidemiology

Colorectal cancer (CRC), counting over 1.8 million new cases in 2018 and comprising 11% of all cancer diagnoses, constitutes the third most frequent cancer worldwide and ranks second in terms of mortality (Bray *et al*, 2018). CRC affects mainly men as the probability of developing CRC is 1.5-fold higher in males than in females. Although the risk to be diagnosed with CRC is correlated with advancing age, in the last decades the incidence rate for this tumour is increasing in young people (under 50 years old) (Rawla *et al*, 2019). The incidence varies geographically: in developed countries, such as Europe, North America, and Oceania, a higher number of CRC diagnoses are reported in comparison with developing countries, probably as a consequence of the "Western" lifestyle. Indeed, is possible to observe a correlation between the risk of developing CRC and factors like a high-fat diet, obesity, smoking, sedentary behaviours, etc (Center *et al*, 2009; Weitz *et al*, 2005). Nevertheless, thanks to efforts in screening programs, like fecal occult blood tests, fecal immunological tests, flexible sigmoidoscopy and colonoscopy, early diagnosis, and improvements in anticancer therapy and perioperative care, incidence and mortality of CRC are decreasing in highly developed countries (Arnold *et al*, 2017; Rawla *et al*, 2019; Ronucci and Mariani, 2015). Besides the aforementioned gender, age, and lifestyle, the risk of developing CRC considerably increases in individuals with a family history of the pathology and patients affected by inflammatory bowel disease (Moore and Aulet, 2017).

Classification

The majority of CRC, 70-80%, are sporadic tumours, the remaining 20-30% of diagnosed cancers are hereditary or familial forms while a little percentage, 1-2%, is represented by cases consequent to inflammatory bowel diseases (Binefa *et al*, 2014; Grady, 2003; Munkholm, 2003).

In sporadic colon neoplasia, the first mutation arises throughout the lifetime and commonly this first step involves APC (Adenomatous Polyposis coli) gene, a tumour suppressor gene. The allelic loss or inactivation of APC drives the formation of a small adenoma or an aberrant crypt focus from normal mucosa (Fodde, 2002). The sequential accumulation of oncogenic alterations, as KRAS mutation or impairments in DNA methylation profiles, drives the progression of the colonic pre-neoplastic lesion to a dysplastic adenoma. In the last phase, the evolution toward carcinoma requires, in most cases, the mutations of p53 with consequent impairments in cell cycle arrest and DNA repair systems. Considering the events at the base of cancer development and progression, this mechanism is also called "Loss of heterozygosity pathway" (Marmol *et al*, 2017; Muller *et al*, 2016; Ponz De Leon and Percesepe, 2000).

Nearly a third of CRC are inherited or familial forms, characterised by a germline mutation of tumour suppressor or DNA repair genes. These genes lose their functionality as a result of a second "hit" that occurs during the lifetime, on the other allele. Inherited forms can be subdivided into polyposis and nonpolyposys syndromes, based on the presence or absence of multiple polyps (Wells and Wise, 2017). The most common hereditary nonpolyposis syndrome is Lynch syndrome, also known as Hereditary Non-Polyposis Colon Cancer (HNPCC), an autosomal dominant disorder (Lynch and de la Chapelle, 2003). Lynch syndrome predisposes to different types of tumours, predominantly colon and endometrial cancer. In HNPCC patients, cancer arises at a younger age and with a more proximal localisation in comparison with colorectal cancer sporadic forms. From the histological point of view, this form generally appears poorly differentiated, mucinous, and with the presence of tumour-infiltrating lymphocytes (Jasperson *et al*, 2010). The tumour develops from an alteration in DNA mismatch repair (MMR) genes, most frequently MLH1 and MSH2, while less common are mutations in MSH6 and PMS2 genes. The deficiency in the DNA repair system causes a particular condition known as "microsatellite instability"

(MSI), defined as frequent alterations in lengths of microsatellites, repeated DNA sequences of some base pairs, from one to six (Sinicrope, 2018; Weitz *et al*, 2005).

The second most common inherited form of CRC is Familial Adenomatous Polyposis coli (FAP), an autosomal dominant syndrome, caused by a germline heterozygous alteration in the APC gene, a tumour suppressor involved in the Wnt signalling pathway. FAP is characterised by the growth of hundreds to thousands of adenomatous polyps, especially in the proximal colon, beginning from a young age and, if untreated, resulting in CRC development in most patients. FAP syndrome can occur also as an attenuated form (AFAP), with at the base the same genetic cause but the adenomatous polyps are less numerous (Brenner *et al*, 2014; Ma *et al*, 2018; Jasperson *et al*, 2010). There are other inherited forms of CRC, which are less common than the ones already mentioned. MUTYH-Associated Polyposis (MAP), where MUTYH represents a protein involved in the repair of DNA damage caused by reactive oxygen species with a base excision mechanism, is an autosomal recessive disease, that mimics phenotypically AFAP with adenomatous polyps located in the proximal colon. Juvenile Polyposis Syndrome (JPS) and Peutz–Jeghers Syndrome (PJS) can be considered as inherited CRC syndromes, and are characterized by the onset of hamartomatous polyps, especially in the small bowel and are caused by mutations in SMAD4 or BMPR1A and STK11 respectively (Brosens *et al*, 2011; Sengupta *et al*, 2019). Hyperplastic Polyposis (HPP) is a rare inherited disorder that occurs with the appearance of several and large hyperplastic polyps whose etiology is still unknown (Gallagher *et al*, 2010; Macaron *et al*, 2015; Wells and Wise, 2017). All these described syndromes are associated with a high risk to develop CRC during the lifetime, since they are determined by highly penetrant inherited mutations, and are clinically well characterised. However, they represent only 5% of inherited forms. The aetiology of remaining inherited CRC is currently not fully understood and probably caused by common mutations in low penetrance genes (Jasperson *et al*, 2010).

Pathogenesis

Tumorigenesis is defined as a multistep process: accumulation of genetic alterations drives the transformation from normal to malignant cells (Hanahan and Weinberg, 2000). Colon cancer progresses through the sequential accumulation of genetic, histological, and morphological alterations (Simon, 2016). The mutations responsible for the onset of CRC affect oncogenes, and tumour suppressor genes (Morris and Chan, 2015). Point mutations, amplifications, rearrangements, or epigenetic changes can alter the ordinary function of proto-oncogenes, that normally orchestrate cell cycle, proliferation, and cell survival (Boland and Goel, 2010). On the other hand, deletions, mutations, or promoter methylation of tumour suppressor genes result in loss of their function as regulators of cell proliferation, cell cycle arrest, and induction of apoptosis (Macleod, 2000). Lack of normal cell growth regulation results in uncontrolled and limitless expansion of neoplastic cells (Boland and Goel, 2010). Different pathways, involved in the development of colorectal cancer, can be distinguished: the traditional pathway, the alternative pathway, the serrated pathway, and the de novo pathway (Yamagishi *et al*, 2016).

According to the classic pathway, the tumour develops with gradual histological modifications that reflect the molecular dysregulation, defined as "adenoma-carcinoma" sequence (De Palma *et al*, 2019). In most cases, the initial event in tumour development is represented by the onset of mutations in the APC gene responsible for the formation of the adenoma (Wu *et al*, 2018). Mutations affecting KRAS, PIK3CA, SMAD4 and TP53 genes frequently accumulate during adenoma formation and often correlate with the stage of development of the process (Smit *et al*, 2020). The APC gene, on chromosome 5q21-q22, codes for a large protein with multiple functional domains and is involved in several cellular processes, including cell adhesion, polarity, migration, apoptosis, microtubule assembly, and chromosomal segregation (Pino and Chung, 2010; Zhang and Shay, 2017). Moreover, APC plays a key role in the degradation of β-catenin, an essential component of Wnt signaling pathway, to regulate its intracellular levels (Arends, 2013). Normally, APC is a fundamental negative regulator of the Wnt signaling pathway (Roberts *et al*, 2012). In the absence of extracellular Wnt ligands, β-catenin is bound and phosphorylated by the "degradation complex", consisting of axis inhibitor (Axin), protein phosphatase 2 A, glycogen synthase kinase 3 beta (GSK3β), CK1α/ε (casein kinase 1α/ε) and the APC

protein: phosphorylation leads to ubiquitination with consequent proteolytic degradation of beta-catenin (Cheng *et al*, 2019). Instead, Wnt ligands interact with Frizzled receptors and low-density-lipoprotein-related protein 5/6 (LRP5/6) co-receptor complex thereby inactivating the destruction complex. Consequently, β-catenin accumulates in the cytoplasm and translocates into the nucleus, where it interacts with the T cell-specific factor (TCF)/lymphoid enhancer-binding factor (LEF), promoting target genes expression (Li *et al*, 2012). Among these genes can be found MYC and cyclin D1, which play an important role in proliferation, apoptosis, and cell cycle progression, as well as the genes coding for the CD44 protein and matrilysins (MMP), which are mainly involved in tumour promotion process (Aoki and Taketo, 2007; Fodde, 2002). Mutations in the APC gene, generally represented by frameshift or nonsense mutations, result in the production of a truncated APC protein, which cannot interact with β-catenin. Consequently, β-catenin cannot be degraded, accumulates in the nucleus, and leads to constitutive transcription of its target genes, even in the absence of Wnt pathway activators (Zhang and Shay, 2017). Tumour development according to the traditional pathway is characterized by chromosomal instability (CIN), which is manifested as numerical and structural chromosomal alterations that can cause aneuploidy, subchromosomal genomic amplifications and loss of heterozygosity (LOH), especially in tumour suppressors (Al-Sohaily, 2012; Carethers and Jung, 2015).

About a quarter of CRCs develops via serrated pathway, which occurs with the onset of serrated precursors: sessile serrated lesions (SSLs), with large size and predominantly located in the proximal colon, and traditional serrated adenomas (TSAs), the less common type of serrated polyps that occurs predominantly in the distal portion of the colon (Amemori *et al*, 2020; Rashtak *et al*, 2017). Often, the development of this type of lesions is a consequence of the acquisition of gene mutations such as BRAF and, less frequently, KRAS (O'Brien *et al*, 2004; Simon, 2016). Subsequently, the progression of tumour lesions occurs through the accumulation of genetic and epigenetic alterations, which are accompanied by histological modifications (Crockett and Nagtegaal, 2019). BRAF gene encodes the serine-threonine kinase proteins of the RAF family: ARAF, BRAF, and CRAF, involved in the MAPKs pathway. Mutations in BRAF, among the most common the replacement in the amino acid chain of a valine residue with a glutamic acid residue in position 600, lead to an overactivation of the MAPK pathway with consequent increased

expression of the genes involved in proliferation, migration, cell survival and angiogenesis (Sanz-Garcia *et al*, 2017). Tumours that develop along this pathway are associated with a CIMP signature (CpG island methylator phenotype), consisting in widespread methylation of CpG islands, situated in promoter regions, thereby silencing gene transcription (Bordaçahar *et al*, 2015). Moreover, in a particular subgroup of CRCs with high CIMPs, originating according to the serrated pathway and characterized by a high degree of methylation of the CpG islands, methylation occurs at the level of the promoter of the MLH1 gene, involved in the mismatch repair system. As a consequence, these tumours are also characterized by MSI (De Palma *et al*, 2019).

The third mechanism of CRC onset is represented by the "alternative pathway", which involves 10-30% of neoplasms (Pancione *et al*, 2012). It arises from villous or serrated adenomas and the predominantly genetic event underlying these lesions is the KRAS gene mutation (Yamagishi *et al*, 2016). KRAS belongs to Ras superfamily Proteins, it exhibits a binding domain that hosts GTP, when it is in the active form, or GDP when it is inactive conformation, and it also shows a domain with hydrolytic function (Soleimani *et al*, 2019). RAS is involved in the activation of the MAPK and the PI3K pathway. Mutations in this gene lead to the loss of the hydrolytic function of the RAS protein, resulting in constitutive activation of the aforementioned pathways involved in proliferation, survival, and cell growth (Cicenas *et al*, 2017). Tumours developed by the alternative pathway show CIMP-low phenotype, no CIN, and are the most heterogeneous. Furthermore, these tumours are associated with a worse prognosis (Pancione *et al*, 2012).

The pathway de novo appears to be less frequent and there are no precursor lesions. Loss of heterozygosity is found in the TP53 gene and in the short arm of chromosome 3 on which FHIT and RASSF1A genes are located (Yamagishi *et al*, 2016).

Cancer cells metabolism

One of the characteristics of cancer cells is the high rate of proliferation and growth when compared to their non-tumoural counterpart (Hanahan and Weinberg, 2000). Uncontrolled proliferation determines an increased energy demand for cellular processes and biosynthetic pathways maintenance, conservation of the redox balance, as well as an enhanced need of metabolic precursors for cellular anabolism (Vazquez *et al*, 2016). Furthermore, the limited vascularisation in the tumour microenvironment forces the cells to a chronic deprivation of oxygen and nutrients and requires an adjustment of metabolism to maximize the intake of metabolites and the catalytic capacity of enzymes (Park *et al*, 2020). The reprogramming of cancer metabolism is a trait shown by most of the cancer cells, and it is well recognised as a hallmark of cancer (Hanahan and Weinberg, 2011). Genetic and epigenetic alteration in cancer cells resulted in deregulated signaling pathways that adjust cell metabolism to fulfil the increasing energy demand of cancer cells (Yuneva *et al.* 2012). Tumour cells reprogram their metabolism on several fronts: first of all, they show an altered glucose metabolism, their lipid metabolism is different from the non-tumoural counterpart, exhibit a marked dependence on glutamine, present an altered redox balance (Luengo *et al*, 2017).

Glucose metabolism: aerobic glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway

In 1924, Otto Warburg discovered a peculiar characteristic common to cancer cells: they metabolise glucose preferentially through glycolysis, even in the presence of oxygen, and despite being a less efficient energy process compared to oxidative phosphorylation (Abdel-Wahab *et al*, 2019; Brown *et al*, 2018). Initially, it was hypothesized that this adaptation was the result of mitochondrial dysfunction and it was noted that cancer cells carry out this metabolic shift despite being able to conduct mitochondrial respiration (Liberti and Locasale, 2016). This state of aerobic glycolysis, also known as the "Warburg effect", is probably preferred by the tumour to produce ATP more rapidly and to provide metabolic intermediates to support the high proliferation rate (Li and Zhang, 2016; Lunt and Vander Heiden, 2011; Vazquez *et al*, 2016). For instance, glucose-6-phosphate enters

into the pentose phosphate pathway (PPP), generating, through the non-oxidative phase, ribose-5-phosphate essential for the synthesis of nucleotides employed in DNA replication. On the other hand, through the oxidative phase of PPP, glucose-6-phosphate utilization produces NADPH, required to regenerate glutathione (GSH) from the glutathione disulfide (GSSG), to preserve cancer cells from oxidative stress and to maintain redox homeostasis. Furthermore, glyceraldehyde-3-phosphate, another intermediate of glycolysis, is useful for membrane synthesis and represents a substrate for the synthesis of phospholipids (Farhadi *et al*, 2020; Ganapathy-kanniappan, 2018; Park *et al*, 2020). Aerobic glycolysis is a rapid but less efficient energetic process compared to oxidative phosphorylation. As a consequence cancer cells increase glucose uptake by upregulating genes and proteins involved in glucose transport, such as glucose transporter 1 (GLUT1), or enzymes such as hexokinase 2 (HKII), which catalyzes the limiting reaction of glycolysis, i.e. the conversion from glucose to glucose-6-phosphate (Brown *et al*, 2018). The PI3K/Akt/mTOR pathway is involved in the metabolic reprogramming of glucose metabolism of tumour cells, both directly through the phosphorylation of metabolic enzymes, and indirectly by acting on the expression of transcription factors (Hoxhaj and Manning, 2020). PI3K/Akt regulates glucose uptake through various mechanisms: it regulates GLUT1 expression, its translocation from the inner portion of the membrane to the cell surface, and by phosphorylation, it inhibits the TXNIP protein, which is responsible for the endocytosis of GLUT1, thus promoting glucose retention (Courtnay *et al*, 2015; Hong *et al*, 2016). Akt regulates also glycolysis through phosphorylation of glycolytic enzymes enhancing the activity of hexokinase, which converts glucose to glucose-6-phosphate and preventing the efflux of glucose from cells (Hoxhaj and Manning, 2020; Pavlova and Thompson, 2016). Akt can adjust glucose metabolism also through the control of downstream transcription factors, which in turn regulate the expression of metabolic enzymes. Among these transcription factors, Hypoxia-inducible factor 1 (HIF1α) can induce the expression of GLUT1 and numerous glycolytic enzymes, as well as lactate dehydrogenase 1 (LDH1) and pyruvate dehydrogenase kinase 1 (PDK1), which divert pyruvate towards conversion to lactate, rather than being oxidized in the Krebs cycle at the mitochondrial level (Robey and Hay, 2009). Another Akt-regulated transcription factor is MYC, which induces the expression of glucose transporters and glycolytic enzymes, as well as stimulates mitochondrial respiration. The action of Akt on MYC is also indirect, indeed the activation of Akt inhibits FOXO, a transcription factor that

antagonizes the activity of MYC promoting the glycolytic activity of the tumour cell (Hoxhaj and Manning, 2020). The aerobic glycolysis pathway is also boosted by KRAS and BRAF mutations, which are associated with enhanced glucose uptake resulting from increased GLUT1 expression, as well as by the tumour suppressor TP53, which controls the expression of glucose transporters and HKII (Neitzel *et al*, 2020).

Although tumour cells prefer aerobic glycolysis to produce energy, the Krebs cycle plays a fundamental role in cancer as it is involved in the production of energy, the synthesis of macromolecules, and the redox balance. More specifically, oncogenes and tumour suppressor genes regulate the uptake and catabolism of the TCA supplying molecules, glucose, and glutamine, by controlling the expression of transporters and enzymes involved in the cycle. Among these, the transcription factor MYC activates glutaminolysis, the anaplerotic flow that produces α-ketoglutarate from glutamine to fuel the TCA cycle. The activation of HIF1α and the loss of function of TP53 lead to an increase in the glycolytic rate, and KRAS mutation induces an increase in glucose anaplerosis to replenish the TCA cycle (Anderson *et al*, 2018). The TCA enzymes are also involved in tumour development and progression. Isocitrate dehydrogenase (IDH), which oxidatively decarboxylates isocitrate to alpha-ketoglutarate, succinate dehydrogenase (SDH), that converts succinate to fumarate, and fumarate hydratase (FH), which produces L-malate from fumarate, are defined as tumor suppressors. At the same time, succinate and fumarate, and 2-hydroxyglutarate are considered oncometabolites. They act as oncogenic signaling molecules in different cellular processes, including epithelial-to-mesenchymal transition, migration, invasion, and angiogenesis. The accumulation of these oncometabolites also causes epigenetic alterations, by inhibiting several αKG-dependent dioxygenases with consequent histone hypermethylation, and metabolic variations by inducing the stabilization of HIF1α with consequent activation of the glycolytic pathway (Dalla Pozza *et al*, 2020).

The pentose phosphate pathway represents an alternative way for glucose catabolism and provides the ribose necessary for the synthesis of nucleotides, as well as NADPH. This pathway plays a pivotal role in tumour growth and survival. In fact, in oxidative stress conditions, glucose-6-phosphate is diverted towards PPP, consequently reducing the glycolytic rate. Under physiological conditions, TP53 inhibits the enzymatic activity of glucose-6- phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of PPP.

Mutations of the tumour suppressor TP53 lack the G6PD-inhibitory activity with consequent activation of the PPP. Among the TP53 target genes, phosphatase and tensin homologue (PTEN) is capable of suppressing the enzymatic activity of G6PD, reducing the flow through PPP, while TIGAR (TP53-induced glycolysis and apoptosis regulator) redirects glucose-6-phosphate to PPP by blocking PFK1, gatekeeper glycolytic enzyme. mTORC1 (the mammalian target of rapamycin complex), often triggered in tumour cells via the PI3K/Akt pathway, activates glycolysis and PPP indistinctly by inducing the expression of glycolytic enzymes and G6PD (Jiang *et al*, 2014; Patra and Hay, 2014; Sun *et al*, 2018).

Lipid metabolism

Lipid metabolism is also deregulated in colorectal cancer. Lipids are used in the cell not only as an energy source but also as structural components of membranes. Furthermore, lipids act as signal molecules and play an important role in the biosynthesis of cytokines. While normal cells predominantly utilize circulating fatty acids (FAs) derived from the dietary fat, cancer cells show increased lipogenesis and cholesterol synthesis, as well as increased extracellular lipid uptake. The increase in lipogenesis, orchestrated by mammalian target of rapamycin kinase (mTOR kinase) signaling pathway, is allowed by an enhanced activity of the FASN (fatty acid synthase) enzyme and is associated with a composition of the saturated FA-rich membranes. These structural changes in cellular membranes make the tumour cells more resistant to ROS action and penetration of chemotherapeutic drugs. In colorectal cancer, the FASN enzyme upregulates not only the synthesis of fatty acids but also glycolysis, oxidative phosphorylation, and β-oxidation of fatty acids, especially in conditions of metabolic stress (Brown *et al*, 2018; Yan *et al*, 2016; Pakiet *et al*, 2019).

Redox balance

ROS are high reactive species resulting from the incomplete reduction of oxygen, among which we can find radical compounds such as the superoxide anion ($O2^{\cdot-}$) and the hydroxyl radical (OH·) and non-radical compounds as hydrogen peroxide (H_2O_2) (Hayes *et al*, 2020). They are produced following enzymatic reactions, which involve for example NADPH oxidase, uncoupled endothelial nitric oxide synthase, xanthine oxidase, lipoxygenase, cyclooxygenase, and cytochrome P450 enzymes, or in a non-enzymatic way in the mitochondrial electron transport chain (Gill *et al*, 2016). The main source of ROS are mitochondria, where in addition to the electron transport chain, the Fenton reaction takes place (Snezhkina *et al*, 2019). ROS are also produced in peroxisomes, during the β-oxidation of fatty acids and following the activity of flavin oxidase, in the endoplasmic reticulum during protein folding (Gill *et al*, 2016; Gorrini *et al*, 2013). At physiological concentrations, these compounds play a role in signal transduction, gene expression, proliferation, and enzyme activation (Sosa *et al*, 2013). However, when the concentration of oxidant species exceeds the antioxidant capacity of the cell, oxidative stress is generated. The excess of reactive species causes damage to cellular molecules, including DNA, RNA, proteins, and lipids, leading to senescence and apoptosis (Sosa *et al*, 2013). To preserve this redox homeostasis, cells exploit ROS detoxification systems, including enzymes such as superoxide dismutases, that convert $O2^{\cdot-}$ into H_2O_2; peroxiredoxins, glutathione peroxidases, and catalase which instead is involved in the hydrogen peroxide detoxification. Furthermore, intracellular antioxidant compounds play a key role in redox balance. Glutathione, a tripeptide composed of glutamate, cysteine, and glycine is considered the most relevant low molecular weight antioxidant in cells (Chio and Tuveson, 2017; Gill *et al*, 2016). In addition, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is essential for maintaining the redox state, representing a co-factor in numerous enzymatic reactions (Xiao *et al*, 2018). It has been shown that tumour cells exhibit an aberrant redox balance when compared with their non-tumour counterparts. In particular, their high proliferative rate is accompanied by a high ROS level. This increase favours tumor development and progression promoting proliferation, cell cycle progression, tumour survival, and growth through the activation of PI3K/Akt, MAPK/ERK1/2, PKD and NFκB signalling pathways. Furthermore, ROS inactivate Bad, Bax, Bim, and Foxo, which act as pro-apoptotic factors, promoting cell survival (Arfin *et*

al, 2021). However, their potential toxicity is readily buffered by an equally high production of antioxidant species. This new redox homeostasis favours the tumour growth avoiding the ROS-dependent oxidative damage (Hayes *et al*, 2020). One of the main systems used to increase the antioxidant capacities of cancer cells is the activation of NRF2, nuclear factor (erythroid-derived 2)–related factor-2. Once activated, NRF2 promotes the transcription of antioxidant proteins, including glutathione peroxidases, enzymes involved in the synthesis of glutathione, and cysteine transporters. NRF2 also favours the increase in cytosolic and mitochondrial NADPH levels, particularly useful for controlling redox homeostasis during hypoxic conditions (DeBeardinis and Chandel, 2016). Commonly altered pathways in tumour cells are also involved in the maintenance of redox homeostasis. For example, glycolysis provides intermediates that are diverted towards metabolic pathways, such as PPP, to produce reducing equivalents, as NADPH. On the other hand, glutaminolysis supplies the glutamate for glutathione synthesis and also promotes the uptake of cysteine through the SLC7A11 transporter (Panieri and Santoro, 2016).

Glutamine addiction in cancer and therapeutic strategies

Glutamine addiction was discovered among the cancer cell metabolic alterations to meet energy needs and anabolic demand (Deberardinis *et al*, 2008; Hammoudi *et al*, 2011; Mazurek *et al*, 2005). Glutamine is defined as a "conditionally essential" amino acid, although it can be synthesized *de novo* in cells. Glutamine becomes essential in pathological catabolic stress conditions such as infections and nutrient starvation (Altman *et al*, 2016; Jiang *et al*, 2019). Cells are supplied with glutamine thanks to numerous transporters, including the most studied solute carrier family 1 neutral amino acid transporter member 5 (SLC1A5; also known as ASCT2), SLC7A5/SLC3, and SN2. Glutamine can be obtained from the degradation of macromolecules, especially in nutrient starvation conditions. Once entered in a cell, glutamine can take part in metabolic reactions, or can be used for the exchange with other amino acids, including leucine via the L-type amino acid transporter 1 (LAT1, a heterodimer of SLC7A5 and SLC3A2) or, indirectly, with cystine which is imported by the xCT transporter (a heterodimer of SLC7A11 and SLC3A2) (Altman *et al*, 2016; Bott *et al*, 2019; Jiang *et al*, 2019; Li and Le; 2018). From a metabolic point of view, glutamine supplies the TCA cycle through anaplerotic flux contributing to the generation of energy required by high-proliferating cancer cells. The enzyme glutaminase (GLS) catabolizes the conversion of glutamine to glutamate and ammonium. Two main isoforms of glutaminase have been described: GLS1, the kidney-type glutaminase, and GLS2, the liver-type glutaminase, that differ for kinetic properties, protein structures, and tissue distribution (Masisi *et al*, 2020). The glutamate is in turn converted to α-ketoglutarate by glutamate dehydrogenase (GLUD1 and GLUD2) or by aminotransferases, including glutamic-oxalacetic transaminase 2 (GOT2), and glutamic-pyruvic transaminase 2 (GPT2) (Jin *et al*, 2016; Yoo *et al*, 2020). The α-ketoglutarate obtained from glutamate can enter the Krebs cycle and thus feed oxidative phosphorylation, with the production of ATP and electron donor species, as NADH and $FADH_2$. This process, called glutaminolysis, has been recognized as a feature of cancer cells (Pavlova and Thompson, 2016). Furthermore, under hypoxic conditions, α-ketoglutarate can be exported from the mitochondrion in the cytosol, where can be converted into citrate by reductive carboxylation, and consequently employed in lipid synthesis (Altman *et al*, 2016). Glutamine represents a source of nitrogen necessary for the biosynthesis of nucleotides. In particular, the cytosolic glutamine participates with its ɣ-

nitrogen in the synthesis of pyrimidines and purines (Cory and Cory, 2006). Moreover, glutamic acid derived from oxidative deamination of glutamine is a source of nitrogen for the synthesis of non-essential amino acids, whose carbon skeleton is obtained from the catabolism of glutamine and glucose. Among these non-essential amino acids, we found aspartate, alanine, serine, and ornithine, which in turn are used in the synthesis of asparagine, cysteine, glycine, and arginine (Wise and Thompson, 2010). Glutamine also plays a fundamental role in controlling the redox state in cells. It is known that ROS contribute to tumour growth by acting as signal molecules, however, if their level increases beyond the cellular redox capacity they can cause irreparable damage to lipids, nucleic acids and proteins (Moloney and Cotter, 2018). Glutamine, as glutamate source, is directly involved in the synthesis of glutathione and is also responsible for the cystine uptake (Shafer and Buettner, 2001; Koppula *et al*, 2018). More recently, it has been highlighted that glutamine may be involved in the metabolic reprogramming that occurs in cancer cells. The amino acid availability activates mTORC1 through adenosine diphosphate ribosylation factor 1 (Jewell *et al*, 2015; Zhu and Wang, 2020). This pathway is in turn responsible for activating aerobic glycolysis and for inducing glucose dependency. Activation of mTORC1 is also responsible for the suppression of autophagic and DNA damage response processes. However, glutamine plays a double role in autophagy: excess of ammonia production probably stimulates autophagy and therefore allows cells to survive under stressful conditions (Yoo *et al*, 2020). Glutamine is also involved in epigenetic processes. More, in particular, there is general hypermethylation in tumour, which contributes to the altered expression of genes involved in cell differentiation and tumour development and progression (Berdasco and Esteller, 2010; Hanahan and Weinberg, 2011). Specifically, α-ketoglutarate derived from glutamine is involved in the regulation of histone and DNA methylation. Indeed, alpha-ketoglutarate is a 2-oxoglurate-dependent dioxygenases (2-OGDDs) cofactor. The 2-OGDD activity is regulated by the intracellular levels of succinate, fumarate and 2-hydroxyglutarate (2-HG), which compete with the cofactor alpha-ketoglutarate. These enzymes also require Fe^{2+} and O_2, respectively as cofactor and cosubstrate, as well as vitamin C as a reducing agent, capable of restoring the enzyme (Crake *et al*, 2021; Kaelin, 2011). Within this enzyme family, α-ketoglutarate is mostly used by Jumonji C domain-containing histone demethylases and ten-eleven translocation demethylases. These enzymes oxidize α-ketoglutarate to succinate (Crake *et al*, 2021). Mutations in IDH lead to the production of the oncometabolite 2-HG, whereas

alterations in FH and SDH genes lead to excessive storage of fumarate and succinate that inhibit the demethylase activity, resulting in DNA hypermethylation (Saggese *et al*, 2020). Furthermore, the central portions of the tumour mass are poorly vascularised and consequently suffer from nutrient and glutamine deficiency. In these conditions, a high methylation rate was observed probably due to the decreased production of α-ketoglutarate (Pan *et al*, 2016). The increased glutamine consumption and dependency are related to oncogene alterations including MYC, KRAS, and PI3KCA. MYC can increase the glutamine uptake and metabolism acting as a transcription factor for glutaminase GLS and transporters like SLC1A5 (Hsieh *et al*, 2015). Cancer cells harbouring KRAS and PI3KCA mutations show an increased expression of SLC transporters and transaminases GOT1 and 2 and GPT2 (Kandasamy *et al* 2021; Hao *et al*, 2016). The importance that glutamine plays in the tumourigenic process renders transporters and enzymes involved in its metabolism good candidates for anticancer therapy, so several compounds are currently being studied. Acivicin, azaserine, and 6-diazo-5-oxo-*L*-norleucine are glutamine analogues that bind covalently the Ser286 position in the GLS1 catalytic site-blocking enzymatic activity. Although these compounds exhibit a wide spectrum of antitumoral activity, they demonstrate a high toxicity degree, causing myelosuppression, and toxicity at the neurological and intestinal level (Ahluwalia *et al*, 1990; Hensley *et al*, 2013). Another promising strategy for anticancer therapy consists in targeting glutamine transporters. In particular ASCT2 inhibitors are studied, as this transporter is overexpressed in different tumour types. Among ASCT2 inhibitors we can find both compounds such as l-γ-Glutamyl-p-nitroanilide, which showed high toxicity and therefore is relegated to in vitro studies, and monoclonal antibodies such as MEDI7247, an innovative pyrrolobenzodiazepine antibody-drug conjugate, which has entered phase I clinical trial for haematological cancer (NCT03102468) (Liu et al, 2018). The role of GLS2 is still debated (Xiang *et al*, 2013; Zhang *et al*, 2016), while GLS1 is widely expressed in various primary tumours and has been recognized as a key enzyme in numerous tumour types (Gross *et al*, 2014; Huang *et al*, 2014; Pan *et al*, 2015; Xiang *et al*, 2015). In the last years, research is currently progressing to identify drugs that can interfere with glutamine metabolism: inhibiting the activity of GLS1 can starve cancer cells by preventing the synthesis of glutamine-derived glutamate which, in turn, is converted in α-ketoglutarate contributing to the replenish of TCA cycle (Akins *et al*, 2018). In this context, three GLS1 inhibitors were studied: bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide (BPTES), CB-839

and 968 compounds (Gross *et al*, 2014; Lee *et al*, 2020; Robinson *et al*, 2007; Wang *et al*, 2010). BPTES, an allosteric inhibitor of the enzyme, despite having shown excellent ability to inhibit tumour growth *in vitro*, is not a good candidate for the clinic. BPTES shows a low solubility, and this, combined with high doses required for its pharmacological activity, renders its bioavailability and delivery complicated (Li *et al*, 2021; Shukla *et al*, 2012). Only CB839, an analogue of BPTES, is currently employed in various clinical trials. (https://clinicaltrials.gov/ct2/results?cond=Cancer&term=CB839&cntry=&state=&city=&dist=). CB839 passed phase I clinical trials in March 2019, for the treatment of patients with renal cell carcinoma (NCT02071862). It has also been used in phase I clinical trials to treat haematological tumours and leukemias (NCT02071888 and NCT02071927). In 2020 it entered phase II studies for the treatment of non-small cell lung cancer (NCT04265534). Furthermore, to block the replenish of the TCA cycle, a possible intervention is to target the enzyme that catalyzes the deamination of glutamate to α-ketoglutarate, GLUD1. Among natural compounds that inhibit this enzyme, we can find epigallocatechin gallate and epicatechin gallate. In 2015, R162, a derivative of purpurin, was discovered (Jin *et al*, 2015). R162 binds selectively to GLUD1 in a competitive manner, resulting in inhibition of tumour growth *in vitro* due to an increase in ROS levels, as well as a decrease in levels and activity of glutathione peroxidase (Li *et al*, 2019). Additionally, aminotransferase inhibitors showed antiproliferative activity *in vitro*, especially through activation of the ER stress pathway, setting the stage for further studies. At the moment, these drugs are currently in clinical use to treat tinnitus (Korangath *et al*, 2015). Finally, an approach that is widely used in clinical trials consists in treating various types of cancer with compounds that cause the depletion of glutamine reserves, in particular L-asparaginase (https://clinicaltrials.gov/ct2/results?cond=cancer&term=asparaginase&cntry=&state=&city=&dist=) and phenylbutyrate (https://clinicaltrials.gov/ct2/results?cond=cancer&term=phenylbutyrate&cntry=&state=&city=&dist=) are being studied. L-Asparaginase has already been used in the treatment of acute lymphoblastic leukemia and Non-Hodgkin Lymphoma. Asparaginase catalyzes the deamination of asparagine to produce aspartate and can remove the amide nitrogen from glutamine to produce glutamate. Its administration causes a decrease in plasma glutamine levels. *In vitro,* it causes cytotoxicity, cell cycle block, and apoptosis, probably

because the cell does not have adequate amounts of glutamine for the *de novo* synthesis of purines and pyrimidines (Cory and Cory, 2006; Lanvers-Kaminsky, 2017). On the other hand, phenylbutyrate works as a "glutamine trap" thanks to its ability to conjugate it, thus causing a decrease in the availability of this amino acid (De Las Heras *et al*, 2017). In vitro studies have shown that this compound can cause apoptosis in colon cancer, lung carcinoma, and prostate cancer cells lines (Feinman *et al*, 2002; Xu *et al*, 2016; Zhang *et al*, 2004), as well as growth arrest in renal cancer cells (Franco *et al*, 2003). Given the role that glutamine plays in tumor proliferation and growth, the use of drugs that target glutamine metabolism appears to be a promising strategy. However, in this perspective, further studies are needed.

Metabolomics

Metabolomics was developed with the purpose to improve the knowledge of disease pathogenesis, to discover new biomarkers useful for early diagnosis, as well as to develop personalized therapies (Klupczy-Ska *et al,* 2015). Metabolomics represents the newest of the "omics" sciences, and studies the metabolome, the set of all small molecules, with a molecular weight between 1 and 1.5 kDa, of a biological system. Metabolome includes different molecules as sugars, amino acids, nucleotides, organic acids but also toxins or drugs (Lains *et al,* 2019). The metabotype can be evaluated at various levels of complexity, at the cellular, tissue, or entire organism level, and represents the result of genetic and environmental factors, such as age, diet, physical activity, stress levels, or latent pathologies, thus representing an extremely complex and dynamic entity (Holmes *et al*, 2008). Metabolomics, therefore, represents the discipline that describes the result of the "-omics cascade", consisting of genomics, transcriptomics, and proteomics, giving an integrated and more complete knowledge of the system (Bujak *et al*, 2015). One of the fundamental metabolomics goals is discovering specific and sensitive biomarkers useful for diagnostic and prognostic assessment of human disease (Baskind *et al*, 2011; Klupczy-Ska *et al*, 2015; Newgard, 2017). To date, although not extensively used in the clinic, several metabolic panels have been identified in various pathologies, such as heart diseases (Albert end Tang, 2018; Griffin *et al*, 2011), endocrine disorders (Piras *et al*, 2021) or neurodegenerative diseases (Murgia *et al*, 2020), and can be useful in characterizing particular stages of disease progression, response to therapy, as well as risk indicators (Emamzadeh and Surguchov, 2018; Nicholson and Lindon, 2008; Zetterberg and Burnham, 2019). This new “-omics” science finds application in cancer. In particular, it is possible to study the metabolic alterations that characterize cancer cells and how the metabolic pathways are involved in the development and maintenance of the tumour mass (Kumar and Misra, 2019). Metabolic reprogramming is considered a hallmark of cancer (Hanahan and Weinberg 2011) and metabolomics represents a powerful strategy to monitor the dynamic alterations in tumor metabolism regarding glutaminolysis, lipid, and amino acid metabolism (Vazquez *et al*, 2016). Furthermore, metabolomic studies identify oncometabolites, which are significantly more abundant molecules in tumour cells than their normal counterparts. Succinate, fumarate, and 2-hydroxyglutarate are oncometabolites believed to be actively involved in cancer development and progression

(Zhou *et al*, 2018). Although the list is still short, further studies may allow identifying other molecules or groups of compounds that can be classified as tumour-specific oncometabolites, necessary for oncogenesis, such as cystathionine in breast cancer (Sen *et al*, 2016) and polyamines in colorectal cancer (Paz *et al*, 2014). A further aim of metabolomics is to identify specific and sensitive biomarkers for the various tumour types in a non-invasive way. However, specific markers for early diagnosis have not yet been identified for colorectal cancer (Snyder *et al*, 2015). Similarly, metabolomics could also be useful to predict individual tumor therapy response, assess drug efficacy and evaluate chemoresistance (Wishart 2019). Metabolomic analysis follows three basic steps: sample collection and preparation, sample analysis and data interpretation. Many sample types can be studied with metabolomic approach as cells, tissues, or biofluids including plasma, urine, cerebrospinal fluid, and saliva. The sample collection method greatly affects the success and repeatability of the experiment (Balashova *et al*, 2018; Zhou *et al*, 2012). Once the metabolites have been extracted from the biological matrix with appropriate protocols, the sample is analyzed using two main techniques: mass spectrometry (MS) or nuclear magnetic resonance techniques (NMR) (Wishart, 2019). NMR shows several advantages: the technique is non-destructive, shows high reproducibility, the experiment is fast, considering that it does not require separation of metabolites, and inexpensive; however, the sensitivity is low and the annotation of metabolites is difficult (Jacob *et al*, 2019). Mass spectrometry, on the other hand, makes it possible to identify metabolites based on the mass/charge ratio, after molecule fragmentation and ionization. MS is more sensitive than NMR technique and allows detecting a wide range of metabolites even if present at low concentrations. However, MS has the disadvantage of being a destructive technique: the molecule is subject to ionization and fragmentation allowing the production of a detectable negative or positive charged ion (Clendinen *et al*, 2017; Wishart, 2019). Furthermore, the metabolites must be primarily separated, so the MS is coupled with chromatographic separation systems such as gas chromatography (GC), liquid chromatography, and capillary electrophoresis (Bujak *et al*, 2015). Generally, the metabolomic analysis is carried out following two different approaches. One is the untargeted approach consisting of the analysis and identification of the highest number of metabolites in a sample. This strategy is especially useful for characterizing the whole metabolome. The other is the targeted approach that investigates specific metabolites to accurately and precisely determine their concentration and abundance and is therefore helpful for the biomarkers

determination (Griffiths *et al*, 2010). Once the data has been acquired and processed, they are subjected to multivariate or univariate statistical analysis, which allows identifying the metabolic profile (Wishart, 2019).

Gas chromatography coupled with Mass spectrometry (GC-MS)

Mass spectrometry coupled with gas chromatography (GC-MS) represents an excellent method for the analysis of metabolites, thanks to its sensitivity, resolution, and reproducibility (Pasikanti *et al*, 2008). The GC-MS consists of a gas chromatograph, mass spectrometer, data collection, and analysis system. The gas chromatograph allows the separation of metabolites based on their different affinity with the carrier, which is represented by gas, and stationary phase, which can be represented by both liquid or solid phase. The sample is conveyed inside the chromatographic column through an injection system and is immediately volatilized so that it can be transported by the gaseous carrier (Dondelinger, 2012). The separation upstream of the spectroscopic analysis allows to improve the performance and prevents quantitative artefacts, which occur when the signal intensity of some molecules is influenced by changes in the concentration of the most abundant species (Jang *et al*, 2018). Although a wide range of metabolites can be covered with this technique, only low molecular weight compounds (50-600 Da) and volatile molecules can be separated and identified. To overcome this limitation, chemical derivatization is necessary to improve the thermal stability, the volatility and to ameliorate the detection capacity of the detector (Beale *et al*, 2018). The separated metabolites are ionized through different ionization methods. The "soft" ionization method, such as atmospheric pressure ionization and electrospray ionization, are characterized by low energy and therefore produce limited molecules fragmentation. The "hard" ionization approach, including electron ionization, has high energy and therefore determines a high degree of fragmentation, but guarantees the success of the process on a wider range of compounds (Li *et al*, 2015). The produced ions are directed to the analyzer, which separates them based on their mass-to-charge ratio. To date, the most common detectors are single quadrupole detectors, linear ion trap or quadrupolar ion trap detectors, time-of-flight (TOF) detectors, Fourier transform cyclotron ion resonance analyzer (FTICR) and the Orbitrap detector. Orbitrap, TOF and FTICR allow metabolite detection at a higher

resolution but low sensitivity, which is instead higher in single quadrupole or ion trap detectors (Gowda and Djukovic, 2014; Li *et al*, 2015; Stettin *et al*, 2020). The analysis produces a mass spectrum, where the abundance of ions is represented as a function of their mass-to-charge ratio. The peaks are integrated, producing numerical matrices that will be subjected to statistical analysis to identify the metabolomic profile (Klupczy and Kokot, 2015).

II. Aim of the project

Glutamine plays a pivotal role in tumour metabolism and "glutamine addiction" represents a distinctive feature of several types of tumour. Our attention has been focused on colorectal cancer which still represents one of the deadliest cancers.

This thesis aimed to evaluate the glutamine dependency in several colorectal cancer cell lines and to identify their metabolic profiles under glutamine deprivation conditions or after pharmacological inhibition of glutaminase (GLS1), the key enzyme in glutamine metabolism. Specifically, an in vitro model consisting of four cell lines, Caco-2, HCT116, HT29 and SW480, harbouring different genetic mutations was used.

We investigated the role of glutamine in colorectal cancer cells and evaluated the effects of its starvation on survival, replicative potential, and redox balance, as well as metabolic alterations induced by glutamine deprivation. Understanding the molecular mechanisms underlying glutamine addiction and the strategies implemented by cells to overcome starvation can be an important tool for identifying new therapeutic targets.

We also investigated whether glutamine deprivation produces synergistic effects with classic chemotherapeutic agents, to reduce the doses necessary to produce the therapeutic effect and therefore limit the side effects. Furthermore, we evaluated the effects of the GLS1 inhibitor, CB839, on colorectal cancer cell proliferation, cell cycle and cell death.

We also wondered which metabolic mechanisms could be affected by this pharmacological treatment. Recognizing metabolic dependencies and interfering with the pathways necessary for the survival and proliferation of cancer cells represents an interesting strategy for recognizing therapeutic targets and for improving cancer therapy.

III. Materials and methods

Cell culture and treatments

Caco-2, HCT116, HT29 and SW480 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines are immortalised: Caco-2 (derived from a 72-year-old male) and HCT116 cells (derived from a 48-year-old male) were established from human colorectal carcinomas, while HT29 (derived from a 44-year-old female) and SW480 cells (derived from a 50-year-old male) from human colorectal adenocarcinomas (Ahmed *et al*, 2013). The cell lines under study are all derived from primary tumours with different tumour stages. In particular, SW480 cells originate from a B tumour with involvement of the muscle layer; HT29 cells derive from a Dukes' stage C tumour with the lymph nodes invasion; HCT116 cells arise from Dukes'stage D cancer with widespread metastases (Ahmed *et al*, 2013; Dukes, 1932). Regarding Caco-2 cell line, this information is not available. Furthermore, these cell lines differed in their genetic background. They present mutations in oncogenes (as BRAF, KRAS, CTNNB1 and PIK3CA) and tumour suppressors (as APC, SMAD4 and TP53) commonly involved in the development and progression of colorectal cancer. The most frequent mutations in colorectal cancer cell lines are reported in Table 1. Cancer cell lines isolated from tumoural masses may be considered representative samples for exploring cancer metabolism, studying regulatory mechanisms in CRC and identifying chemoprotective and therapeutic agents. All cell lines used in this work were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose supplemented with L-glutamine 4 mM (Euroclone, Milan, Italy), sodium pyruvate 1 mM (Euroclone, Milan, Italy), 10% heat-inactivated bovine serum (FBS, Life Technologies, Milan, Italy), 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich, Milan, Italy) (complete medium). Cell lines were grown in monolayers at 37°C in a humidified 5% CO2 atmosphere, replacing the medium twice a week and subcultured once a week by trypsinization. Cell lines were seeded in appropriate support (6, 24, 96 well plates), depending on the assay, at least 24 hours before treatment. 5-fluorouracil, Camptothecin and CB839 were purchased from Sigma-Aldrich (Milan, Italy) and stored at 4°C. The substances were solubilized in dimethyl sulfoxide (DMSO) and subsequently diluted at the indicated concentration for each treatment. For glutamine starvation conditions, colorectal cancer cells were cultured in a complete medium without glutamine (DMEM high glucose + Sodium pyruvate 1 mM + 10% FBS + penicillin and streptomycin).

Cell line	Caco-2	HCT116	HT29	SW480	References
APC	c.4099C>T	Wild type	c.2557G>T	c.4012C>T	ATCC; Mouradov *et al*, 2014
BRAF	Wild type	Wild type	c.1799T>A	Wild type	ATCC
CTNNB1	c.734G>C	c.133_135delTCT	Wild type	Wild type	ATCC; Ilyas *et al*, 1997
KRAS	Wild type	c.38G>A	Wild type	c.35G>T	ATCC; Mouradov *et al*, 2014
PIK3CA	Wild type	c.3140A>G	c.1345C>A	Wild type	ATCC
SMAD4	c.1051G>C	Wild type	c.931C>T	Wild type	ATCC; Mouradov *et al*, 2014
TP53	c.610G>T	Wild type	c.818G>A	c.818G>A	ATCC; Liu *et al*, 2006
Histotype	Colorectal carcinoma	Colorectal carcinoma	Colorectal adenocarcinoma	Colorectal adenocarcinoma	Ahmed *et al*, 2013
Duke's stage	/	D	C	B	Ahmed *et al*, 2013

Table 1. Colorectal cancer cell lines, genetic background and classification.

Evaluation of vitality and cytotoxicity

The MTT assay ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium) was assessed on cell lines to evaluate the viability or the cytotoxicity of studied compounds. It is based on the ability of vital cells to convert MTT, which is yellow, into a water-insoluble blue-violet formazan salt thanks to the action of mitochondrial reductases. Since the amount of reduced formazan salt is proportional to the viable and metabolically active cells, measuring the optical density (OD) of the purple formazan allows estimating cell viability (Kumar *et al*, 2018). Cells were seeded in complete medium in 96-well plates (1×10^5 cells/mL, 100 µL/well), after 24 h cell medium was replaced with complete medium (with glutamine 4mM) or with glutamine deprived medium (complete medium without glutamine) for additional 48h. For antitumoral drugs combined with glutamine deprivation, cells were also treated with different compounds concentrations (5-fluoruracil, campothecin and CB839) or with DMSO 0.5% (≈ 3 mM) (Controls) and incubated for 48 h in the presence or the absence of glutamine in the medium. For MTT assay, after incubation, the medium was removed, cells washed twice with PBS, and subsequently incubated for 2 hours at 37°C with 50 µL of MTT (2.5 mg/mL) dissolved in PBS. 100 µL of DMSO were used to solubilise the resulting formazan crystals and the absorbance was measured at 570 nm, after 15 seconds

of shaking, using a microplate reader (Infinite 200, Tecan, Salzburg, Austria). Cell viability was expressed as a percentage (± SD) respect to the control, represented as DMSO-treated cells.

Growth curves

The MTT assay, given its ability to estimate viable cells, was exploited to build growth curves in the presence or absence of glutamine or after treatment with antitumoural drugs. Briefly, cells were seeded in 96-well plates to the density of 1×10^5 cells/mL in complete medium. After the time necessary for attachment, a basal MTT assay was performed. At the same time, cells were deprived of glutamine or treated with the drug. After 48 hours of treatment, MTT assays were performed every day for a total of 4 days, the time necessary to evaluate the exponential growth rate of the tested cancer cell lines. The assay was performed in the same way as described in the previous paragraph. The data were expressed as OD at 570 nm (± SD).

Colony forming assay

The colony forming assay can be used to evaluate the proliferative capacity of adherent cells exploiting the capacity of the single cell to form colonies (at least 50 units) (Crowley *et al*, 2016; Li *et al*, 2016). Cells were seeded at low concentration in 24-well plates, (1×10^4 cells/mL, 500 µL per well) in complete medium and left growing 4 days before being cultured in complete medium or deprived medium conditions for a total of 6-10 days. At this time, cells were fixed with 200 µL of methanol for 20 minutes, rinsed with water, and stained with a solution of 5% crystal violet in 80% methanol. After 5 minutes, cells were washed and air-dried overnight. Then a picture of each well has been taken.

Determination of intracellular aminothyol levels

Determination of intracellular aminothiols, in particular the ratio between the reduced and oxidized form of glutathione (GSH/GSSG), was performed following the method developed by Khan and colleagues (Khan et al. 2011), using a High-performance liquid chromatography linked with an electrochemical detector (HPLC–ECD). Cells were seeded, in complete medium, in 6-well plates at a concentration of 1 x 10^5 cells/ml. After 4 days of growth, the medium was replaced, and cells were cultured in the presence or the absence of glutamine for an additional 48h. Then cells were washed twice with PBS and extracted with 150 µl of 10% meta-phosphoric acid and 150µl of 0.05% trifluoroacetic acid (Sigma-Aldrich, Milan, Italy) solution. Once centrifugated, the supernatant was injected into the HPLC system. Glutathione and its oxidised form were identified by electrochemical detection, employing an HPLC (Agilent 1260 infinity, Agilent Technologies, Palo Alto, USA) supplied with an electrochemical detector (DECADE II Antec, Leyden, The Netherlands) and an Agilent interface 35900E. The used column was a C-18 Phenomenex Luna, 5 µm particle size, 150 × 4.5 mm. The mobile phase consisted of 99% water with 0.05% TFA (v/v) and 1% MeOH with a flow rate of 1 mL/min. The oxidising potential of the electrochemical detector was set at 0.74 V. Before and after the samples run, GSH and GSSH standards were injected.

Glucose uptake assay

Glucose uptake assay was performed by using the fluorescent 2- deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG), a glucose analogue that can be uptaken by cells via glucose transporters (Zou et al, 2005). The fluorescence produced by 2-NBDG, which is proportional to glucose uptake, can be measured with a fluorescent microplate reader. Cells were seeded to the concentrations of 1 x 10^5 cells/ml in 96-well plate in a complete medium. After 24 h the medium was replaced, and cells were cultured in the presence or the absence of glutamine for 48 h. Subsequently, cells were washed twice with PBS to remove growth medium and especially glucose, then 100 µL 2-NBDG (fluorescently-tagged glucose derivative, N13195; Invitrogen) 50 µM were added and incubated for 30 minutes, in the presence or absence of glutamine. Following the

incubation, cells were washed and 100 µL of PBS were added. The fluorescence emitted by the 2-NBDG in cells was measured with a microplate reader (Infinite 200, Tecan, Salzburg, Austria), at 37°C, employing an excitation of 485 nm and an emission of 530 nm. Glucose uptake was expressed as a percentage (± SD) of the control (cells cultured in the presence of glutamine).

Immunofluorescence

Cells were enzymatically dissociated, cultured on previously sterilized slides, and placed in square tissue culture dishes (quadriPERM®, Sarstedt AG & Co, Nümbrecht, Germany). Cell cultures were maintained for 24 hours in a complete medium at 37°C in a humidified 5% CO_2 atmosphere to allow the cells to attach. After that, the medium was replaced and cells were cultured in the presence or absence of glutamine for 48 hours and subsequently fixed with 4% paraformaldehyde for 10 minutes at room temperature. Immunostaining was performed as previously described (Caria *et al*, 2017) using rabbit polyclonal anti-GLUT1 (1：200; Abcam, CA, USA) antibody. Alexa-conjugated (Alexa Fluor 488 or 594, Life Technologies) goat anti-rabbit IgG was used as secondary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were obtained with an epifluorescence microscope (Olympus BX41) and charge-coupled device camera (Cohu), interfaced with the CytoVysion system (software 2.81 Applied Imaging, Pittsburg, PA, USA). For each cell line, twenty randomly selected fields were acquired with a 20X objective. The Image J software (US National Institutes of Health, USA) was used to determine the fluorescence intensity as previously described (Rangel-Pozzo *et al*, 2021).

Fluorescence-activated cell sorting (FACS) analysis

To investigate cell death, we performed a flow cytometric analysis using the cell apoptosis kit Annexin V/Propidium Iodide (PI) double staining uptake (Invitrogen, Life Technologies, Italy). Colorectal cancer cell lines (Caco-2, HCT116, HT29 and SW480) were seeded at 5×10^4 cells/mL in 6-well plates (Euroclone) and cultured in DMEM High

glucose medium, added with 10% FBS and in the presence of glutamine 4 mM (complete medium) for 48h. Subsequently, the cancer cells were exposed to a complete medium, or glutamine deprived medium, or treated with CB839 0.25 μM for 48h. Next, cancer cells were washed with PBS 1X, added with 100 μL of annexin binding buffer plus 1 μL of Annexin V fluorescein isothiocyanate and 1 μL of PI, and incubated in the dark for 15 min at room temperature. Stained cells were then subjected to flow cytometric analysis, by measuring the fluorescence emission at 530 and 620 nm using a 488 nm excitation laser (MoFloAstrios EQ, Beckman Coulter). The evaluation of apoptosis was performed using Software Summit Version 6.3.1.1, Beckman Coulter.

The cell cycle phases were evaluated in colorectal cancer cells in the same conditions as described above for cell death analysis. The cells were cultured in the presence or the absence of glutamine, or with CB839 0.25 μM for 48h, and then harvested after 48h. Subsequently, the cells were resuspended in TBS buffer (200 μL) and stained with PI (Invitrogen, Life Technologies, Italy) for 30 min in the dark at room temperature. The cell cycle was evaluated with a flow cytometric analysis, by measuring the fluorescence emission at 530 and 620 nm using a 488 nm excitation laser (MoFloAstrios EQ, Beckman Coulter). The percentage of G0-G1, S and G2/M phases were obtained with FlowJo® software (Version 7.1. Becton, Dickinson & Company, Ashland, OR, USA),

Sample preparation for metabolomics analysis

The intracellular metabolites of Caco-2, HCT116, HT29, SW480 cell lines were extracted to perform metabolomic analysis by GC-MS approach. Briefly, cells were seeded in 6-well plates (5 x 10^5 cells/ml) in a complete medium. After 4 days, cells were cultured in the presence or the absence of glutamine for an additional 48h. Cells were washed twice with a physiological solution and 500 μL of an ice-cold 80% methanol solution was added. After 10 minutes at 4°C, cells were scraped and transferred in Eppendorf™ tubes. Subsequently, samples underwent 10 minutes of ultrasonication at 4°C, to ensure complete lysis of cell membranes. Then, centrifugation at 4500 rpm for 30 minutes at 4°C was carried out. In the end, 400 μL of the upper aqueous phases were collected in Eppendorf™ tubes and dried overnight in an Eppendorf™ Concentrator Plus. Afterwards, samples were derivatised. 50

μL of a solution of methoxyamine in pyridine (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were added to the dried pellet for 1 h at 70°C. The second step consisted of adding 100 μL of N-Methyl-N-(trimethylsilyl)-trifluoroacetamide, MSTFA, (Sigma-Aldrich, St. Louis, MO, USA) and incubating samples at room temperature for one hour. Subsequently, 50 μL of hexane were added and the solution was transferred in a vial for the GC-MS analysis. 10 μL from each sample were collected in a pool and used as quality control (QC).

Gas chromatography-mass spectrometry analysis

Samples were injected splitless mode into a 7890A gas chromatograph coupled with a 5975C Network mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 0.25 mm ID, fused silica capillary column, packaged with a 0.25 μM TG-5MS stationary phase (Thermo Fisher Scientific, Waltham, MA, USA). The injector and transfer line temperatures were 250°C and 280°C, respectively. The gas flow rate through the column was 1 ml/min. Initially, the column temperature was set at 60 °C for 3 min. After, the temperature was increased up to 140°C at 7°C/min and held at 140°C for 4 minutes. Subsequently, the temperature was raised to 300°C at 5°C/min and kept in isocratic mode at 300°C for 1 minute (Santoru *et al*, 2020). Identification of metabolites was performed using the standard NIST 08 (http://www.nist.gov/srd/mslist.cfm), GMD (http://gmd.mpimp/golm.mpg.de) mass spectra libraries and AMDIS software (Automated Mass Spectral Deconvolution and Identification System), freely available on www.amdis.net. Peak deconvolution, filtering, and normalization were performed using MassHunter Profinder Software from Agilent Technologies (La Jolla, CA, USA).

Statistical Analysis

Two main types of statistical analysis were performed:

- Multivariate statistical analysis

Multivariate statistical data analysis was performed using SIMCA (version 14.0, Umetrics, Umea, Sweden) program. The GC-MS chromatograms were processed using the Profinder program, and the output data were organized into data matrices where the rows represent the samples (observations) and the columns represent the variables (area of the chromatographic peak). The final dataset was normalized to the total area to minimize the effects of variable dilution of the samples. The generated matrix was imported into SIMCA software and submitted to UV-scaling before the multivariate statistical analysis. An unsupervised analysis, Principal component analysis (PCA), was performed to identify any possible trends and/or outliers. The original variables are transformed into a set of new variables, the principal components (PCs), which are uncorrelated and ordered in descending order of explained variance. Graphically, the output from the PCA analysis consists of a score plot, giving an indication of any grouping in the data sets in terms of metabolomic similarity, and loadings plot, indicating which variables are important for the patterns obtained in the scores plot (Bro and Smilde, 2014).

- Univariate statistical analysis

The most significant variables were extracted by the loadings plot of each model and undergo the univariate statistical analysis using GraphPad Prism software (version 7.01, GraphPad Software, Inc., CA, USA). Statistical significance was assessed using Student t-Test and a p-value of < 0.05 was considered statistically significant.

IV. Results

Sensitivity to glutamine starvation of colorectal cancer cell lines

To evaluate the cell response to glutamine deprivation, the growth rate of four colorectal cancer cell lines was analysed by MTT assay at different time points. After seeding, cancer cells were maintained for 48h in a complete medium (DMEM high glucose added with glutamine 4 mM and sodium pyruvate 1mM) to allow cell attachment and growth and then were cultured in complete medium with glutamine 4 mM (Cont) or in a complete medium without glutamine (-Gln) for additional 4 days. The results showed that glutamine starvation significantly affected cell growth and viability of Caco-2, HT-29, SW480 and HCT116 cells (Fig. 1).

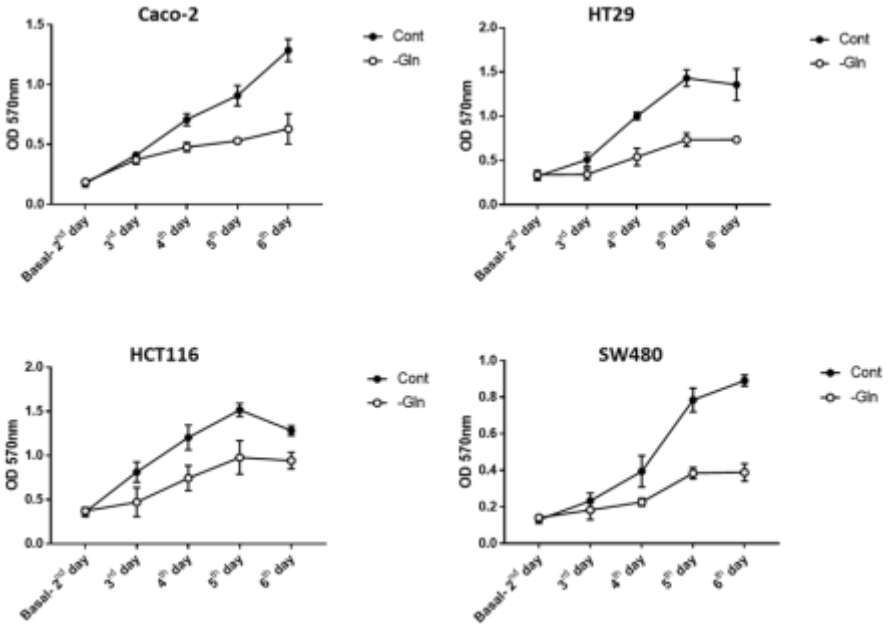


Figure 1. Growth curves of colon cancer cell lines under glutamine starvation condition for 4 days. Cell viability was assessed by MTT assay at the indicated time points in the presence or absence of glutamine in the medium. Representative growth curves of an independent experiment are shown. Data are presented as means ± Standard Deviation (n=3).

Interestingly, HCT116 cells showed the lowest sensitivity to glutamine deprivation. Particularly, after 4 days of glutamine withdrawal, the viability of HCT116 cells in glutamine-deprived condition was 75% respect to the same cells grew in complete medium (Cont), whereas the others cell lines displayed a growth rate of about 50-58% compared to the same cells cultured in complete medium (Fig. 1 and Tab. 2).

	Cell viability in -Gln condition (% of controls)	±SD
Caco-2	57.76	7.97
HCT116	74.78	3.48
HT29	50.7	5.34
SW480	49.79	8.78

Table 2. Cell viability in colorectal cancer cell lines after 4 days of glutamine starvation. Cell viability was assessed by MTT assay in the presence or absence of glutamine in the medium (at 6th day of Fig. 1). Data represent the cell viability of cancer cells under glutamine deprivation condition expressed as percentage of control cells (grew in the presence of glutamine) and represents the average of at least three independent experiments ± Standard Deviation (SD).

These observations were also confirmed by colony forming assay: a significant reduction in colony forming ability was observed in all cell lines (Fig. 2). These results confirmed that glutamine represented an important amino acid, and its deprivation altered the proliferation rate and growth capacity of these colorectal cancer cell lines.

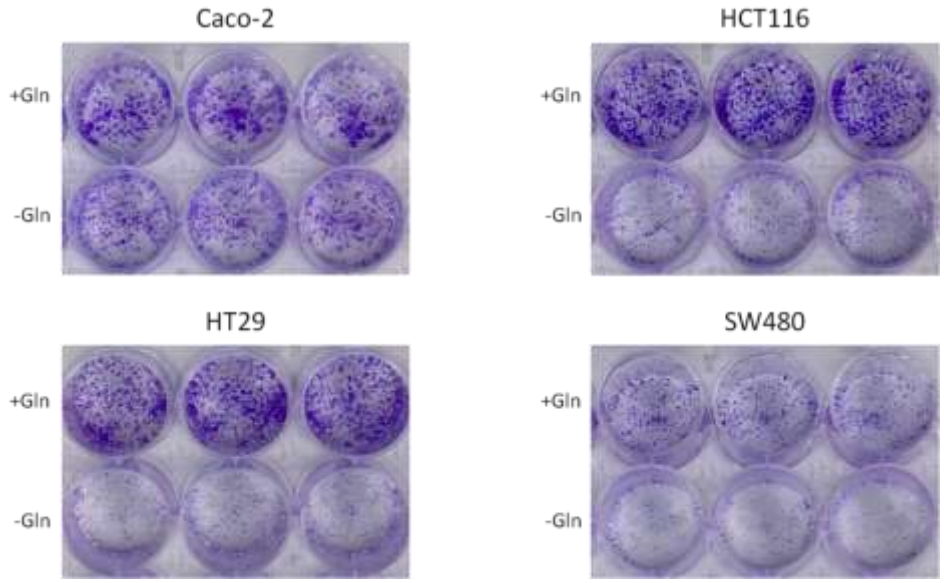
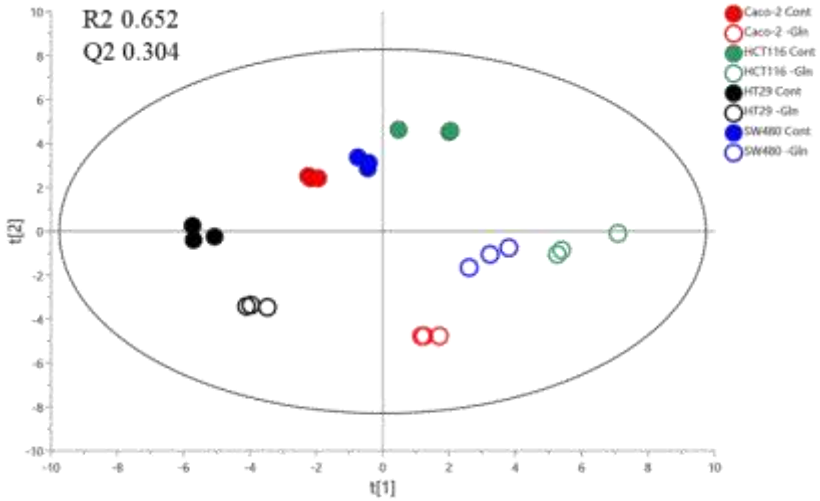


Figure 2. Glutamine deprivation reduces the colony forming ability of colorectal cancer cells. Cells were seeded in 6 well plate (500 cell/well) and left to grow for 48h in complete medium (+Gln). Cells were then shifted to glutamine deprived medium (-Gln) or to glutamine-added medium (+Gln) and were fixed with crystal violet after 6 days for Caco-2, HCT116 and HT29 and 10 days for SW480.

Metabolomic analysis of cancer cells under glutamine deprivation condition

The metabolic alterations that characterize cancer cell growth under glutamine starvation conditions were dissected using a GC-MS metabolomic approach. Colorectal cancer cell lines were seeded and cultured in the presence of glutamine for 4 days to allow cell proliferation under optimal growth conditions and then exposed to glutamine starvation for 48h. Intracellular hydrophilic metabolites were collected and analyzed by GC-MS and metabolomic data were subjected Principal Component Analysis (PCA) (Fig. 3A). Eight different groups were generated in the score scatter plot with a good separation (R2X 0.652), highlighting a different metabolic profile among cell lines and, more interestingly, between cells deprived of glutamine and their respective control. This data suggested that glutamine starvation induced alterations in the metabolic profile of cancer cell lines. A univariate statistical analysis was performed for each cell line cultured in complete medium or in glutamine-free medium to identify significantly altered metabolites under glutamine depletion (Fig. 3B). According to the relevant role of glutamine in energetic and anabolic process (Zhang *et al*, 2017), a significant alteration in carbohydrates (glucose and galactose), aminoacids (serine, glycine, threonine, glutamate, beta-alanine), organic acid (pyroglutamic acid) and polyol (myo-inositol) was observed in all cell lines after glutamine withdrawal. Furthermore, an elevated number of metabolic compounds were significantly deregulated in at least three cell lines: glycerol 3-phosphate, valine, phenylalanine, tyrosine, and amminomalonic-acid were upregulated whereas, aspartic acid, gamma-amminobutirric acid and lactic acid were down-regulated after glutamine withdrawal, indicating that glutamine starvation induced a similar metabolic reprogramming in these cancer cells (Fig. 3B).

A



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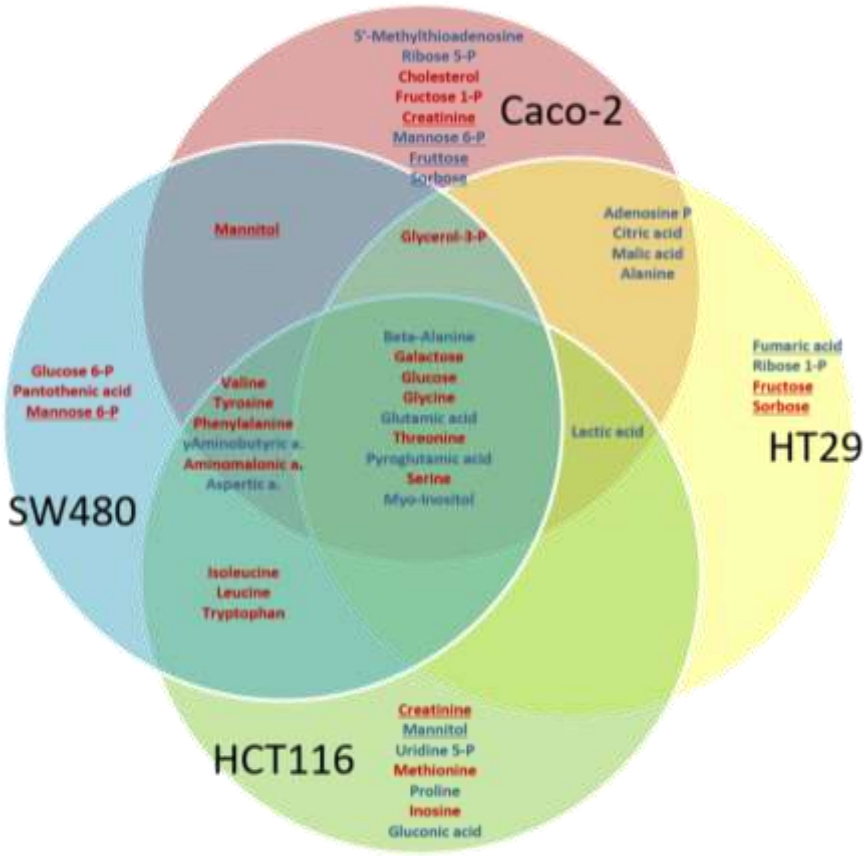
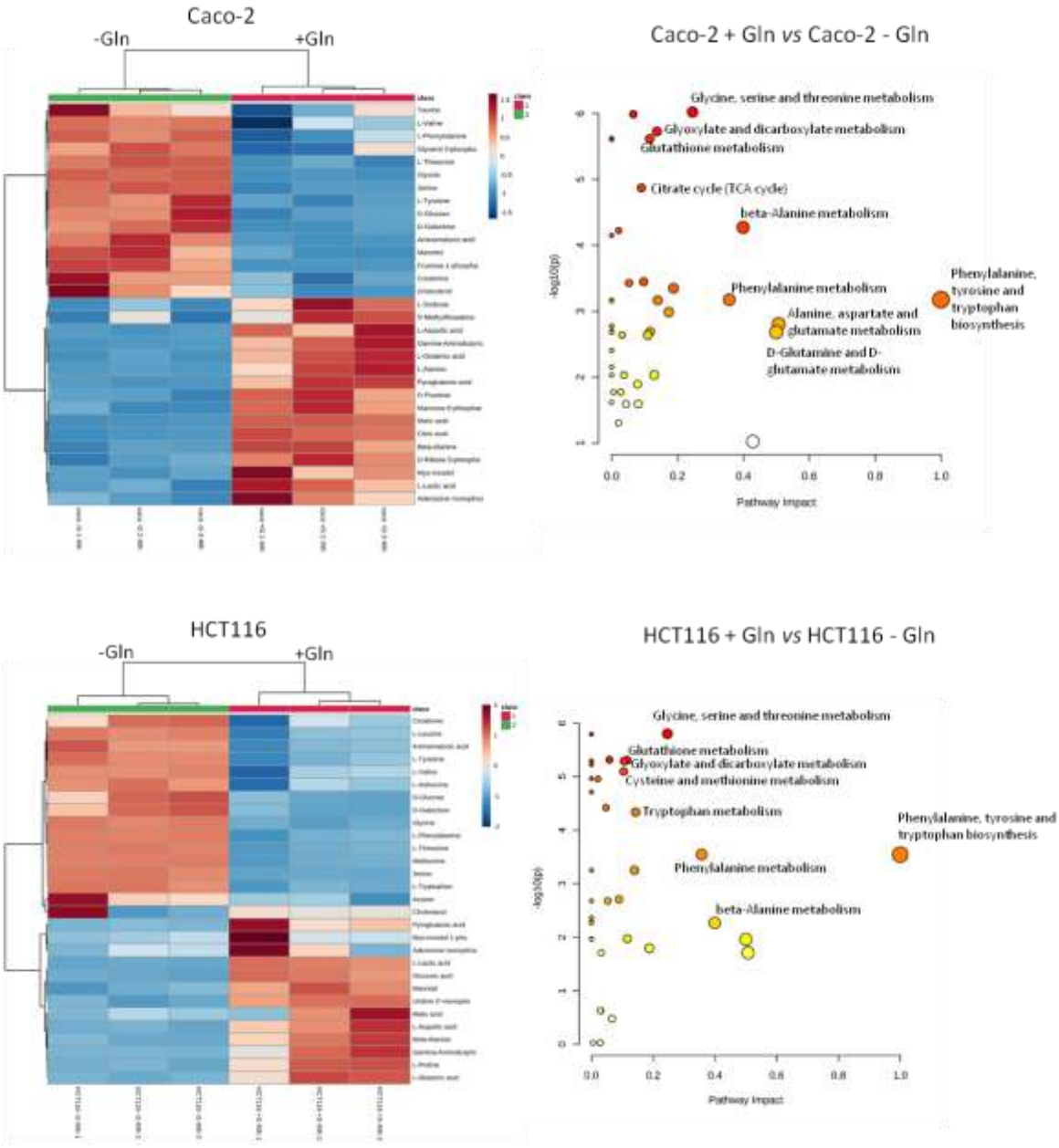


Figure 3. Metabolomic analysis of colorectal cancer cell lines under glutamine starvation revealed significant variations of metabolic profiles. A) PCA of cancer cells in the presence (Cont) or absence (-Gln) of glutamine. **B)** Schematic representation of significantly altered metabolites in glutamine deprivation. Red and blue colours indicated upregulated or deregulated metabolite levels, respectively, in glutamine deprived conditions. Statistical analysis was performed by Student t-Test. Results were considered significant when *P<0.05.

Consequently, Heatmap and Pathway Analysis of differential metabolites displayed an elevated number of common features among the different cell lines. Glutamine deprivation induced significant alteration in numerous metabolic products involved in different pathways (glyoxylate and dicarboxylate metabolism; glycine, serine and threonine metabolism; glutathione metabolism; alanine, aspartate and glutamate metabolism; beta-alanine metabolism) (Fig. 4).



(Figure 4 is divided in two pages)

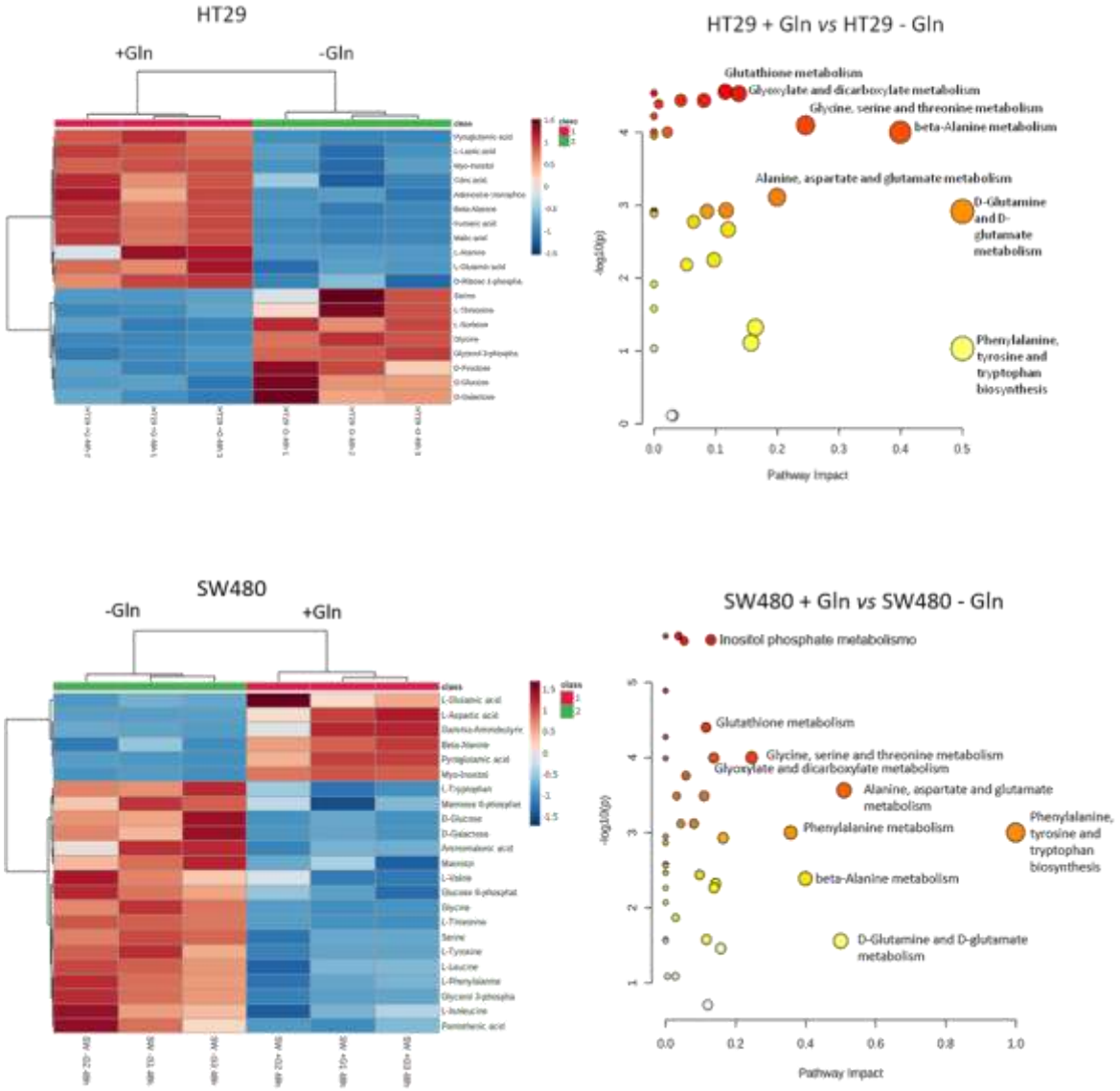


Figure 4. Differential metabolites induced by glutamine starvation are associated to bioenergetic and biosynthetic pathways. Heatmaps (on the left) and Pathway analysis (on the right) of differential metabolites were obtained with the Metaboanalyst 5.0 platform. Class 1 (bright red block) indicates cell lines cultured in the presence of glutamine (4mM) whereas class 2 (green block) indicates cell lines grew in the absence of glutamine in the medium (DMEM High glucose without glutamine). The dark red and blue color scale indicate the relative concentration for each metabolite. Expression of the significant metabolites is shown as a result of triplicate experiments.

The altered pathways in glutamine deprivation conditions reflected the pivotal role of glutamine in energetic and biosynthetic pathways and redox homeostasis. However, differences were also found between the colorectal cancer cell lines that could be

associated with glutamine dependency. Particularly, a significant reduction of TCA cycle intermediates was observed in Caco-2, HT29, and SW480 but not in HCT116 cell lines under glutamine deprivation. Additionally, a significant reduction of uridine 5-phosphate, proline, and gluconic acid and a significant increase of methionine and inosine were observed only in HCT116 cell line. Furthermore, an increase in glucose 6-phosphate and pantothenic acid were detected in SW480 cells and an enhanced level of intracellular cholesterol and reduced levels of 5-methylthioadenosine and ribose 5-phosphate were found in Caco-2 cells. Interestingly, ribose 1-phosphate, an intermediate in purine metabolism, was exclusively detected in HT29 cells, and was reduced in glutamine starvation (Fig. 3 and 4). All metabolic compounds detected by GC-MS analysis, their profiles, and statistical significance levels were reported in table 3.

Metabolites	Caco-2		HCT116		HT29		SW480	
	Trend	P value	trend	P value	trend	P value	trend	P value
Adenosine	/	/	/	/	↑	ns	/	/
Adenosine monophosphate	↓	0.0135	↓	ns	↓	0.0017	↓	ns
Aminomalonc acid	↑	0.0003	↑	0.0011	/	/	↑	0.0154
Beta-Alanine	↓	0.0007	↓	0.0054	↓	<0.0001	↓	0.0016
Beta-Glycerophosphoric acid	=	ns	=	ns	/	/	/	/
Cholesterol	↑	0.0169	=	ns	↓	ns	↓	ns
Citric acid	↓	<0.0001	↓	ns	↓	0.0029	↓	ns
Creatinine	↑	0.0059	↑	0.0342	/	/	/	/
D-Fructose	↓	0.0010	=	ns	↑	0.0062	/	/
D-Galactose	↑	0.0004	↑	0.0021	↑	0.0065	↑	0.0042
D-Glucose	↑	0.0011	↑	0.0040	↑	0.0049	↑	0.0030
D-Glucose 6-phosphate	↑	ns	↑	ns	=	ns	↑	0.0036
D-Ribose 1-phosphate	/	/	/	/	↓	0.0013		/
D-Ribose 5-phosphate	↓	0.0010	↓	ns	↓	ns	↑	ns
Fructose 1-phosphate	↑	0.0005	/	/	/	/	/	/
Fumaric acid	↓	ns	↓	ns	↓	<0.0001	↓	0.0003
Gamma-Aminobutyric acid	↓	0.0025	↓	0.0210	↑	ns	↓	0.0199
Gluconic acid	/	/	↓	<0.0001	/	/	/	/
Glycerol 3-phosphate	↑	0.0256	↑	ns	↑	<0.0001	↑	0.0008
Glycine	↑	<0.0001	↑	<0.0001	↑	<0.0001	↑	0.0002
Inosine	/	/	↑	0.0443	/	/	/	/
L-Alanine	↓	0.0070	↓	ns	↓	0.0262	↓	ns
L-Aspartic acid	↓	0.0039	↓	0.0050	/	/	↓	0.0091
L-Glutamic acid	↓	0.0021	↓	0.0109	↓	0.0012	↓	0.0270
L-Isoluecine	↑	ns	↑	0.0068	/	/	↑	0.0193
L-Lactic acid	↓	0.0039	↓	<0.0001	↓	0.0001	↓	ns
L-Leucine	↑	ns	↑	0.0011	/	/	↑	0.0024
L-Methionine	/	/	↑	<0.0001	/	/	/	/
L-Phenylalanine	↑	0.0064	↑	<0.0001	/	/	↑	0.0016
L-Proline	=	ns	↓	0.0068	/	/	↓	ns
L-Sorbose	↑	0.0114	=	ns	↑	0.0004	/	/
L-Threonine	↑	<0.0001	↑	<0.0001	↑	0.0121	↑	<0.0001
L-Tryptophan	↑	ns	↑	<0.0001	/	/	↑	0.0030
L-Tyrosine	↑	0.0007	↑	0.0006	↑	ns	↑	0.0014
L-Valine	↑	0.0242	↑	0.0093	↑	ns	↑	0.0244
Malic acid	↓	<0.0001	↓	ns	↓	<0.0001	↓	ns
Mannitol	↑	0.0022	↓	0.0006	/	/	↑	0.0095
Mannose 6-phosphate	↓	0.001	↑	ns	↓	0.0775	↑	0.0180
Myo-Inositol	↓	0.0093	↓	0.0010	↓	0.0001	↓	<0.0001
Myo-Inositol 1-phosphate	=	ns	↓	ns	=	ns	↓	ns
Niacinamide	/	/	=	ns	/	/	↓	ns
Pantothenic acid	↓	ns	=	ns	=	ns	↑	0.0112
Pyroglutamic acid	↓	0.0022	↓	0.0333	↓	<0.0001	↓	0.0011
Serine	↑	<0.0001	↑	<0.0001	↑	0.03052	↑	0.0004
Taurine	↑	ns	↓	ns	/	/	↑	ns
Uridine 5-monophosphate	↑	ns	↓	0.0001	↓	ns	/	/
5'Methylthioadenosine	↓	0.0500	=	ns	=	ns	=	ns

Table 3. Statistical parameters of metabolites detected with GC-MS analysis after 48 hours of glutamine deprivation. Trends were expressed in comparison with the control (+Gln), red arrows for increased levels and blue arrows for decreased ones. The slash indicates that the metabolite was not identified in that sample.

Glutamine starvation induced an increase in glucose uptake

Based on the metabolomics analysis, a significant increase of intracellular glucose was observed in all cell lines by glutamine starvation (Fig. 3 and 4). To clarify if this observation was related to enhanced uptake of glucose through the plasma membrane, the evaluation of 2-NBDG uptake, a fluorescent analogue of glucose, and the expression of GLUT1 were performed under glutamine deprivation conditions. A significant increase of 2-NBDG uptake was detected in Caco-2 and SW480 cells whereas in HCT116 and HT29 cells only a rising trend was observed without reaching statistical significance (Fig. 5).

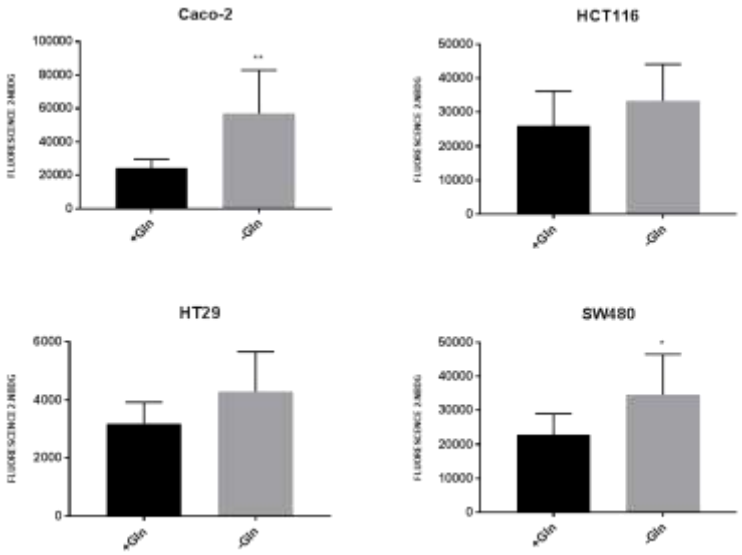
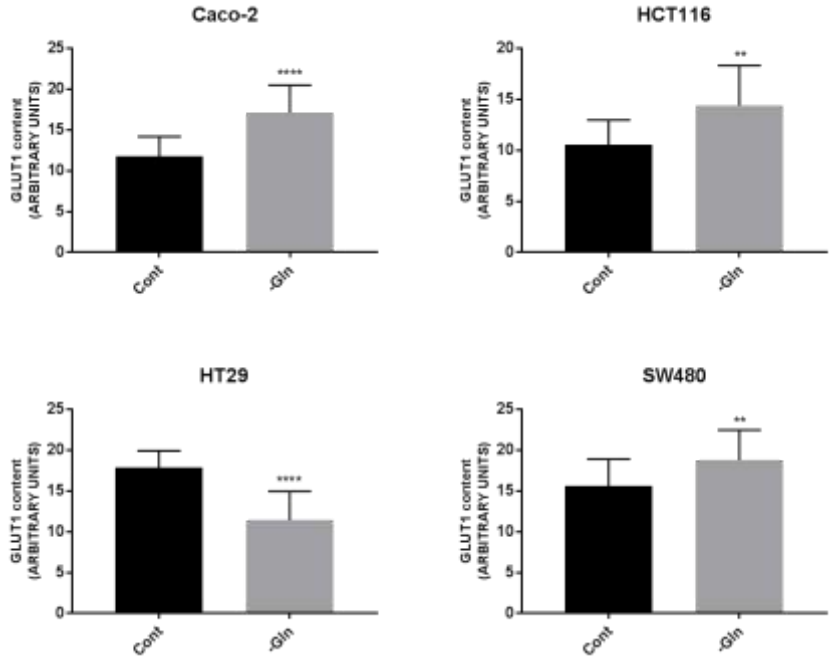


Figure 5. Colorectal cancer cells lines exhibited an increased glucose uptake under glutamine deprivation. Glucose uptake was measured through the quantification of the fluorescent glucose analog 2-NBDG in cancer cells cultured in the presence or the absence of glutamine 4 mM for 48h. Data are expressed as media ± SD (n=3). Statistical analysis was performed by Student t-Test. Results were considered significant when * P<0.05

We also analyze the glucose transporter GLUT1, which is a glucose transporter frequently upregulated in cancer cells (Ancey *et al*, 2018) by immunofluorescence. We observed a significantly increased expression of GLUT1 in Caco-2, HCT116, and SW480 cell lines under glutamine deprivation conditions compared to relative controls (Fig. 6). Surprisingly, HT29 cells showed a significant reduction of GLUT1 expression after 48h of glutamine withdrawal, indicating that in this cell line other mechanisms contributed to the increase in intracellular glucose levels (Fig. 6).

A



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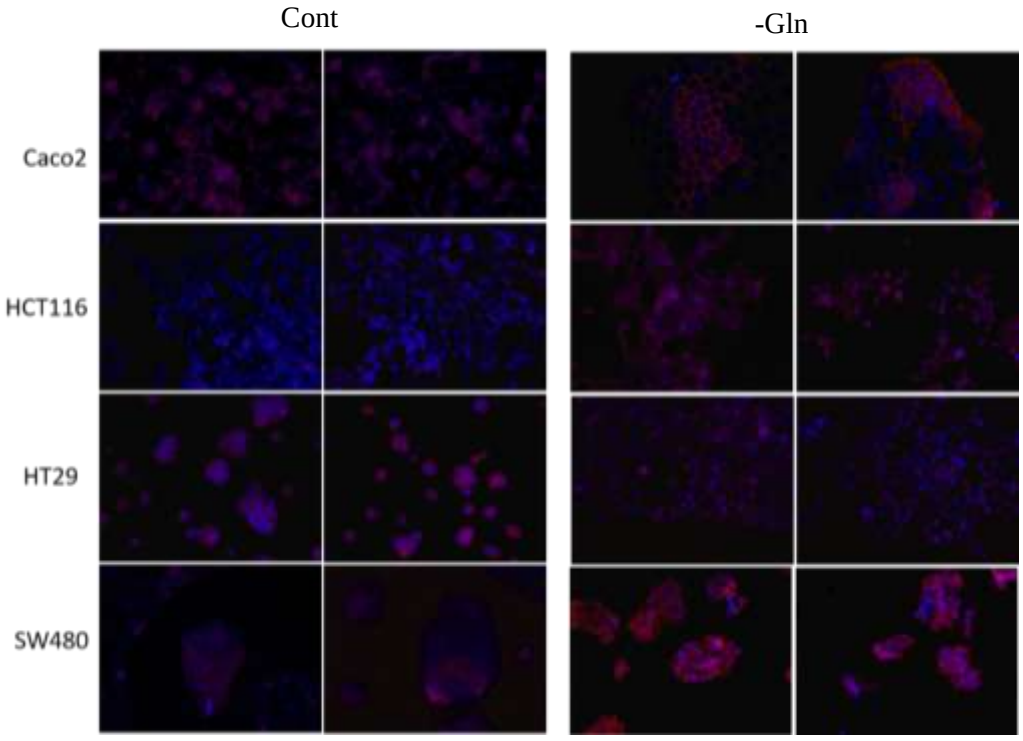


Figure 6. Glutamine starvation altered the expression of glucose transporter GLUT1. A) Quantification of immune fluorescent pattern in the colorectal cancer cell lines after 48h of glutamine deprivation condition (-Gln) and relative controls (Cont) grew in the presence of glutamine. **B)** Microphotographs of colorectal cancer cells with GLUT1 stained in red and nuclei stained with DAPI in blue.

Glutamine deprivation induced oxidative stress in colorectal cancer cell lines

Glutamine has an important role in redox homeostasis (Amores-Sanchez and Medina, 1999) so to assess the changes in redox balance induced by glutamine starvation, the reduced and oxidized glutathione forms (GSH and GSSG, respectively) were evaluated by HPLC. A significant reduction in GSH/GSSG ratios was observed in all cell lines after 48 hours of glutamine starvation (-Gln) compared to the same cell lines grown in complete medium (+Gln) (Fig. 7). This indicated that glutamine fundamentally contributed to maintaining proper antioxidant defences. Interestingly, GSH levels are significantly higher in the HCT116 than the other cell lines and this could suggest a possible role of glutathione levels in the lower sensitivity after 48 hours of deprivation of this cell line to glutamine deprivation.

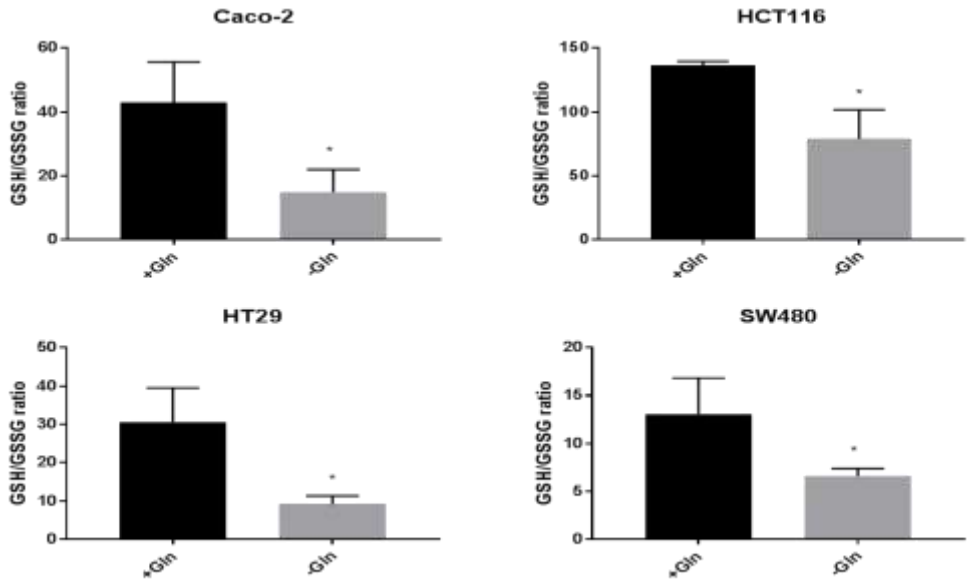


Figure 7. Glutamine starvation significantly decreased intracellular glutathione levels. GSH and GSSG levels were measured by HPLC in colorectal cancer cells after 48h of glutamine deprivation condition. Experiments were performed in triplicate and data were expressed as mean ± SD.

Evaluation of the combined effect of 5-Fu or campothecin and glutamine deprivation on colorectal cancer cells

We next sought to analyse whether glutamine deprivation can sensitize cells to anticancer molecules conventionally used for colorectal cancer therapy. Cell lines were cultured in the presence or the absence of glutamine (+Gln or –Gln, respectively) and exposed to the different doses of 5-Fluorouracil (5-FU) and Camptothecin (CPT) for 48h. Glutamine deprivation failed to sensitize colorectal cancer cells to 5-FU: the inhibition of cell proliferation was quite similar in the presence or absence of glutamine for the reported drug concentrations (Fig. 8).

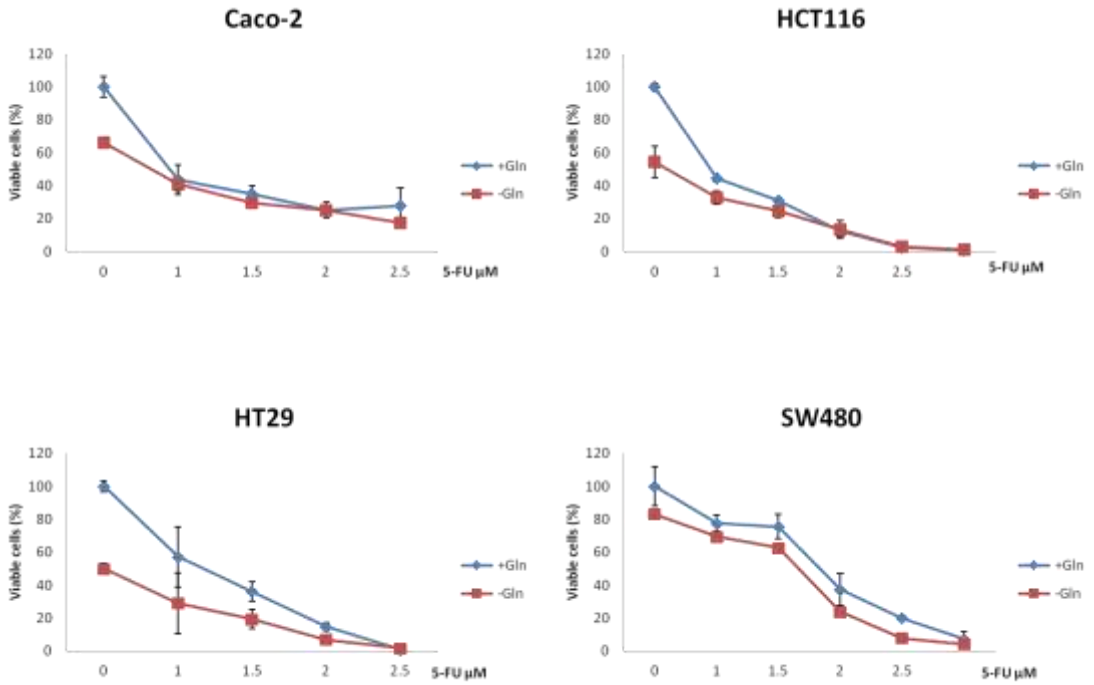


Figure 8. Growth curves of colorectal cancer cells treated with different concentrations of 5-FU in the presence or absence of glutamine. Colorectal cancer cells were initially cultured in complete medium (+Gln) for 48h, then shifted to glutamine-deprived medium or glutamine-supplemented medium (+Gln) for 48h and treated with 5-FU at indicated concentrations for an additional 48h. Experiments were performed in triplicate and expressed as mean ±SD.

On the other hand, camptothecin exerted a greater antiproliferative effect when colorectal cells were cultured in the absence of glutamine compared to cells that grew in the presence of glutamine (Fig. 9) suggesting a synergistic inhibitory effect of the combined treatments.

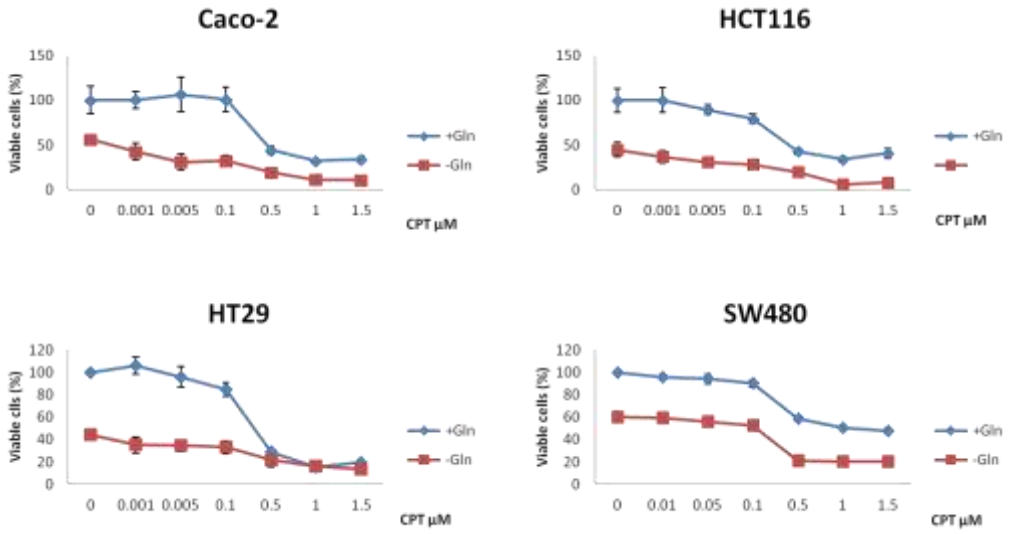


Figure 9. Growth curves of colorectal cancer cells treated with different concentrations of CPT in the presence or absence of glutamine. Colorectal cancer cells were initially cultured in complete medium (+Gln) for 48h, then shifted to glutamine-deprived medium or glutamine-supplemented medium (+Gln) and treated with CPT at indicated concentrations for 48h. Experiments were performed in triplicate and expressed as mean ±SD.

Glutaminase Inhibitor CB389 exerted antiproliferative activity in colorectal cancer cell lines

The results presented so far have shown how glutamine deprivation induces a reduction in cell growth and survival. Recently, numerous compounds that interfere with the glutamine metabolic pathway have also been tested in clinical trials (Jin *et al*, 2016). In addition, one of the key enzymes in glutamine metabolism, the mitochondrial glutaminase 1 (GLS1), has been found to increase in several cancers (Mates *et al*, 2020) and its expression is associated with tumor progression and the development of colorectal cancer metastases (Huang *et al*, 2014; Xiang *et al*, 2019). CB839 is a non-competitive allosteric inhibitor of glutaminase 1 tested in phases I and II of clinical trials for the treatment of solid tumors. To determine whether CB839 was able to inhibit the proliferation of colorectal cancer lines, we evaluated the growth rate in the presence of different doses of the inhibitor by MTT assay (Fig. 10). The results showed significant dose-dependent inhibition of cell proliferation in colorectal cancer cells. Cytotoxicity data showed that Caco-2 and SW480 cells were slightly less sensitive to CB389 inhibition, with an IC50 of about 0.3 μM respect to HCT116 and HT29 cells (IC50 ≈ 0.2 μM). Furthermore, quite surprisingly, HCT116 cell growth was severely impaired by 0.5 μM CB389 treatment for 48h resulting in low cell viability (about 5%), whereas the other cell lines displayed a lower sensitivity with cell viability of about 20-25% (Fig. 10).

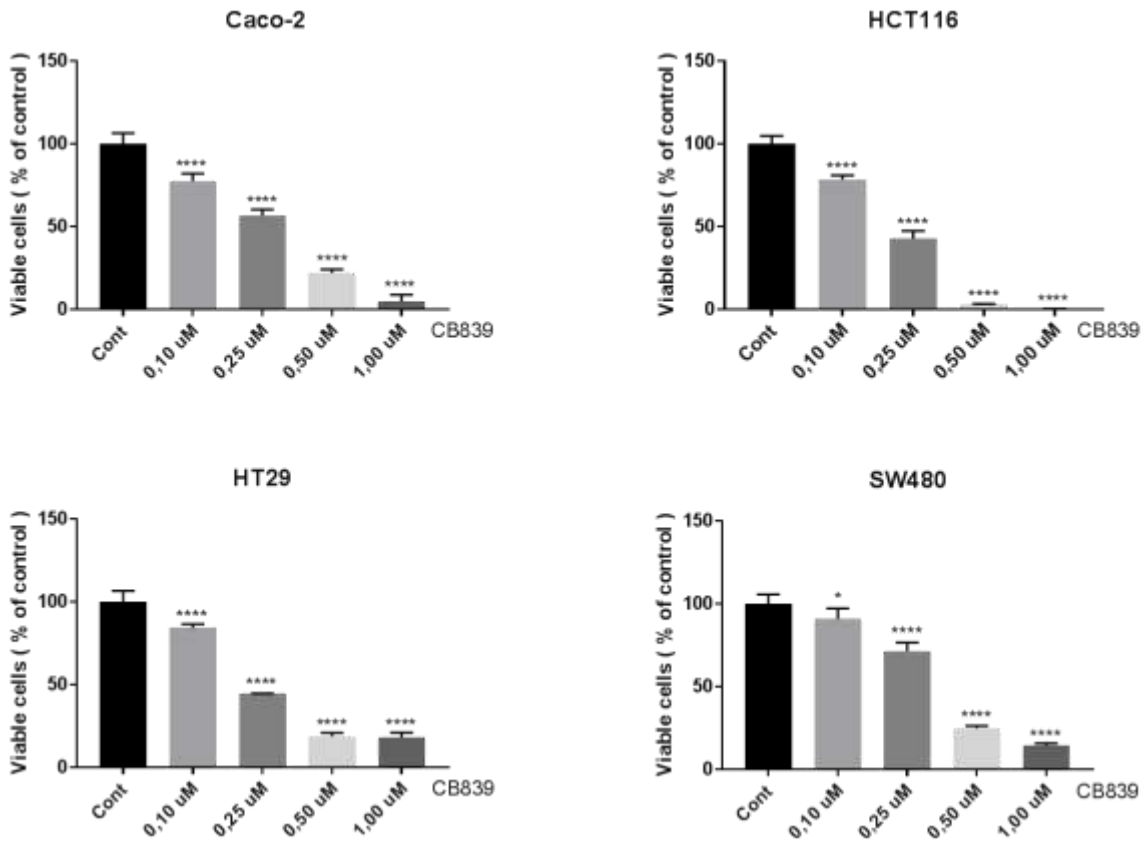


Figure 10. Viability of cells treated with CB389 (0.1 – 1 µM) for 48 h. Data are expressed as % of the control (untreated cells) ± SD. All experiments were performed three times independently.

To explore the activity of CB389 on cancer cells proliferation over a longer period we performed a growth curve and colony forming assay. We decided to use an intermediate concentration of inhibitor (0.25 µM) which inhibited growth by 30-60% in cancer cells. Colorectal cancer cells were seeded and cultured with complete medium (+Gln) for 48h and then treated with CB389 0.25 µM for 4 days. MTT assay was performed at indicated time points (Fig. 11). These results showed that CB839 treatment for 4 days induced a marked reduction of the proliferative capacity of colorectal cancer cells. Most of the tested cell lines displayed decreased cell proliferation by more than observed in glutamine deprivation condition (Fig. 11). In particular, HT29 cells were the most sensitive to the inhibitory effect, with cell viability of 12% respect to the same line grew in complete medium (Cont) whereas Caco-2 and HCT116 display an intermediate inhibitory effect with a 34% and 55% of cell viability, respectively. SW480 cell lines were the least sensitive to the antiproliferative effect of CB839 with cell viability of 66% compared to controls (Tab. 4).

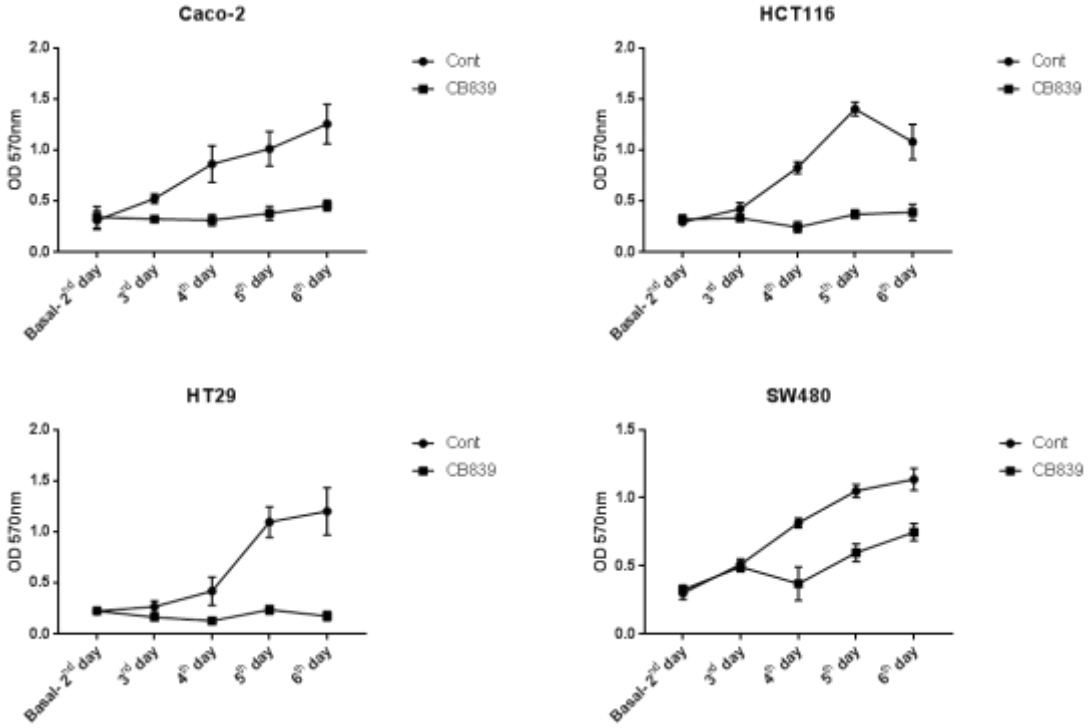


Figure 11. Glutaminase inhibitor CB389 severely affected colorectal cancer growth. Representative graphs of cell growth in the presence of CB389 0.25 μM for 4 days.

	Cell viability in CB839 treated cells (% of controls)	±SD
Caco-2	33.75	3.64
HCT116	55.16	15.01
HT29	12.38	3.06
SW480	61.74	5.68

Table 4. Cell viability of colorectal cancer cell lines treated with CB839 0.25 μM for 4 days. Data represent the cell viability percentage of colorectal cancer cells treated with CB839 compared to their control and are expressed as mean ± SD of at least three experiments.

Glutaminase Inhibitor CB389 exerted antiproliferative activity in colorectal cancer cell lines

The antiproliferative effect of the GLS inhibitor, CB839 was also analyzed by colony forming assay (Fig. 12). These results showed a marked reduction in the growth forming capacity of colorectal cancer cells exposed to CB389 (0.25 µM) for 6-10 days compared to those grown in complete medium (+ Gln).

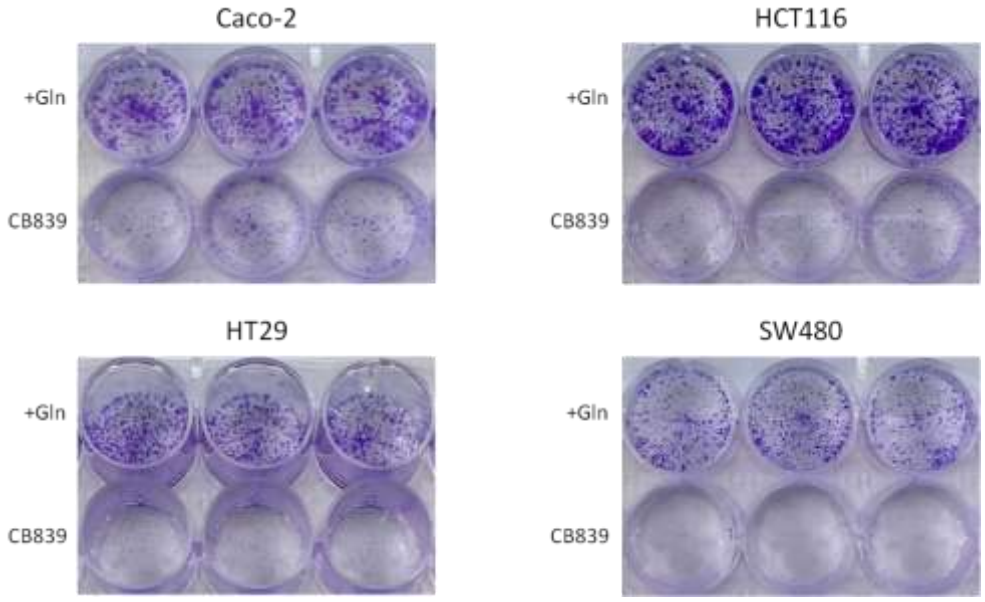


Figure 12. The GLS1 inhibitor CB839 reduced the colony forming ability of colorectal cancer cells. Cells were seeded in 6-well plates (500 cell/well) and left to grow for 48h in complete medium (+Gln). Cells were then treated with CB839 (0.25 µM) and were fixed with crystal violet after 6 days for Caco-2, HCT116 and HT29 and10 days for SW480.

CB839 altered the metabolomic profiles of colorectal cancer cell lines

To study the metabolic changes induced by the glutaminase inhibitor, we obtained the metabolic profile of cell lines treated with CB839 for 48h by a GC-MS approach. Metabolomic data were evaluated through multivariate statistical analysis and a PCA model was applied to the entire dataset of different cell lines (Fig. 13). The plot showed a clear separation between CB839 treated cancer cells and their untreated counterpart. We also observed a strong similarity between the Caco-2 and HT29 lines (both treated and untreated were very close in the plot) while the HCT116 and SW480 cells seem to be quite different from a metabolic point of view. Individual PCA analysis of the cell lines (Fig. 14) displayed a clear separation between CB839 treated and untreated cells indicating that the GLS1 inhibitor significantly affected cancer cell metabolism.

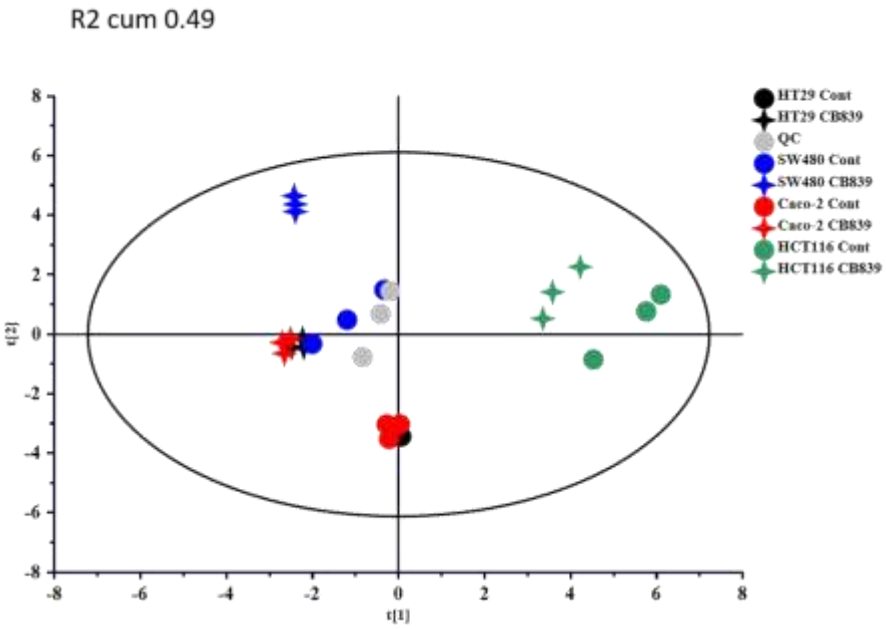


Figure 13. PCA scores plot of colorectal cancer cell lines. The colorectal cancer cell lines untreated (Cont) or treated (CB839) with CB839 (0.25 µM) for 48h were plotted, QC: quality control. Each cell line was analyzed in triplicate for the two conditions.

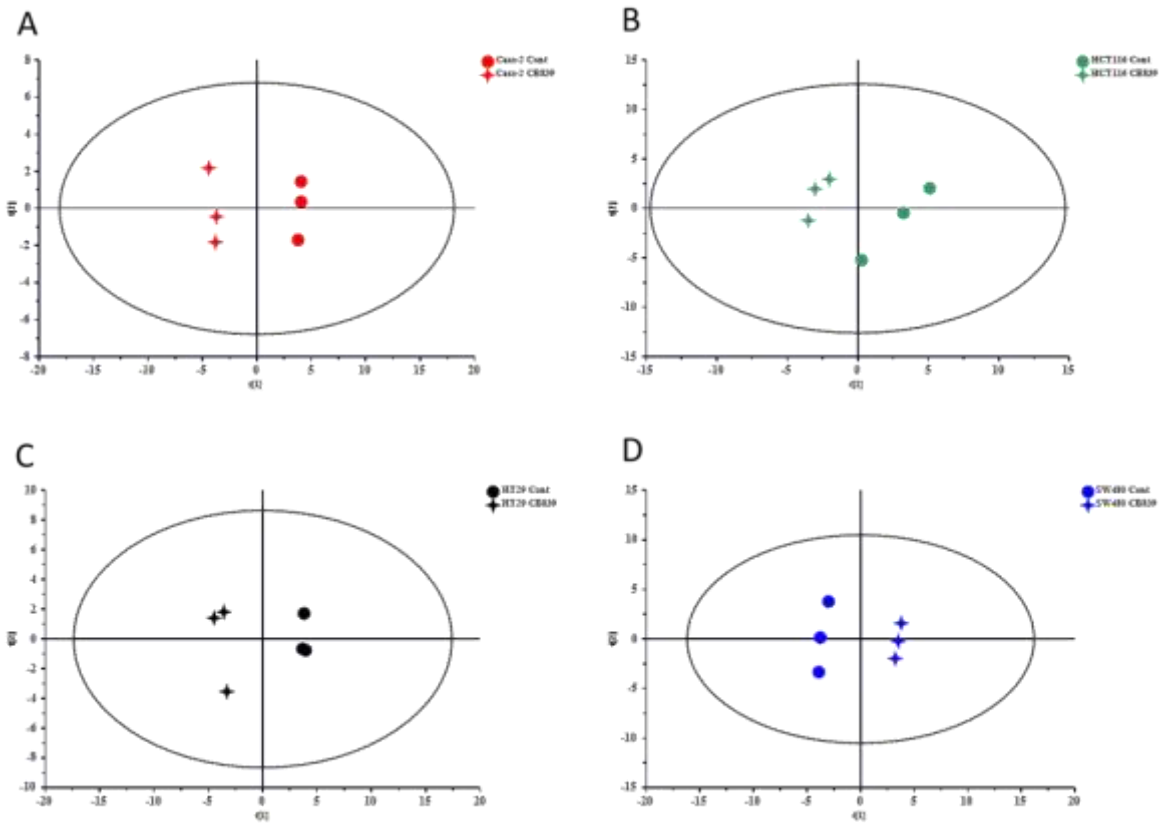
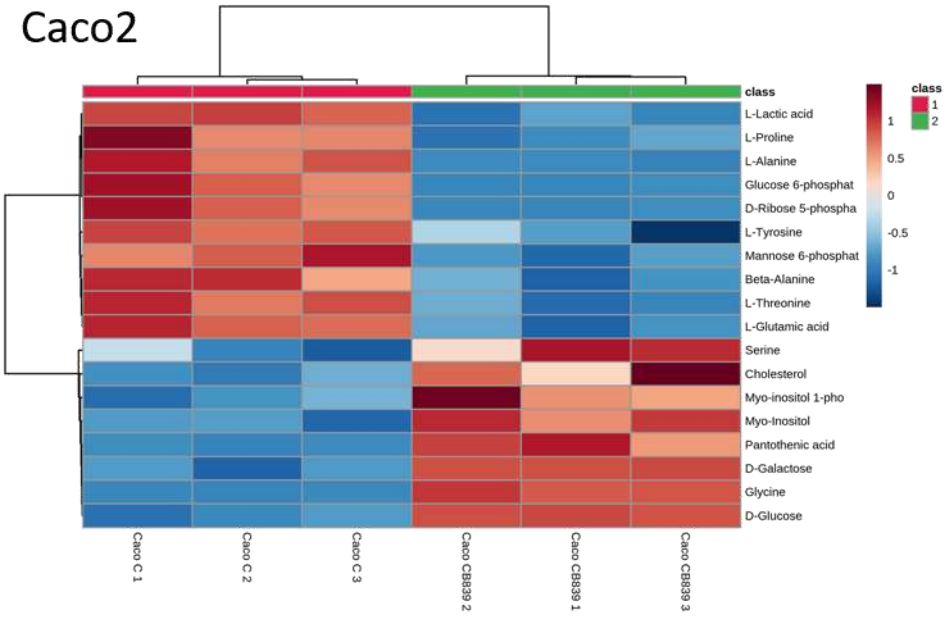
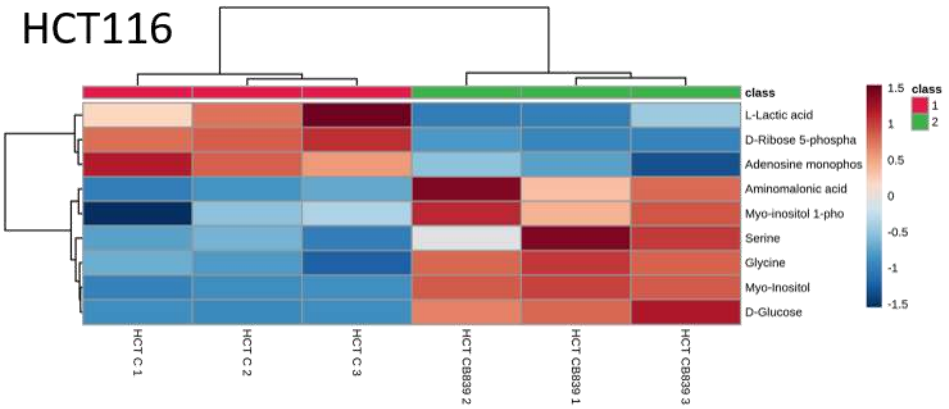


Figure 14. PCA analysis of colorectal cancer cells treated with CB839. The colorectal cancer cell lines untreated (Cont) or treated (CB839) or with CB839 (0.25 μM) for 48h were plotted: A) Caco-2, B) HCT116, C) HT29 and D) SW480 cell lines. Each cell line was analyzed in triplicate for the two conditions.

The univariate statistical analysis was then applied to identify the differences in metabolic profile induced by CB839 treatment in the colorectal cancer cell line. Furthermore, heatmaps and pathway analysis of significantly altered metabolites were constructed by Metaboanayst 5.0 platform (Fig. 15). HT29 and Caco-2 cells showed the highest number of significantly altered metabolites (19 and 18 compounds, respectively), whereas SW480 and HCT116 cell lines exhibited 12 and 9 differentially regulated metabolic products after CB839 treatment (Fig. 15).





(Figure 15 is divided in two pages)

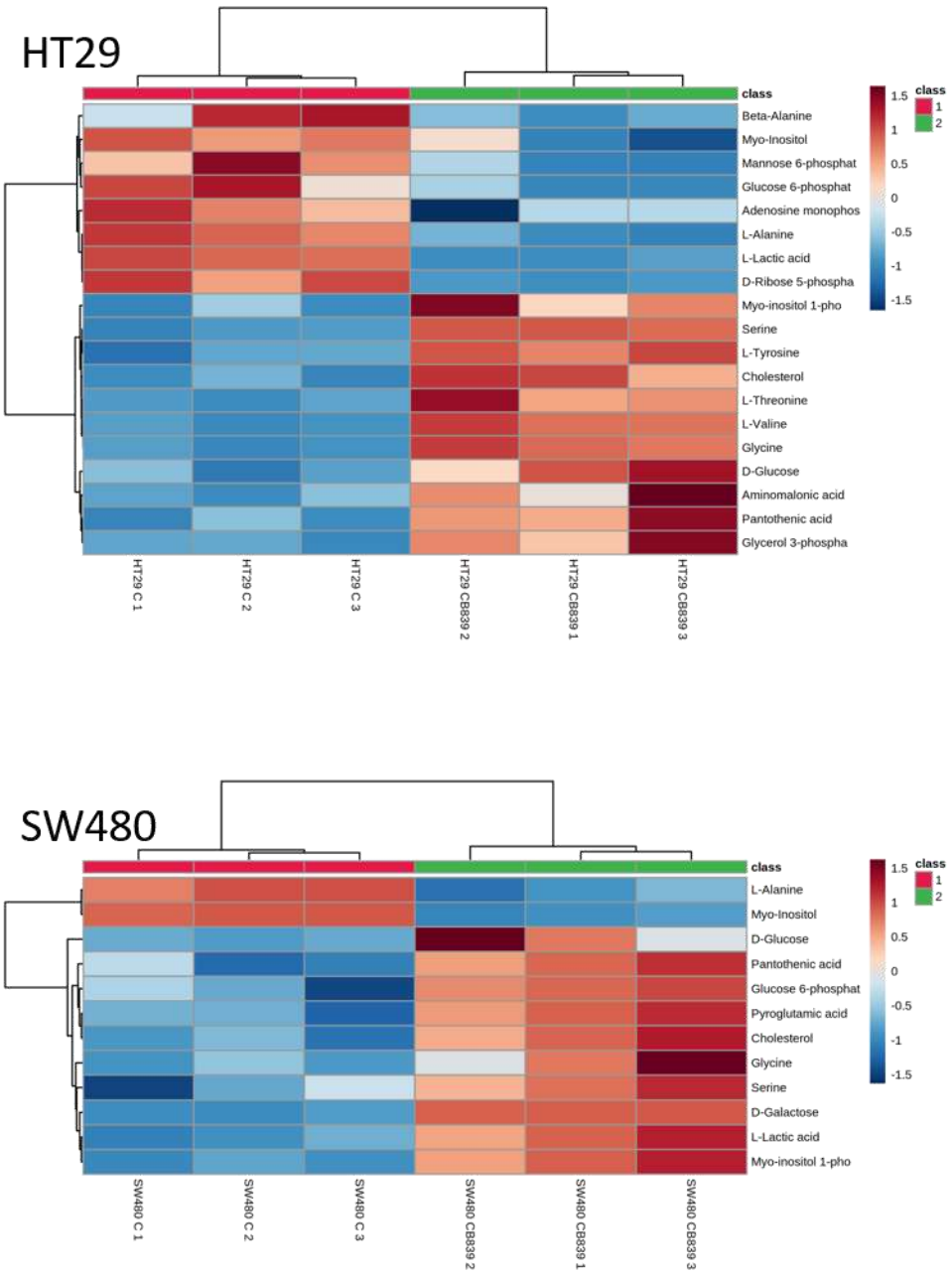


Figure 15. Differential metabolites induced by CB839 are associated with bioenergetic and biosynthetic pathways. Heatmaps and Pathway analysis of differential metabolites after CB treatment (2.5 µM) for 48h was obtained with Metaboanalyst 5.0 platform. Class 1(bright red box in the heatmap) represented untreated cells (Cont) whereas class 2 (green box in the heatmap) represented CB839 treated cells. The dark red to the blue graduated line indicated the high or low metabolite levels, respectively.

Significantly increased levels of glucose, serine, glycine, and myo-inositol 1-phosphate was observed in all cell lines. Furthermore, an elevated number of metabolic compounds were significantly upregulated in at least three cell lines after CB839 treatment, such as

pantothenic acid and cholesterol while ribose 5-phosphate, alanine, and lactic acid were found significantly downregulated in cell lines treated with glutaminase inhibitor. These data suggested that GLS1 inhibition induced similar metabolic changes in these cancer cells (Fig. 16). As already observed in glutamine deprived condition, only a few metabolites were found to be exclusively altered in each cell line after CB839 treatment (Fig. 15 and 16). Reduced levels of different amino acids (glutamic acid, proline, threonine, and tyrosine) were found in Caco-2 cells, while glycerol 3-phosphate, valine, threonine, and tyrosine were increased in HT29 (Fig. 15 and 16).

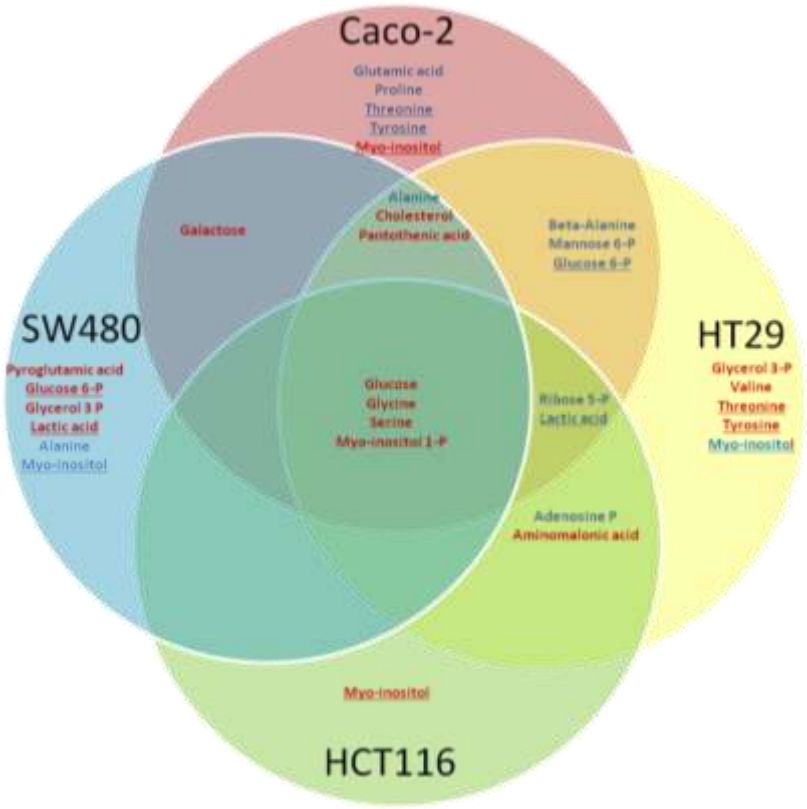
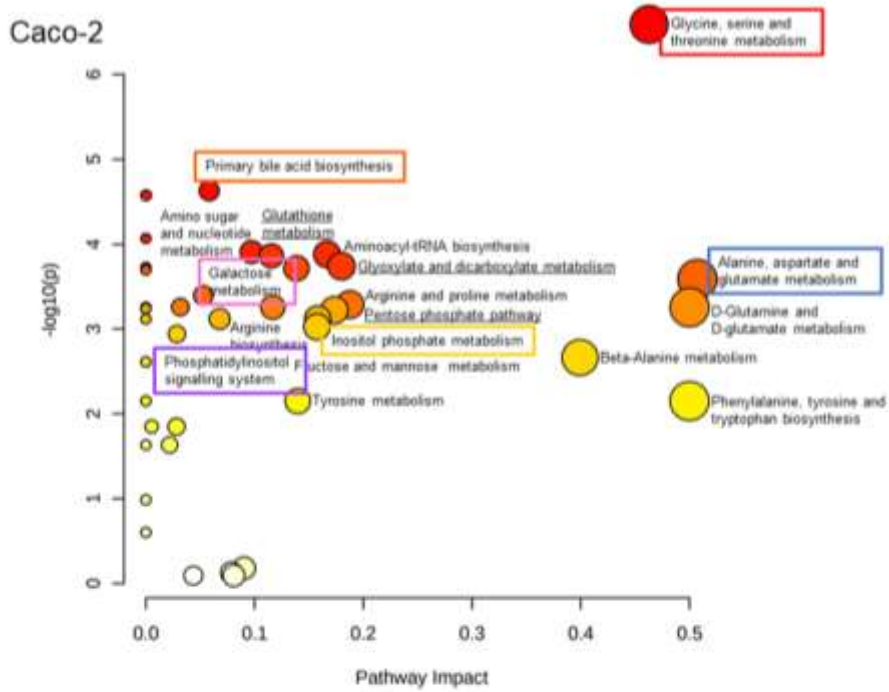
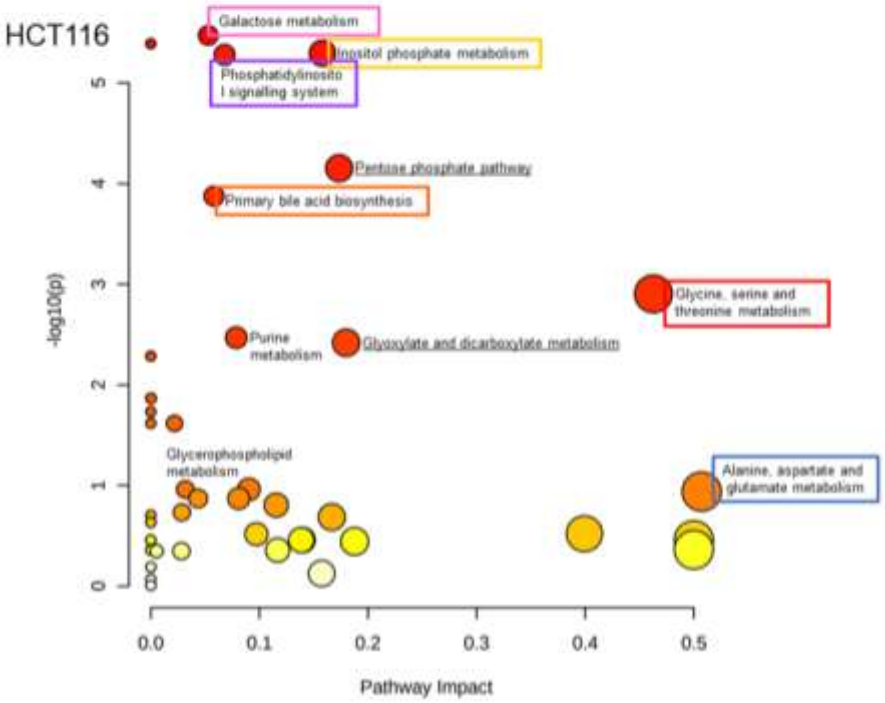


Figure 16. Metabolic profile of colorectal cancer cell lines treated with GLS1 inhibitor CB839. Differentially regulated metabolites in the analyzed cancer cells: red and blue colours indicated upregulated and downregulated compounds after CB839 treatment. Metabolites that showed an inverse trend in different cells were underlined.

Contrary to what was observed in the other cell lines, a significant increase in glucose 6-phosphate and lactic acid as well as an exclusive increase in pyroglutamic acid was found in SW480 cells treated with CB839. Furthermore, a significant increase in myo-inositol levels was observed in Caco-2 and HCT116 lines, while the same compound was found to be significantly downregulated in the other cell lines (Fig. 15 and 16). To explore CB839-

induced alterations in key metabolic pathways we performed a pathway analysis using Metaboanalyst 5.0 platform. The pharmacological inhibition of glutaminase induced a significant deregulation of metabolic compounds involved in different metabolic pathways (Fig. 17). We observed that several metabolic pathways were significantly enriched in most of the colorectal cancer cell lines: glycine, serine, and threonine metabolism; alanine, aspartate and glutamate metabolism; primary bile acid biosynthesis; inositol phosphate metabolism; phosphatidil inositol signaling system; galactose metabolism; glyoxylate and dicarboxylate metabolism; glutathione metabolism and pentose phosphate pathway. A complete list of significantly changed metabolites identified by GC-MS approach after CB839 treatment was reported in table 5.


Caco-2
Glycine, serine and threonine metabolism
Primary bile acid biosynthesis
Amino sugar and nucleotide metabolism
Glutathione metabolism
Aminoacyl-tRNA biosynthesis
Glyoxylate and dicarboxylate metabolism
Alanine, aspartate and glutamate metabolism
Galactose metabolism
Arginine and proline metabolism
D-Glutamine and D-glutamate metabolism
Pentose phosphate pathway
Arginine biosynthesis
Inositol phosphate metabolism
Beta-Alanine metabolism
Phosphatidylinositol signaling system
Fructose and mannose metabolism
Tyrosine metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
-log10(p)
0
1
2
3
4
5
6
0.0
0.1
0.2
0.3
0.4
0.5
Pathway Impact


HCT116
Galactose metabolism
Inositol phosphate metabolism
Phosphatidylinositol signaling system
Pentose phosphate pathway
Primary bile acid biosynthesis
Glycine, serine and threonine metabolism
Purine metabolism
Glyoxylate and dicarboxylate metabolism
Glycerophospholipid metabolism
Alanine, aspartate and glutamate metabolism
-log10(p)
0
1
2
3
4
5
0.0
0.1
0.2
0.3
0.4
0.5
Pathway Impact

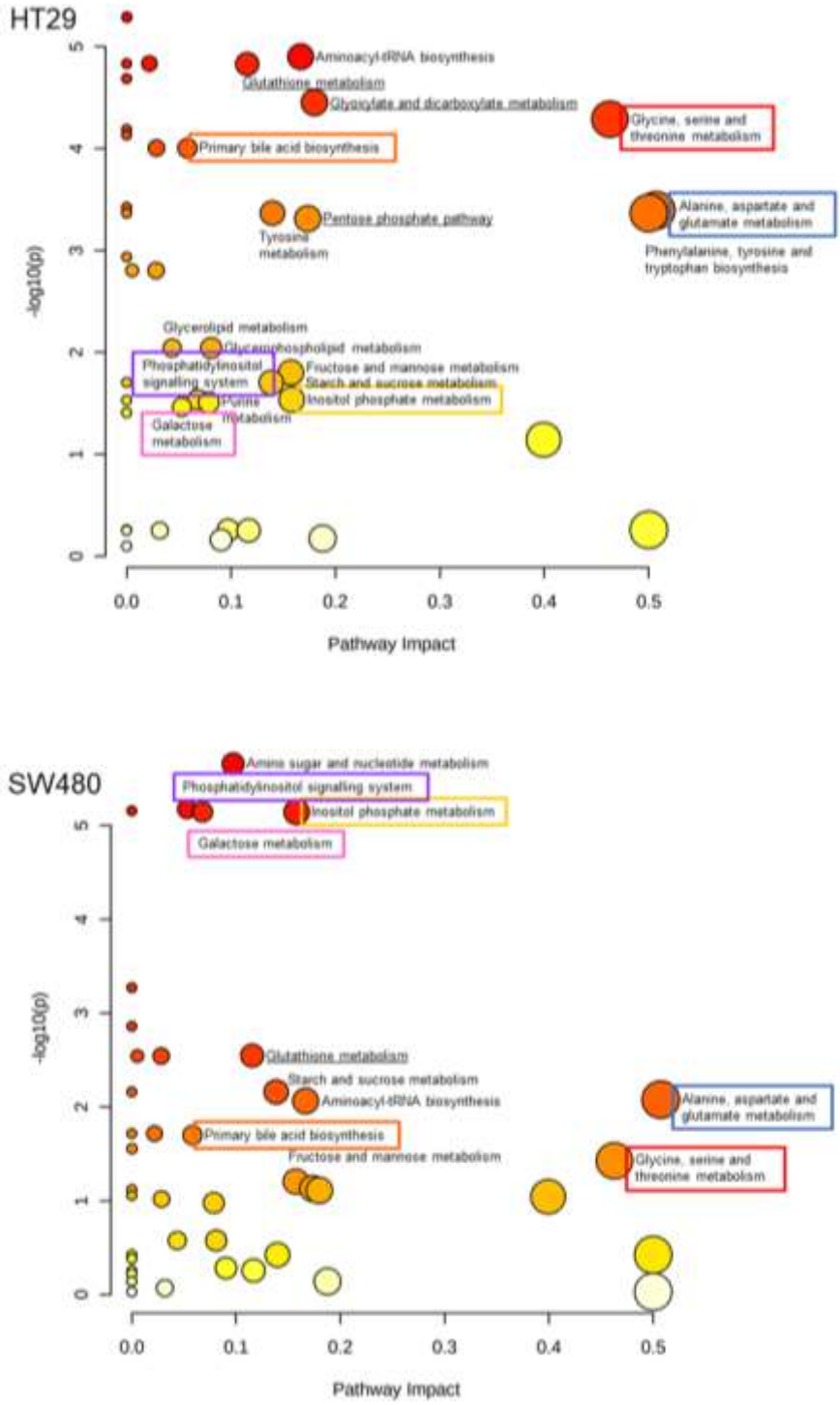


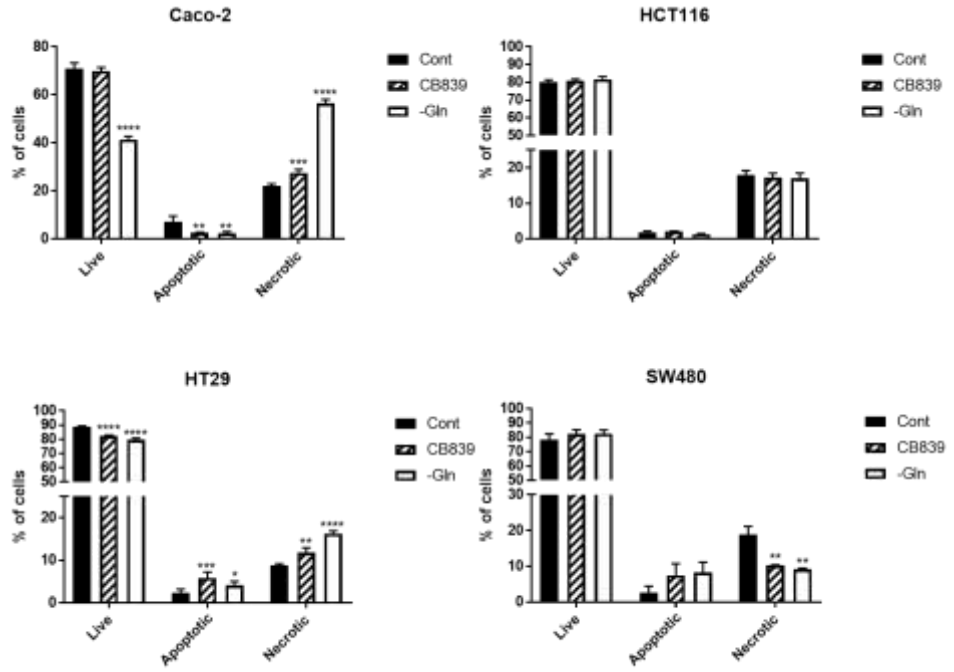
Figure 17. Metabolic pathways altered in colorectal cancer cells by GLS1 inhibitor CB839. The metabolomic profile of each cell line was analyzed through Metaboanalyst 5.0 platform. Coloured rectangles represented the common modified pathways in all cell lines whereas underlined pathways represent altered pathways found in at least three cell lines.

Metabolites	Caco-2		HCT116		HT29		SW480	
	trend	P value	trend	P value	trend	P value	trend	P value
Adenosine monophosphate	↓	ns	⬇	0.0043	⬇	0.0365	↓	ns
Aminomalonc acid	↑	ns	⬆	0.0061	⬆	0.0331	↑	ns
Beta-Alanine	⬇	0.0024	↓	ns	⬇	0.0388	↓	ns
Cholesterol	⬆	0.0141	↓	ns	⬆	0.0015	⬆	0.0029
Citric acid	↓	ns	↑	ns	↑	ns	↓	ns
D-Galactose	⬆	0.0001	↑	ns	=	ns	⬆	0.0046
D-Glucose	⬆	<0.0001	⬆	0.0003	⬆	0.0108	⬆	0.0032
D-Glucose 6-phosphate	⬇	0.0001	↑	ns	⬇	0.0197	⬆	0.0069
D-Ribose 5-phosphate	⬇	0.0006	⬇	<0.0001	⬇	0.0004	=	ns
Gamma-Aminobutyric acid	↓	ns	↓	ns	↓	ns	↓	ns
Glycerol 3-phosphate	↑	ns	↓	ns	⬆	0.0090	↑	ns
Glycine	⬆	<0.0001	⬆	0.0006	⬆	<0.0001	⬆	0.0365
L-Alanine	⬇	0.0002	↓	ns	⬇	0.0003	⬇	0.0308
L-Aspartic acid	↑	ns	=	ns	↓	ns	↓	ns
L-Glutamic acid	⬇	0.0005	↑	ns	↓	ns	↓	ns
L-Lactic acid	⬇	<0.0001	⬇	0.0184	⬇	<0.0001	⬇	0.0014
L-Proline	⬇	0.0027	↓	ns	↑	ns	↓	ns
L-Threonine	⬇	0.0005	↑	ns	⬆	0.0033	↑	ns
L-Tyrosine	⬇	0.0070	↓	ns	⬆	0.0004	↓	ns
L-Valine	↓	ns	↑	ns	⬆	<0.0001	↓	ns
Mannose 6-phosphate	⬇	0.0009	↓	ns	⬇	0.0157	↓	ns
Myo-Inositol	⬆	0.0007	⬆	<0.0001	⬇	0.0294	⬇	<0.0001
Myo-Inositol 1-phosphate	⬆	0.0073	⬆	0.0188	⬆	0.0183	⬆	0.0010
Pantothenic acid	⬆	0.0006	=	ns	⬆	0.0069	⬆	0.0063
Pyroglutamic acid	↓	ns	↓	ns	↑	ns	⬆	0.0023
Serine	⬆	0.0233	⬆	0.0240	⬆	<0.0001	⬆	0.0192

Table 5. Statistical parameters of metabolites detected with GC-MS analysis after 48 hours of CB839 0.25 µM treatment. Trends were expressed in comparison with the untreated cells, red arrows for increased levels and blue arrows for decreased ones. The slash indicates that the metabolite was not identified in that sample.

Analysis of cell death and cell cycle progression in colorectal cancer cell lines after glutamine starvation or GLS1 inhibitor treatment

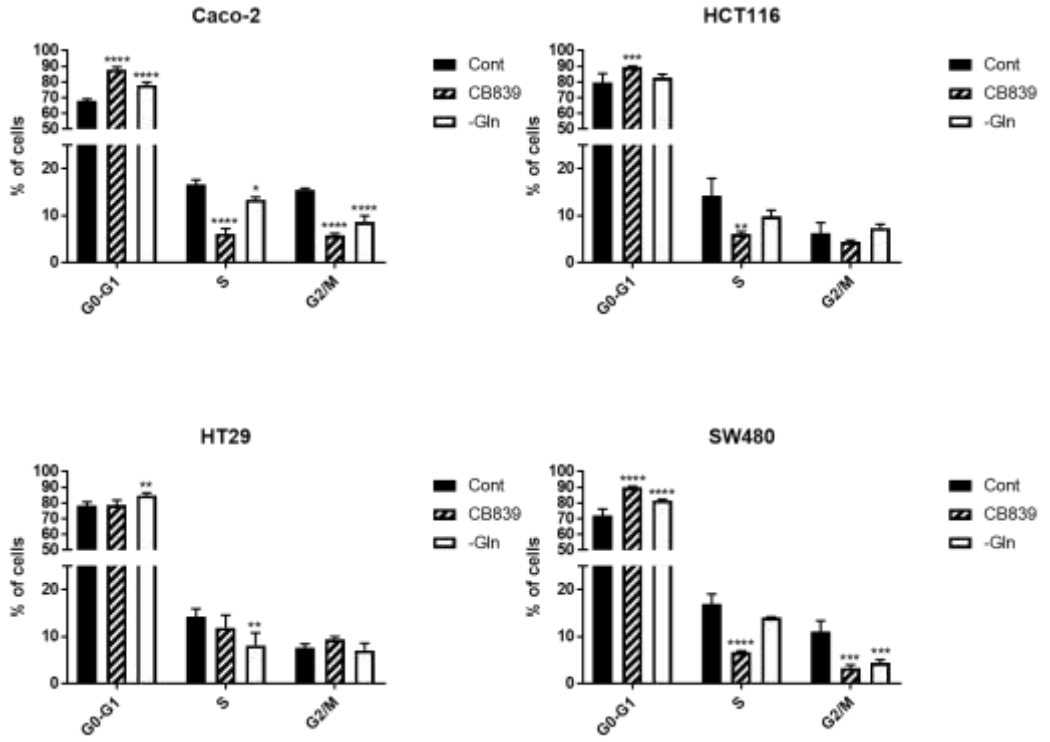
To investigate the role of cell death in the antiproliferative mechanism induced by glutamine deprivation or GLS-mediated pharmacological inhibition, we performed Annexin V/Propidium Iodide assay. Colorectal cancer cell lines were exposed to glutamine deprivation or CB839 treatment (0.25 μM) for 48h and then subjected to flow cytometric analysis with Annexin V and Propidium Iodide staining. As shown in fig. 18, a significant reduction in the percentage of live (41%) and apoptotic cells (2%) and an increase of necrotic cells (56%) was observed in Caco-2 cells after glutamine starvation compared to relative cells grown in the presence of glutamine (71% live, 7% apoptotic and 22% necrotic cells). In the same cell line, CB839 treatment did not induce changes in the percentage of live cells compared to controls, while resulting in a marked reduction in the percentage of apoptotic cells (3%) and a significant increase in the necrotic ones (28%). In HT29 cells, the percentage of live cells was significantly reduced by glutamine starvation (80%) or after CB839 treatment (82%) compared to control cells, cultured in the presence of glutamine (89% live, 2% apoptotic and 9% necrotic cells) while there is a significant increase in apoptotic and necrotic cells after glutamine deprivation or CB839 treatment (Fig. 18). Surprisingly, no significant changes in the percentage of living or dead cells are observed for the HCT116 cell line. Furthermore, for SW480 cells a significant reduction in necrotic cells was observed after glutamine deprivation or metabolism inhibition (10% and 9%, respectively) compared to controls (19%). The data reported for Caco-2 and HT29 cell lines could explain at least in part the strong antiproliferative effect exerted by glutamine deprivation or the inhibition of the glutaminase-1 mediated by CB839. In the remaining cell lines, HCT116 and SW480, however, the growth inhibition does not appear to be associated with induction of cell death in the first 48h after glutamine withdrawal or GLS-1 blocking.



Cell line		Live		Apoptotic		Necrotic	
		% of cells ±SD	P value (vs cont)	% of cells ±SD	P value (vs cont)	% of cells ±SD	P value (vs cont)
Caco-2	Cont	70.93 ±2.39	/	7.05±2.48	/	22.02±0.86	/
	CB839	69.92±1.50	ns	2.57±0.21	0.0047	27.51±1.39	0.0008
	-Gln	41.36±1.27	0.0001	2.20±0.86	0.0025	56.44±1.67	0.0001
HCT116	Cont	80.24±1.15	/	1.84±0.33	/	17.93±1.28	/
	CB839	80.71±1.12	ns	2.04±0.16	ns	17.21±1.24	ns
	-Gln	81.73±1.46	ns	1.24±0.21	ns	17.02±1.48	ns
HT29	Cont	88.92±0.49	/	2.32±0.90	/	8.77±0.40	/
	CB839	82.36±0.56	0.0001	5.84±1.39	0.0003	11.79±1.14	0.0012
	-Gln	79.60±1.14	0.0001	4.16±0.88	0.0395	16.24±0.63	0.0001
SW480	Cont	78.43±4.00	/	2.66±1.80	/	18.91±2.22	/
	CB839	82.30±3.08	ns	7.5±3.22	ns	10.20±0.21	0.0063
	-Gln	82.48±2.60	ns	8.33±2.83	ns	9.19±0.23	0.0030

Figure 18. The influence of glutamine deprivation and pharmacological inhibition of GLS1 on colorectal cancer cell death. Cell death was evaluated in colorectal cancer cells in the presence (Cont) or absence (-Gln) of glutamine or after CB839 treatment (0.25 μM) for 48h using flow cytometry combined with Annexin V and Propidium Iodide staining. Data are expressed as cell percentage; the experiment was performed in triplicate and represents mean ± SD.

To clarify the mechanisms responsible for the antiproliferative effect induced by glutamine deprivation or by blocking glutamine metabolism, we evaluated cell cycle alterations in colon cancer cell lines in these two conditions. We observed a significant increase of cells in G0-G1 phases and a significant reduction of cell percentage in S or G2-M phases after glutamine starvation in Caco-2, HT29 and SW480 cell lines (Fig. 19). To define the proliferative capacity of these cells, we calculate the proliferation index, a measure of the percentage of cells in proliferative phases (PI= (S+G2-M)/(G0-G1+S+G2-M)) (Fig.19). A marked reduction of proliferation index was reported for Caco-2, HT29, and SW480 cell lines under glutamine deprivation conditions compared to their respective controls, while HCT116 cell lines displayed no changes in cell cycle phases after 48h of glutamine withdrawal (Fig. 19). Furthermore, a significant increase in the percentage of cells in the G0-G1 phase and a significant reduction in those in the S or G2-M phase was reported for Caco-2, HCT116 and SW480 cell lines after CB839 treatment with a marked decrease in the proliferation index (Fig. 19). Conversely, no changes in cell cycle phases were observed in HT29 cell lines after glutaminase 1-inhibitory treatment. These results suggest that both the proliferation arrest and cell death induction may be responsible for the antiproliferative effect induced by glutamine deprivation or the pharmacological inhibition of glutaminase. Furthermore, the prevalence of one mechanism over the other may be specific for the different cell lines.



Cell line		G0/G1		S		G2/M		PI
		Cell percentage Mean % ±SD	P value (vs cont)	Cell percentage Mean % ±SD	P value (vs cont)	Cell percentage Mean % ±SD	P value (vs cont)	Cell percentage Mean % ±SD
Caco-2	Cont	67.89±1.27	/	16.63±0.98	/	15.49±0.29	/	32.11±1.27
	CB839	88.06±1.49	0.0001	6.13±1.08	0.0001	5.81±0.42	0.0001	11.94±1.49
	-Gln	78.00±1.75	0.0001	13.38±0.53	0.0144	8.62±1.33	0.0001	22.00±1.75
HCT116	Cont	79.53±5.93	/	14.19±3.69	/	6.28±2.24	/	20.47±5.93
	CB839	89.45±0.76	0.0003	6.12±0.46	0.0018	4.44±0.37	ns	10.55±0.76
	-Gln	82.80±2.11	ns	9.83±1.32	ns	7.37±0.81	ns	17.20±2.11
HT29	Cont	78.22±2.51	/	14.19±1.72	/	7.60±0.82	/	21.78±2.51
	CB839	78.74±3.05	ns	11.87±2.69	ns	9.39±0.62	ns	21.26±3.05
	-Gln	84.80±1.59	0.0021	8.11±2.66	0.0040	7.08±1.50	ns	15.20±1.59
SW480	Cont	72.09±4.10	/	16.88±2.13	/	11.02±2.32	/	27.91±4.10
	CB839	89.98±0.58	0.0001	6.68±0.27	0.0001	3.34±0.65	0.0001	10.02±0.58
	-Gln	81.57±0.83	0.0001	14.01±0.16	ns	4.42±0.67	0.0005	18.43±0.83

Figure 19. Glutamine starvation and inhibition of glutaminase 1 induced cell cycle arrest. Colorectal cancer cell lines were exposed to glutamine deprived medium or treated with CB839 (2.5 μ) for 48h, stained with propidium iodide, and subjected to flow cytometric analysis. The proliferating index was calculated as PI=(S+G2/M)/(G0/G1+S+G2/M)*100. Experiments were performed in triplicate and data are expressed as cell percentage and represent mean ± SD.

V. Discussion

Tumorigenesis requires metabolic reprogramming to support tumour growth and glutamine represents a pivotal metabolite in highly proliferating cells fuelling energetic and biosynthetic processes and ensuring redox homeostasis (Gwangwa *et al*, 2019; Lee *et al*, 2020). Numerous studies highlighted that different types of cancer cells rewire glutamine metabolism and become addicted to this amino acid, despite being a non-essential amino acid (Màrquez *et al*, 2017; Mishra and Ambs, 2015; Vanhove et al, 2019). On this basis, glutamine metabolism represents an attractive target for investigation. The examined colorectal cancer cell lines are sensitive to glutamine deprivation: glutamine starvation resulted in a marked antiproliferative effect. Growth curves showed a decrease in growth rate close to 50% for three of the four cell lines studied, only the HCT116 cells displayed a lower sensitivity to the starvation of this amino acid. The antiproliferative effects of glutamine deprivation were also confirmed by colony forming assay: colonies formed by cells cultured without glutamine were visibly fewer and smaller.
Specifically, glutamine deprivation determines a significant increase in the rate of necrosis in Caco-2 and HT29 cells, in the latter, we can also note an increase in the percentage of cells undergoing apoptosis. However, glutamine starvation did not affect cell death percentage in HCT116 and SW480 cells. Rather, glutamine deprivation causes a decrease in proliferating cells, expressed in terms of proliferative index, which is decreased in all cell lines. Recently, it has been reported that glutamine plays a role in the regulation of cell cycle phases. Indeed, its deprivation causes an arrest in the G1 phase (Saqcena *et al*, 2013; Bernfeld and Foster, 2019) and this could explain the increased arrest in the G1 phase observed in colorectal cancer cell lines, after glutamine deprivation.

Several papers explored the effect of glutamine deprivation in different colorectal cancer cell lines, but the molecular basis of this dependence remains still controversial (Hao *et al*, 2016; Kandasamy *et al*, 2021, Li *et al*, 2017; Polat *et al*, 2021; Toda *et al*, 2021; Wong *et al*, 2016). Some authors hypothesized that glutamine dependency is associated with the presence or the absence of mutations in specific genes, such as PIK3CA or KRAS. In our experiments, all the analyzed colorectal cancer cells exhibited a significant glutamine dependency, irrespective of their oncogenic mutation status. These discrepancies in terms of glutamine dependence could be due, in some cases, to differences in the culture media, or in the time of analysis, or the glutamine dependence assay (proliferation or cell death evaluation) or could be due to the use of isogenic cell lines. Our data also indicated that

deprivation of glutamine does not completely inhibit the growth of these cancer cells, as revealed by the colony assay. Recent reports indicated that cancer cells could adapt to glutamine starvation through different mechanisms such as nutrient accessibility (Zhang *et al*, 2014) or regulation of glutamine synthetase (Tardito *et al*, 2015), or arginine uptake (Lowman *et al*, 2019).

Our data demonstrated that glutamine starvation induces changes at the metabolic level: cells adapt their metabolism to supply the lacking of this fundamental nutrient. This was highlighted by the multivariate statistical analysis of the metabolites identified in GC-MS analysis: samples cultured in the absence of glutamine show a good separation from the respective controls. Although each cell line retains its identity in the absence of glutamine, most of the metabolites vary following the same trend between the various cell lines under study, as shown in the Venn diagram (Fig. 3). Before proceeding with the exploration of the data obtained by GC-MS analysis, it should be highlighted that the detection of glutamine and glutamic acid is difficult with this technique: these compounds undergo spontaneous or enzyme-assisted cyclization to pyroglutamic acid in both acidic and basic environments and this process is favoured at high temperatures (Nagana Gowda *et al*, 2015; Purwaha *et al* 2014). Furthermore, this artefact could be caused by the derivatization process (Kanani and Klapa, 2007), necessary for GC-MS analysis to improve volatility and reduce polarity and chemical instability of metabolites (Halket *et al*, 2005). A large amount of pyroglutamic acid has been detected in our samples, which could therefore be derived from the cyclisation of glutamine and glutamic acid. We also observed that its concentrations decrease in glutamine-deprived samples, as expected for glutamine and glutamic acid, the latter directly produced by glutamine. Furthermore, we must specify that despite this process, glutamic acid is still visible and quantifiable in our spectra, therefore its trend will be discussed considering both forms, unlike glutamine which is not detectable in GC-MS chromatogram.

Notably, glutamine is involved in the maintenance of the redox homeostasis, as a precursor of glutamate, which in turn represents a component of glutathione, one of the main antioxidant species used by the cell to counteract oxidative stress (Bansal and Simon, 2018). As evidenced by the GC-MS metabolomic analysis, glutamate levels (and its cyclised form, pyroglutamic acid) significantly decreased in the absence of glutamine in all cell lines. Furthermore, the evaluation of antioxidant species, expressed as the ratio

between the reduced and oxidized form of glutathione (GSH/GSSG), showed how glutamine deprivation determines a significant decline in the cell's antioxidant defences. The decreased cell proliferation could be correlated with oxidative stress damage, even if the cell proliferation mechanism regulated by glutathione levels remains still unknown (Traverso *et al,* 2013). The HCT116 cell line showed the highest levels (in basal conditions) of antioxidant species, expressed as GSH/GSSG, compared to the other cell lines (Fig.7). This could explain, at least in part, the lower sensitivity of HCT116 cells to glutamine deprivation that we observed in the first 48h after glutamine withdrawal. Based on these observations, we hypothesized that the reduction of the antioxidant defences induced by glutamine deprivation would have only a marginal inhibitory effect on HCT116 cell proliferation compared to the other cell lines which displayed lower antioxidants levels. The evaluation of the GSH/GSSG ratio has highlighted the possible involvement of glutamine in the cellular redox balance. However, further experiments are necessary to have a complete picture of the role of glutamine in cellular antioxidant capacity.

Glutamine is an anaplerotic substrate of the Krebs cycle: once it has been deaminated to glutamate, it is converted to α-ketoglutarate which enters the cycle of tricarboxylic acids contributing to the energy production (Zhao *et al*, 2019). Therefore, the absence of glutamine could be responsible for the decrease in the concentration of TCA cycle intermediates identified by GC-MS analysis: the levels of citrate, fumarate and malate are indeed significantly decreased in almost all cell lines cultured without glutamine. GC-MS analysis also shows higher glucose levels in glutamine-deprived cells. To find out the cause of the increase of this sugar level, its uptake was evaluated through the use of a fluorescent analogue, as well as the expression of GLUT1, which represents one of the most important glucose transporters. We observe a marked enhanced uptake in both Caco-2 and SW480 cells, while in HCT116 and HT29 cells we can only notice an increasing trend. Furthermore, the expression of GLUT1 changes in the absence of glutamine: 48 hours of deprivation lead to a significant increase in its expression in three cell lines (Caco-2, HCT116 and SW480), while surprisingly its expression decreases in HT29 cells. This increase in glucose uptake could be exploited to produce NAPDH, through PPP (Cairns *et al*, 2011), to regenerate GSH starting from GSSG, whose ratio is decreased in the absence of glutamine as already pointed out, and counteract oxidative stress, rather than to replenish the TCA cycle, as already demonstrated by Cetinbas and colleagues (Cetinbas *et*

al, 2016). Furthermore, GLUT1 expression was found upregulated in a variety of solid and haematological malignant neoplasia (Adekola *et al*, 2012), including colorectal cancers (Shen *et al*, 2011; Yang *et al*, 2017), and there is evidence that this overexpression was associated with poor prognosis in colorectal cancer patients. Additionally, combined inhibition of glucose transporters (GLUT1-3) and glutamine metabolism has been demonstrated to inhibit cancer cell growth and may be used as a powerful strategy for cancer therapy (Lee *et al*, 2016; Reckzeh *et al*, 2019). In the HT29 cell line, we observed a significant reduction of GLUT1 expression despite increased glucose uptake and its intracellular levels. Glucose can also be transported by other members of the GLUT family and in particular, the glucose transporter GLUT3 is expressed at high levels in the HT29 cell line (Kuo *et al*, 2019). Furthermore, some authors have shown an increased expression of GLUT3 in patients with colorectal cancer and this overexpression correlates with the development of metastases and a poor outcome (Dai *et al*, 2020; Kim *et al*, 2019; Kuo *et al*, 2019). Notwithstanding the high glucose uptake, a decrease in lactate levels following glutamine deprivation was reported. This is probably due to a decrease in glycolytic rate, so glucose could be used for biosynthetic rather than energy purposes. In the pancreatic β-cell line in the absence of glutamine, carbons from glucose were directed to the synthesis of glutamate, which is synthesized only starting from glutamine when it is present in appropriate concentrations (Brennan *et al*, 2002; Carlessi *et al*, 2019). Moreover, in glutamine sufficiency conditions, the majority of glutamine is converted to alanine and lactate and then transported into the extracellular space (DeBerardinis et al, 2007; Shanware et al, 2011). In glutamine deprived conditions, the induction of a compensatory anaplerotic response regarding the utilization of pyruvate or alanine to refuel the TCA cycle and to compensate for reduced glutamate levels has been proposed as a resistant mechanism to glutaminase pharmacological inhibition in cancer cells (Caiola *et al*, 2020; Cheng *et al*, 2011; Singleton *et al*, 2020). These observations would explain the significant decrease of these metabolites, alanine, and lactate, in our glutamine-deprived samples. It is, therefore, possible to hypothesize that in our cell lines sensitive to glutamine deprivation an adaptation mechanism may try to provide energetic substrates and biosynthetic intermediates without overcoming the inhibition induced by the absence of glutamine.

Focusing our attention to the amino acid content, we can notice how glutamate levels (and its cyclised form, pyroglutamic acid) significantly decrease in all cell lines in glutamine

deprivation condition, indeed it is directly produced by glutamine through deamination by GLS (Xiang *et al*, 2019). Conversely, the levels of threonine, tryptophan, tyrosine, and valine increased significantly in all cell lines. It may be assumed that glutamine deprivation triggers uptake of exogenous amino acids, as also reported Chen and colleagues (Chen *et al*, 2014). From the metabolomics analysis, we can see how glutamine deprivation causes a significant increase in the intracellular concentrations of serine and glycine in all cell lines. These amino acids perform numerous functions, including the participation in the synthesis of other amino acids, purines, and antioxidants, and feed the folate cycle through the one-carbon metabolism (Geck and Toker, 2016; Locasale, 2013). Also, Tanaka and colleagues found that serine level is markedly elevated in glutamine-deprived GBM cells (Tanaka *et al*, 2021). The enhanced levels of glycine and serine could be a strategy adopted by cells in glutamine deprivation conditions to maintain redox homeostasis, which we have seen to be compromised by evaluating antioxidant species.

Notably, in all studied cell lines, we observed a significant decrease in myo-inositol levels in the absence of glutamine. This metabolite, produced from glucose 6-phosphate or by *de novo* synthesis, plays an important role in cell survival as well as cell proliferation (Croze and Soulage, 2013). This should be consistent with what has been observed in the growth curves and colony forming assay, where it is possible to notice a decrease in the proliferation capacity.

Considering the pivotal role that glutamine plays in tumour metabolism, we wondered if its deprivation could act synergistically with drugs commonly used in chemotherapy. Specifically, glutamine deprivation was combined with the use of 5-fluorouracil, an antimetabolite (Longley *et al*, 2003), and camptothecin, a topoisomerase I inhibitor (Liu *et al*, 2000). Surprisingly, the antitumoural effect exerted by 5-FU is no greater in glutamine-deprived cells than in those grown in a complete medium. We can hypothesize that since cell replication is arrested, as indicated by the proliferation index, using a nucleotide analogue, which is incorporated during the replicative phases of the cell, does not represent a winning strategy. On the other hand, glutamine deprivation sensitizes cells to camptothecin with greater cytotoxic consequences. It happens with drugs that induce DNA damage such as etoposide or cisplatin (Chen *et al*, 2016), therefore we can bring forth the hypothesis that this also happens with a genotoxic agent such as camptothecin (Hong *et al*, 2013). We hypothesized that glutamine is necessary for the maintenance of the redox

balance, its deprivation causes a decrease in GSH which could lead to oxidative stress responsible for the sensitization of cells to the drug.

As demonstrated above, glutamine deprivation exerted antiproliferative effects on the examined colorectal tumour cells and therefore represents a possible therapeutic strategy. In this regard, the CB839 an allosteric inhibitor of GLS1, a key enzyme in glutaminolysis (Chen *et al*, 2020), showed promising clinical responses (Harding *et al*, 2021). As reported in the literature, metastatic cell lines like HCT116 and HT29 highly express GLS1, whereas GLS1 expression was relatively lower in Caco-2 and SW480 (Xiang *et al*, 2019). CB839 was shown to be effective in limiting cell survival and proliferation of the colorectal tumour cell lines under investigation. The inhibitory effect is evident at the concentration of 0.25 μM (dose chosen based on the results obtained in the MTT cytotoxicity assay) both from the growth curves and from the colony forming assay. CB839 proved to be effective in reducing cell viability also in other cell lines, such as acute myeloid leukemia cells (Zacharias *et al*, 2019), triple-negative breast cancer cells (Gross *et al*, 2014) and non small cell lung cancer (Caiola *et al*, 2020). More specifically, if we look at the data obtained by FACS analysis (Fig. 18) we can observe how the drug induces a significant increase in necrotic cells in Caco-2 and HT29 lines and an increase in the percentage of apoptotic cells only in HT29. While we have no increase of cell death percentage in SW480 and HCT116 cells after CB839 treatment. However, as highlighted above, also these cells are sensitive to the drug. In agreement with the results of the cell cycle assay (Fig. 19), these data indicated that the effect of CB839 on HCT116 and SW480 cells is at the proliferative level: 48 hours of drug treatment causes an accumulation of cells in G0/G1 phase, while the percentage of cells in phase S and G2/M decreased. This can also be summarized with the decreasing proliferative index in these two cell lines, as well as in Caco-2. Although all four cell lines are sensitive to CB839 growth inhibition, treatment with this drug for 48 hours leads to different results: while in HT29 and Caco-2 this time interval is sufficient to induce cell death by apoptosis (HT29 cells) and necrosis, in HCT116 and SW480 cells we notice only a cell cycle arrest. Moreover, for the above-mentioned cell lines, we cannot exclude that the cell death process may occur after a longer period of treatment.

The multivariate statistical analysis of metabolomic data obtained by colorectal cancer cell lines treated with glutaminase inhibitor CB839 displayed a separation along the [t]1

component: the HCT116 cells are separated from the other cell lines examined. The reasons for this separation remain unclear although we cannot exclude the possibility that this could depend on the metastatic capacity of this cell line: the HCT116 cells originates from Dukes'stage D primary tumor, while the other lines derive from less invasive primary cancers as reported in table 1. The PCA analysis of each colorectal cancer cell line showed good separation between CB839 treated cells and their respective controls (Fig. 14), this highlights that CB839 induced marked alterations in colorectal cancer cell metabolism. The significantly altered metabolites, like glucose, serine, and glycine, resulted upregulated after CB839 treatment and glutamine withdrawal in all colorectal cancer cell lines. Furthermore, other metabolites share a similar variation profile in the two conditions: amino malonic acid, beta-alanine, alanine, lactic acid, galactose, ribose 5-phosphate, and glycerol 3-phosphate. These data suggest that both glutamine deprivation and enzymatic inhibition of its metabolism similarly affect the same metabolic pathways (alanine, aspartate and glutamate metabolism; glycine, serine and threonine metabolism; phenylalanine, tyrosine, and tryptophan metabolism; glutathione metabolism and glyoxylate and dicarboxylate metabolism). The univariate analysis shows that the metabolites significantly altered following treatment with CB839 are more numerous in Caco-2 and HT29 cells, compared to HCT116 and SW480, suggesting a lower metabolic impact of the drug in these latter cell lines. Nevertheless, proliferation, cell cycle, and cell death assays underline the activity of CB839 also on HCT116 and SW480 cells. We could hypothesize that the 48-hour period of treatment is not sufficient to highlight the metabolic changes and that a longer period is required to observe the metabolic effect of the drug. This would be in line with what was observed at the FACS analysis: 48 hours of treatment are sufficient for the induction of cell death only in Caco-2 and HT29 cells, while HCT116 and SW480 cells only show an arrest of the cell cycle. This hypothesis can be corroborated by the fact that the differences in terms of vitality showed by the growth curves are emphasized on the fourth day of treatment.

VI. Conclusions

As with many other types of cancer, also different colorectal cancer cells are dependent on glutamine for survival and proliferation, even if not all of them showed the same sensitivity to the deprivation of this important amino acid. Glutamine starvation deeply alters energy metabolism and aminoacidic content. Furthermore, glutamine deprivation significantly affects the redox state, and antioxidant defences. Moreover, we can notice that glutamine deprivation induced common metabolic changes in all cell lines, underlining that cancer cells try to overcome starvation by a similar metabolic rewiring. Glutamine deprivation has also been shown to be effective in sensitizing colorectal cancer cells to drugs commonly used in chemotherapy such as camptothecin, offering a possible strategy to be adopted in clinical practice to reduce the doses of anticancer drugs necessary to obtain the therapeutic effect.

Considering the crucial role played by glutamine in colorectal cancer cells, we investigated the effect of CB839 which inhibits GLS1, a key enzyme of glutaminolysis. The drug CB839 results highly effective in decreasing cell proliferation and is also capable of inducing cell death in some tumour cell lines. The identification of metabolic alterations induced GLS1 inhibitor could improve our knowledge of its action on cancer metabolism and could shed the light on promising pathways that could be targeted to potentiate cancer therapy.

VII. Bibliography

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