Metabolic phenotype of bladder cancer

METABOLIC PHENOTYPE OF BLADDER CANCER

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ABSTRACT

Metabolism of bladder cancer represents a key issue for cancer research. Several metabolic altered pathways are involved in bladder tumorigenesis, representing therefore interesting targets for therapy.

Tumor cells, including urothelial cancer cells, rely on a peculiar shift to aerobic glycolysis-dependent metabolism (the Warburg-effect) as the main energy source to sustain their uncontrolled growth and proliferation. Therefore, the high glycolytic flux depends on the overexpression of glycolysis-related genes (SRC-3, glucose transporter type 1 [GLUT1], GLUT3, lactic dehydrogenase A [LDHA], LDHB, hexokinase 1 [HK1], HK2, pyruvate kinase type M [PKM], and hypoxia-inducible factor 1-alpha [HIF-1α]), resulting in an overproduction of pyruvate, alanine and lactate. Concurrently, bladder cancer metabolism displays an increased expression of genes favoring the pentose phosphate pathway (glucose-6-phosphate dehydrogenase [G6PD]) and the fatty-acid synthesis (fatty acid synthase [FASN]), along with a decrease of AMP-activated protein kinase (AMPK) and Krebs cycle activities. Moreover, the PTEN/ PI3K/AKT/mTOR pathway, hyper-activated in bladder cancer, acts as central regulator of aerobic glycolysis, hence contributing to cancer metabolic switch and tumor cell proliferation.

Besides glycolysis, glycogen metabolism pathway plays a robust role in bladder cancer development. In particular, the overexpression of GLUT-1, the loss of the tumor suppressor glycogen debranching enzyme amylo-α-1,6-glucosidase, 4-α-glucanotransferase (AGL), and the increased activity of the tumor promoter enzyme glycogen phosphorylase impair glycogen metabolism. An increase in glucose uptake, decrease in normal cellular glycogen storage, and overproduction of lactate are consequences of decreased oxidative phosphorylation and inability to reuse glucose into the pentose phosphate and de novo fatty acid synthesis pathways. Moreover, AGL loss determines augmented levels of the serine-to-glycine enzyme serine hydroxymethyltransferase-2 (SHMT2), resulting in an increased glycine and purine ring of nucleotides synthesis, thus supporting cells proliferation.

A deep understanding of the metabolic phenotype of bladder cancer will provide novel opportunities for targeted therapeutic strategies.

Keywords: bladder cancer, metabolism, metabolic pathway, novel target

INTRODUCTION

Muscle-invasive bladder cancer (BC) is the 9th most common cause of death worldwide, and the 4th most common type of cancer among men in developed countries [1]. The poor survival of BC patients depends on an intrinsic biologic aggressiveness of this tumor and a peculiar radio- and chemo-resistance. Therefore, efforts to deeply understand the pathogenic mechanisms that support bladder carcinogenesis, so as to identify new and effective targets for therapy are strongly suggested.

Cancer development and progression is characterized by a unique reprogramming of energy metabolism, required to sustain highly proliferating cancer cells. Hence, understanding cancer metabolism represents a major topic of cancer research.

Bladder cancer has profound metabolic abnormalities. Several altered metabolic pathways play a role in bladder tumorigenesis. Moreover, metabolomics contributed substantially in understanding relevant alterations of catabolic and anabolic metabolic processes impaired in cancer, through the identification of tumor-specific metabolic biomarkers with a potential diagnostic, prognostic or predictive value [2]. Metabolomics studies identified various metabolites of diverse metabolic pathways (glucose, lipid, amino acid, nucleotide metabolites) as probable biomarkers for BC [3].

In this review we retraced the main metabolic pathways involved in the pathogenesis of bladder cancer.

- **Glucose metabolism pathway**

A peculiar shift to aerobic glycolysis-dependent metabolism (the Warburg-effect) is the hallmark of tumor cells, including BC.

Glucose catabolism produces: 1) energy in the form of ATP (glycolysis); 2) reducing equivalents in the form of NADH (anaerobic glycolysis); 3) intermediate metabolites used as precursors for the biosynthesis of non-glucidic compounds (amino acids and lipids).

Glycolysis (anaerobic degradation of glucose to pyruvic acid) is the binding process of glucose utilization for energy production. Glycolytic pyruvate can be transformed/reduced to lactic acid in the cytoplasm (anaerobic conditions), or oxidized in the mitochondria (via tricarboxylic acid [TCA] cycle) into carbon dioxide and water (aerobic status). Glucose can also be metabolized with non-energy purposes, via the pentose phosphate cycle (PPP) to produce pentoses necessary for the synthesis of nucleotides and nucleic acids, and most of the NADPH required to promote reductive processes (i.e. fatty acids biosynthesis).

Unlike healthy human tissues, cancer cells metabolism is marked by a peculiar dependence on anaerobic breakdown of glucose (glucose fermentation into lactate), reduced AMPK and TCA activity, non-energy utilization of glucose through the PPP, and increased fatty acids synthesis even under normoxic conditions for mitochondrial oxidative phosphorylation. This neoplastic preferential metabolic switch to anaerobic glycolytic flux (instead of oxidative phosphorylation) in normoxia is termed “aerobic glycolysis” or Warburg effect [4]. Glycolysis, although less efficient than mitochondrial respiration in energy yield, allows obtaining intermediates of other key metabolic pathways. Hence, glucose is diverted from oxidative phosphorylation (resulting in less energy yield due to reduced ATP production) towards the biosynthesis of macromolecular precursors (acetyl-CoA for fatty acids, glycolytic intermediates for non-essential amino acids, and ribose for nucleotides) required to sustain cell growth and division [5]. Therefore, cancer metabolism is profoundly altered to support the demands of proliferating tumor cells. Several alterations in metabolic genes directly or indirectly involved in the Warburg-effect and supporting a glycolytic profile are implicated in cancer progression, including over-expression of glucose transporters [6], up-regulation of both oxidative and non-oxidative branches of the PPP (via increased activity of *glucose-6-phosphate-dehydrogenase* [G6PD] and transketolase-like-1 [TKTL1] protein, respectively) [7], increased ATP citrate lyase (ACL) activity (key enzyme linking glucose metabolism to lipid synthesis, releasing acetyl-CoA from citrate) [8].

Moreover, the high lactate amount and subsequent acidification due to cancer cells reliance on glycolytic metabolic shift might promote carcinogenesis, favoring immune escape, acid-mediated matrix degradation, invasiveness and metastasis, and chemo-radio-therapy resistance [9,10].

The Warburg effect is strongly implicated in BC, being associated with aggressive tumor behavior. In particular, the main alterations in glucose metabolism reported in urothelial bladder cancer patients include:

- Stimulated GLUT-1 activity. As discussed in more detail later, glucose transporters (GLUT) activity undergoes relevant changes in tumors, including BC, since cancer cells survival and proliferation is strictly associated with glucose uptake [11].

- Up-regulation of glycolysis.

  Three key rate-limiting enzymes control glycolysis: hexokinase (HK), 6-phosphofructokinase (PFK) and pyruvate kinase (PK). First, HK phosphorylates glucose in glucose-6-phosphate; the HK type II isoenzyme is over-expressed in cancers. PFK catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, a key regulatory step of the glycolytic pathway. PFK activity is elevated in cancer cell lines particularly in response to proliferating signals secondary to activation of RAS and SRC or HIF-1α. PK (especially PK type M2) controls the final step of glycolysis, dephosphorylating phosphoenolpyruvate to generate pyruvate and ATP, therefore contributing to the glycolytic flux in PKM2- expressing cancer cells. Given the up-regulation of glycolytic pathway enzymes, glycolytic inhibitors could be an effective anticancer strategy, but there are some concerns with their use (low potency, low selectivity for the target, resulting in significant toxicity) [12].

  A proteomic analysis of isobaric tags for relative and absolute quantification (iTRAQ) identified down-regulation of PFK (along with proliferating cell nuclear antigen [PCNA], PKM2, HK-1and cell surface glycoprotein [CD146]) in bladder cancer after Bifidobacterium infantis-

mediated HSV-TK/GCV suicide gene treatment, indirectly demonstrating the role of PKF in promoting BC carcinogenesis [13]. Interestingly, in bladder cancer PFK protein expression levels significantly decreased with increased tumor stage and grade [14-16], assuming a role in early phases of carcinogenesis.

- Increased pyruvate metabolism, and augmented lactated and alanine production.

  Pyruvate is the terminal product of anaerobic glycolysis. Under physiological aerobic conditions, pyruvate is oxidized to carbon dioxide to efficiently produce energy, and serves as a precursor for different biosynthetic pathways. As previously stated, tumor cells exhibit a distinctive high glycolytic flux even in conditions of adequate availability of oxygen to perform mithocondrial oxidative phosphorylation for the Warburg effect.

  Accordingly, BC shows a shift in pyruvate metabolism, which is more pronounced in advanced disease stages. BC progression from a less to a highly invasive stage is associated with increased of pyruvate levels [16].

  Interestingly, pyruvate becomes the main energy fuel (instead of glucose) for highly proliferating BC cells, which consume most of the available pyruvate [16]. This can explain the down-regulation of pyruvic acid reported in BC cell lines, which could be related to high synthetic rate of lactate [17].

  Therefore, in cancer cells pyruvate is usually reduced to lactate. Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate, as it converts NADH to NAD+. Pyruvate is, in fact, used as acceptor of reducing equivalents of NADH(H+) that are formed in the oxidative reaction of the glyceric aldehyde-3-phosphate. If NADH(H+) is not re-oxidized to NAD+ the glycolytic flow decreases up to run out. The continuous oxidation of NADH(H+) in the reaction catalyzed by LDH allows the glycolytic flux.

Cancer cells rely on fermentative glycolysis, where LDH isoform A converts the majority of glucose stores into lactate regardless of oxygen availability, shifting use of glucose from simple energy production to the promotion of enhanced cell growth [18]. Bladder cancer cells showed an over-production of lactate, with lactate levels rising concurrent to progression to highly proliferative stage [16]. LDH-A over-expression has been demonstrated to promote cell proliferation, invasion and migration in invasive bladder cancer cell line in vitro. LDH-A seemed to promote malignant progression by stimulating epithelial-to-mesenchymal transition (EMT) and conferring stemness in muscle-invasive bladder cancer [19]. Consistent with these data, metabolomic analyses revealed up-regulation of glycolysis-related metabolites (enhanced acid lactic production), and a corresponding down-regulation of TCA-related metabolites (reduced citric acid synthesis) [3].

The lactate is extruded to the extracellular space by the action of monocarboxylate transporters (MCTs) that play a crucial role in regulating intracellular pH homeostasis. MCT1 and MCT4 are involved in the metabolic reprogramming of cancer cells, contributing to BC aggressive behavior. The immunohistochemical analysis of MCT1 and MCT4 expression in BC tumor cells revealed a diffuse membranous staining (while normal urothelial cells showed negative or weak staining), which correlated with poor overall survival and poor recurrence-free survival, respectively [20]. Of note, the expression of MCTs within bladder tumor shows peculiar patterns, which differ from tumor cells to tumor stroma and from hypoxic areas to normoxic regions. In particular, a significant decrease in MCT1 and MCT4 positivity occurred from normoxic to hypoxic regions, and a correlation between MCT4 concomitant staining in hypoxic tumor cells and in the tumor stroma and MCT1 positivity in normoxic tumor cells with poor prognosis and chemoresistance was observed [21].

Interestingly, up-regulation of MCT1 and MCT4 expression has been described also in cancer-associated fibroblasts (CAFs), which undergo aerobic glycolysis and lactate over-production

providing energy for supporting BC cell growth. This corroborates the essential role of tumor microenvironment in sustaining tumor proliferation and invasion [22].

Moreover, BC tissues express higher levels of the ABCC3 (ATP-binding cassette, subfamily C, member 3) transporter compared to normal urothelium, with a positive correlation with LDHA expression, advanced BC stage and poor overall survival. Therefore, ABCC3 could be another potential prognostic biomarker and promising target for BC therapy [23].

The elevated lactate production creates an acidic extracellular environment that is thought to promote tumor invasion and metastatic spread by exerting an inhibitory action on anticancer immune effectors [24,25].

Pyruvate can also be used in biosynthetic pathways. Instead, part of pyruvate derived from anaerobic glycolysis can be converted to alanine in a process (trans-deamination) catalyzed by the enzymes glutamate dehydrogenase and glutamic-pyruvate transaminase (GPT). This reaction requires NADPH(H+) to be converted into NADP+. The carbon skeleton of alanine may be then used for gluconeogenesis, while the amino group can be incorporated in the urea cycle. The production of alanine has been reported to be enhanced in highly invasive bladder cancer cells, while the levels of GPT are decreased [16]. The conversion of pyruvate to lactate or alanine is coupled with the oxidation of NADH/NADPH to NAD+/ NADP+. Therefore, the lactate/alanine ratio is an indicator of the cell's redox state, reflecting the equilibrium between those two intermediates. The lactate/alanine ratio is higher in advanced BC stage, suggesting that cancer progression is associated with higher oxidative stress due to increased pyruvate metabolism and lactate production [16].

- Increased expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3).

The 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB1-4) family encompasses bifunctional proteins that are involved in both the synthesis (6-phosphofructo-2-kinase activity)

and degradation (fructose-2,6-biphosphatase activity) of fructose-2,6-bisphosphate (F2,6BP), a potent regulatory molecule that activates 6-phosphofructo-1-kinase (Pfk-1), thus controlling glycolysis.

PFKFB3, which has a high ratio of kinase to phosphatase activity, is over-expressed in human cancers, regulated by HIF-1a, Akt and PTEN, and stimulates the survival and growth of several malignancies [26]. An inhibitor of PFKFB3 (3PO) demonstrated to reduce BC cell lines proliferation, indirectly suggesting the role of PFKFB3 in BC progression [27].

- Over-expression of Steroid receptor coactivator-3 (SRC-3).

  SRC-3 is overexpressed and/or amplified in various cancer types, both steroid (where it exerts a steroid receptor coactivator function) and non-steroid targeted tumors, including BC [28,29]. Zhao and colleagues demonstrated a correlation between SRC-3 overexpression and increased BC cell proliferation, assuming a role of SRC-3 in reprogramming cancer cell metabolism. In fact, SRC-3 hyper-expression caused over-expression of genes involved in hypoxia-induced glycolysis (HIF1α-target genes, including glut1 and pgk1), thus favoring BC development via enhanced glycolytic rate [30].

- Increased pentose phosphate pathway: up-regulation of TKTL1 protein.

  Transketolase enzyme reactions are crucial steps of the non-oxidative part of the PPP, allowing oxygen-independent glucose conversion to ribose for nucleic acid synthesis and generating reduced NADPH required for synthesis reactions in tumor cells [31].

  Langbein et al. reported a correlation between TKTL1 over-expression and invasive behavior of colon and urothelial cancer leading to poor patient survival [32]. TKTL1 upregulation in tumors leads to increased, oxygen-independent glucose catabolism and a lactate-dependent matrix degradation. Several transketolase inhibitors have demonstrated to dramatically inhibit cell

proliferation and suppress tumor growth in preclinical cancer cells models [33,34], supporting TKTL1 as a potential target for anticancer therapy.

Indirect evidence of the pro-carcinogenic role of PPP hyperactivation comes from the demonstration of the inhibitory activity of zoledronic acid on the G6PD enzyme (through mitigation of the Ras-TAp73-G6PD pathway), resulting in the inhibition of the PPP and BC cell proliferation [35].

- **PI3K/AKT/mTOR pathway**

Aberrant activation of the PI3K-Akt-mTOR pathway is implicated in bladder carcinogenesis. In particular, activating mutations can involve PI3K α-subunit (PIK3CA), Akt1, and TSC1, while inactivation of PTEN (loss of heterozygosity on chromosome 10q, homozygous deletion of the PTEN locus, and occasional inactivating mutations in the PTEN coding region) can occur in approximately 30% of BC, correlating with poor outcomes [36-38]. In addition, inactivation of both p53 and PTEN may promote BC invasiveness via up-regulation of mTOR signaling, with a correlation with aggressive disease and poor survival in human urothelial tumors [39].

The mTOR pathway has been reported to act as a key regulator of cancer cells energy metabolism through the fundamental role of Akt (the "Warburg kinase") that stimulates aerobic glycolysis [40].

- <u>Activation of the oncogenic kinase Akt.</u>

The serine/threonine kinase Akt is commonly activated in cancer cells, acting as an oncogene promoting cell survival. Frequently, Akt hyper-activation can indirectly result from amplification of the upstream Akt activator phosphatidylinositide-3-kinase (PI3K), or deletion of the PI3K inhibitor PTEN.

Akt exerts a direct influence on glucose metabolism by promoting the shift to aerobic glycolysis, rendering cancer cells dependent on glucose consumption and aerobic glycolysis for growth and

survival, thus contributing to a more aggressive cancer behavior [41]. Several molecular mechanisms are implicated in Akt-dependent shift to aerobic glycolytic metabolism:

- Akt directly stimulates glycolytic enzymes such as glucose transporters, HK, and LDH [40].
- Activated Akt impairs the ability to induce fatty acid oxidation in response to glucose deprivation [42].
- mTOR (downstream effector of Akt) induces the expression of glycolytic enzymes, including GLUT1, LDHB, HK2 and PKM2 [43,44].
- mTOR directly stimulates the translational machinery by phosphorylating eIF4E binding proteins (eIF4E-BPs) and ribosomal protein S6 kinases (S6K), enhancing expression levels of glycolytic proteins.
- mTOR complex 1 enhances the normoxic upregulation of the HIF-1α transcription factor, inducing the expression of genes involved in glycolysis [45].

- Hyper-expression of miR-21.

MicroRNAs (miRNAs) are a class of endogenous non-coding single-stranded RNA molecules, usually 17-27 nucleotides in length, which modulate gene expression post-transcriptionally, by binding to the 3' untranslated regions of target messenger RNAs (mRNAs) of protein-coding genes. miRNAs are implicated in vital cellular processes: control of normal development, cell growth, differentiation and apoptosis [46].

Altered miRNAs expression plays a crucial role in cancer development [47]. Of note, aberrant miRNAs expression regulates aerobic glycolysis [48]. Several genome-wide profiling analyses identified specific miRNAs alterations occurring in BC [49-52]. Among these, miR-21 hyper-expression has a carcinogenic role in BC, inducing cell proliferation by inhibiting PTEN expression and promoting AKT hyperactivation [53]. Moreover, miR-21 expression level has been described to positively correlate with expression of key aerobic glycolytic genes (GLUT-1, GLUT-3, LDHa,

LDHB, PKM2, HK1 and HK2) via the induction of PI3K/AKT/mTOR pathway, strongly suggesting that miR-21 is involved in aerobic glycolysis switch (Figure 1). Therefore, miR-21 inhibition may impair high glycolysis levels in bladder cancer cells via the miR-21/PTEN/AKT/ mTOR cascade [54].

- **Glycogen metabolism pathway**

Cancer cells undergo specific metabolic reprogramming in response to the hypoxic environment of solid tumors.

Besides aerobic glycolysis, which has a fundamental role in providing energy and multiple intermediates critical for cell proliferation, glycogen metabolism is altered in tumor cells.

Glycogen, a highly branched polysaccharide composed of many monomers of alpha-glucose containing both α-1,4 and α-1,6 glycosidic linkages (the latter at branch points), is the main storage form of glucose in human tissues (liver, skeletal muscles, heart), since the tissue accumulation of monosaccharides would create an untenable osmotic pressure. The enzymatic processes of glycogen synthesis and breakdown are subjected to a complex and delicate regulation system sensitive to variables metabolic conditions, in order to adapt reserves to the organism demands. This is made possible primarily through the differentiation of biosynthetic (glycogen synthesis) and catabolic (glycogenolysis) pathways, and thanks to the regulating the activity of two fundamental enzymes: *glycogen phosphorylase* and *glycogen synthase*.

Glucose enters the neoplastic urothelial cell mainly through glucose transporter type 1 (GLUT-1). Intracellular glucose is converted to glucose-6-phosphate (G6P) by *hexokinase* and transformed in glucose-1-phosphate (G1P) by the serine enzyme *phosphoglucomutase*. *Phosphoglucomutase* requires catalytic quantities of glucose-1,6-bisphosphate, derived from the phosphorylation of glucose-1-phosphate by *phospho-glucokinase*. G1P reacts with uridine triphosphate (UTP) to form uridine-diphosphate-glucose (UDPG) and inorganic pyrophosphate (PPi), which is in turn demolished into two molecules of inorganic phosphate (Pi) making the reaction irreversible. The

first reaction is catalyzed by the *UDPG pyrophosphorylase*, while the second by the enzyme *pyrophosphatase*. UDPG is the immediate precursor for glycogen synthesis. *Glycogen synthase* catalyzes the elongation of glycogen chains by incorporating glucose residues of UDPG to the hydroxyl at C4 of the terminal residue of the growing glycogen chain forming an α-1,4 glycosidic bond, with release of UDP as a reaction product. To increase the solubility of the glycogen molecule and create a greater number of non-reducing terminal glucose [C4] residues elongated chains of glycogen are branched. A branching enzyme transfers a segment of at least 4 glucose units from the end of a glycogen chain to the C6 hydroxyl of a glucose residue of glycogen to yield a branch with an α-1,6 linkage.

When the cell requires glucose, and exogenous glucose lacks, glycogen is degraded (glycogenolysis). *Glycogen phosphorylase* catalyzes glycogen breakdown via phosphorolytic cleavage of the α-1,4 glycosidic bonds, releasing G1P as the reaction product. However, *glycogen phosphorylase* is not able to disrupt the α-1,6 glycosidic linkages at branch points. Therefore, when 4 glucose monomers remain before the branch point, the further degradation of glycogen requires the intervention of the debranching enzyme (amylo-α-1,6-glucosidase, 4-α-glucanotransferase [AGL]). AGL catalyzes two different reactions: 1) The transferase activity transfers three glucose monomers from a 4-residue limit branch to the end of another glycogen chain, reducing the limit branch to a single glucose monomer. 2) The α-1,6 glucosidase activity catalyzes hydrolysis of the α-1,6 linkage, yielding one free glucose residue and removing the branch point [55].

An altered turnover of glycogen with high levels of glycogen stores (a reserve to be used as a glucose source for anaerobic glycolysis under transient energy shortage conditions) are observed in several cancer cells models (breast, kidney, uterus, ovary, skin, and brain cancer cell lines, including also urothelial cancer), emerging as a metabolic survival pathway [56,57].

The intracellular increase in glycogen accumulation occurs under hypoxic conditions, and seems to be dependent on the hypoxia-inducible factor 1 (HIF-1). The transcription factor HIF-1 promotes tumor cell proliferation altering cell metabolism, not only favoring a metabolic shift from oxidative

phosphorylation to glycolysis and lactic acid production, but also stimulating glycogen synthesis and accumulation by the induction of *phosphoglucomutase* activity [58], glycogen synthase 1 [59], protein phosphatase 1 regulatory subunit 3C gene (PPP1R3C) [60], and other proteins involved in glycogen synthesis. Therefore, glycogen storage represents an adaptive survival mechanism to overcome microenvironment glucose and oxygen paucity, hence assuring tumor cell proliferation and survival [58,59].

Interestingly, glycogen concentrations in tumor cells inversely correlate with proliferation rate via modulation of glycogen phosphorylase activity. Maximal activity of glycogen phosphorylase (causing low intracellular glycogen content) coincided with the period of high division rate. Conversely, when the rate of tumor cell division decreased, phosphorylase activity consensually decreased leading to increased glycogen levels [56,61]. The high proliferation rate of cancer cells needs augmented macromolecule synthesis (mainly RNA, DNA and fatty acids), which requires an increased demand for glucose to be shunted into ribose-phosphate and acetyl-CoA precursor synthesis pathways. Glycogen breakdown (releasing glucose) represents therefore a rapid energy source. Evidence of a key role of glycogen in promoting cancer cell survival under hypoxic conditions comes from preclinical data of a glycogen phosphorylase inhibitor (CP-320626), which impairs the glycogen homeostasis by blocking glycogenolysis, reducing the availability of the energy substrate glucose with subsequent arrest of proliferation and increase in apoptosis of pancreatic cancer cells [62].

Glycogen metabolism pathway plays a robust role also in bladder cancer development. Therefore, a deep understanding of the glycogen metabolism and its metabolic alterations in urothelial malignancies represents a key topic of cancer research, hypothesizing novel effective targets for therapy [63].

Several aberrations of the glycogen metabolic pathway have been observed in urothelial tumors:

1. The overexpression of GLUT-1. Neoplastic cells distinctively display accelerated metabolism, high glucose requirement, and increased glucose intake. The critical step limiting intracellular glucose availability is the trans-membrane transport of glucose, which is mediated by facilitative glucose transporter (GLUT) proteins. Increased expression of GLUT proteins (mainly GLUT-1 and/or GLUT-3) in cancer cells leads to augmented intracellular glucose uptake, thus supporting cells proliferation in human tumors [6].

   Interestingly, GLUT-1 is selectively expressed in neoplastic urothelial tissue (of both non-muscle invasive and muscle-invasive bladder carcinoma), but not in normal urothelium or benign papillomas of the bladder [64,65]. Moreover, the grade of GLUT-1 expression correlates with bladder cancer progression (grater GLUT-1 expression in muscule-invasive and high nuclear grade cancers compared to superficial and low-grade tumors) [65]. Finally, the overexpression of GLUT1 seems to confer poor prognosis, correlating with worse overall survival, supporting its potential role as a marker of biologically aggressive disease [11, 66].

2. The loss of the tumor suppressor glycogen *debranching enzyme AGL*.

   A recently published analysis used human xenograft models of bladder cancer and a genome-wide lentiviral short-hairpin RNA (shRNA) library coupled with next-generation sequencing (NGS) to discover *in vivo* novel genes functionally important in bladder carcinogenesis. AGL was identified as a crucial player in negatively regulating bladder cancer growth. Indeed, loss of AGL was strongly associated with tumor growth and clinically aggressive disease [67].

   AGL contributes to complete breakdown of glycogen to glucose-6-phosphate (glycogenolysis) removing branch points. Tumor cells with AGL loss showed a reduced content of normal glycogen, and a reciprocal accumulation of abnormally branched glycogen (such as limit dextrin) [67]. Analogous biochemical sequelae are observed in glycogen storage disease type III (GSDIII – Cori disease), an autosomal recessive hereditary syndrome marked by germline mutation of AGL, compromising glycogen breakdown with subsequent intracellular storage of

abnormal limit dextrin. Impaired liver function, cardiomyopathy and rapid muscular exhaustion are clinical consequences of the accumulation of abnormal glycogen in the liver, heart and skeletal muscle, respectively [68].

However, the precise mechanism by which AGL depletion acts as tumor suppressor is not yet entirely understood. It has been supposed that the role of AGL in promoting tumor growth cannot be attributed to its enzymatic activities in glycogenolysis.

AGL loss impairs glycogen breakdown and favors bladder cancer growth via independent mechanisms. Indeed, in vitro inhibition of glycogenolysis through depletion of glycogen phosphorylase (another crucial enzyme in the glycogen breakdown process) does not affect (induce) bladder cancer cell proliferation, suggesting that the glycogenolysis inhibition is not a necessary step of bladder cancerogenesis and strengthening the absolute independence of the pro-oncogenic role of AGL-loss from glycogenolysis. Moreover, enzymatically inactive AGL mutants (lacking both the glucotransferase and glucosidase activities) preserve the tumor-suppressive function of wild-type AGL in bladder cancer models. Indeed, bladder cancer cells transduced with shRNA against AGL (shAGL) showed AGL depletion and subsequent enhanced proliferation. Conversely, transfection with enzymatically inactive AGL variants (and shAGL-insensitive wild-type AGL) reversed the increased tumor growth observed with AGL loss. These data demonstrate that loss of AGL promotes bladder cell growth independently from reduced glycogen breakdown. Inhibition of glycogenolysis is not the key driver of bladder cancer growth. Thus, the enzymatic activities of AGL are not required for its growth-suppressive function in bladder carcinogenesis. Increased tumor cell proliferation observed with AGL loss is not correlated to its role in glycogenolysis [67].

Therefore, we should assume that AGL carries unknown non-enzymatic properties (not yet entirely known) that influence tumor growth. Among non-metabolic functions, it seems that AGL loss leads to a metabolic reprogramming of cancer cells.

In particular, AGL loss reprograms the serine-to-glycine conversion pathway. In fact, cells with diminished AGL levels exhibit increased glycine amount, but unchanged concentrations of the glycine precursor (serine) [67]. Augmented mitochondrial glycine biosynthesis strongly correlates with rapid cancer cells proliferation [69]. AGL depletion determines augmented levels of the serine-to-glycine enzyme serine hydroxymethyltransferase-2 (SHMT2), resulting in an increased glycine synthesis and purines synthesis (for which glycine is a precursor), thus supporting nucleotides synthesis required for DNA synthesis and subsequent cancer cells proliferation in vitro and in vivo. Therefore, AGL loss induces tumor growth via promoting increased glycine-driven synthesis of nucleotides from non-glucose (serine) sources [67].

In summary, AGL loss causes high SHMT2 expression, and consequently increased glycine-dependent nucleotide synthesis leading to bladder cancer growth.

Additional metabolic pathways are compromised by AGL loss. In particular, an augmented glucose uptake, due to increased GLUT-1 translocation to the membrane, does not result in increased glucose-to-lactate conversion (which is, on the contrary, decreased), suggesting a glucose shift towards metabolic pathways involved in the synthesis of macromolecules required for cell proliferation [67]. Moreover, AGL depleted-cells have increased anabolic processes, including increased protein synthesis due to increased amino acids (alanine, aspartate, and glutamate) formation, consistent with an increased contribution of glucose from glycolytic and tricarboxylic acid cycle (TCA) flux followed by transamination of the intermediates oxaloacetate, pyruvate, and α−ketoglutarate. Similarly, in AGL depleted-cells, glucose represents an important carbon source for nucleotide ring and ribose moiety de novo synthesis (increased glycine synthesis from glucose), both necessary for RNA and DNA formation in proliferating cells, via an increased flux through the pentose phosphate pathway and increased incorporation of ribose into purine and pyrimidine nucleotides [67].

3. <u>The increased activity of the tumor promoter enzyme *glycogen phosphorylase*</u>.

Glycogen turnover undergoes significant alterations in cancer cells, breaking the physiological equilibrium between synthesis and breakdown, thus resulting in temporal changes of intracellular glycogen concentration. Initially, a rapid hypoxia-driven induction of *glycogen synthase* leads to an early glycogen storage. The accumulation of glycogen promotes cancer cell survival as an adaptive way to ensure an energy source in a status of glucose and oxygen deprivation [58,59]. Subsequently, an increase of *glycogen phosphorylase* activity (mainly of the *liver isoform of glycogen phosphorylase* [PYGL]) causes a slow decline of glycogen stores, raising the proportion of glucose that is diverted to the pentose phosphate and de novo fatty acid synthesis pathways, which are necessary to support elevated synthesis of macromolecules (nucleotides, amino acids, and fatty acids) required by rapidly proliferating cells. Therefore, *glycogen phosphorylase* plays a crucial role in cancer cell biology, regulating the glucose availability to sustain tumor cell proliferation and prevent premature senescence [70]. Depletion (or pharmacological inhibition [62,71]) of *glycogen phosphorylase* determines glycogen accumulation, limits the synthesis of nucleotides (decreasing the glucose substrate source for the pentose phosphate pathway), induces premature senescence by increasing ROS levels, and impairs tumor cells proliferation due to p53-dependent growth arrest [70,72]. *Glycogen phosphorylase* might therefore be a possible therapeutic target for inhibition in bladder cancer with overactive glycogen and glucose metabolism.

- **Lipid metabolism pathway**

Alterations in lipid metabolism are involved in bladder carcinogenesis [73].

Lipids are molecules composed of fatty acids insoluble in polar solvents (hydrophobicity) and soluble in apolar solvents (lipophilicity). Physiologically, lipids can be divided into storage lipids (with protective and energy functions - triglycerides) and structural lipids (the main component of cell membranes and responsible of inter-cellular transporting systems and intra-cellular signal transmission- phospholipids, glycolipids, cholesterol).

The high energy yield of fatty acids is the result of oxidative demolition processes (lipid breakdown), which consist of two main phases: 1) the mitochondrial β-oxidation – a catabolic process entailing the gradual oxidative cleavage of two-carbon fragments in the form of acetyl-CoA from fatty acids previously activated to acyl-CoA. 2) The subsequent oxidation of acetyl-CoA to carbon dioxide by the citric acid cycle (TCA/Krebs cycle), and then by the mitochondrial electron transport chain to release energy.

While breakdown of lipids takes place in the inner space of the mitochondria (matrix), their activation to acyl-CoA occurs outside of the internal mitochondrial membrane. Carnitine is essential in mediating the transport of acyl groups across the mitochondrial inner membrane. The enzyme *carnitine palmitoyltransferase I* (located on the outer surface of the inner mitochondrial membrane) catalyzes the transfer of acyl groups to the hydroxyl group of carnitine. Unlike acyl-CoA molecules (insoluble), acyl-carnitines are able to cross the inner mitochondrial membrane (by *carnitine translocase*), reaching the mitochondrial matrix. Here, the acyl groups are transferred to the mitochondrial CoA-SH by *carnitine palmitoyltransferase II* (localized on the inner surface of the inner mitochondrial membrane), with acyl-CoA and carnitine as terminal products.

Several studies have suggested a possible carcinogenic role of elevated levels of carnitine in bladder cancer. Metabolomic profiling revealed potential urine metabolites altered in bladder cancer progression (including carnitine), therefore helpful distinguishing bladder cancer from normal controls and also non-muscle from muscle-invasive bladder cancer [74]. Jin and Colleagues used high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (HPLC-QTOFMS) to identify different urine metabolomic profiles that allow distinguishing bladder cancer patients from healthy control groups, and muscle invasive from non-muscle-invasive bladder cancer patients. Several cancer-specific metabolites were identified, including carnitine and acyl-carnitines (carnitine, isovalerylcarnitine, glutarylcarnitine, octenoylcarnitine, and decanoylcarnitine). Moreover, bladder cancer groups (and particularly muscle-invasive tumors) expressed significantly higher levels of *carnitine palmitoyltransferase 1A* (CPT1A) than control tissues. These results

suggest a key role of fatty acid oxidation in promoting bladder cancer development and progression [75]. Similar findings derived from metabonomic profiling analysis that identified urinary carnitine C9:1 (combined with component I) as a viable biomarker for discriminating bladder cancer patients [76].

Under conditions of limited glucose availability (i.e. fasted state or rapidly proliferating cancer cells), fatty acid β-oxidation becomes the primary energy source. However, when the amount of acetyl-CoA generated in β-oxidation exceeds its utilization in the Krebs cycle (for lack of oxaloacetate - derived from glucose via pyruvate), ketogenesis take place. Acetyl-CoA is then used in biosynthesis of ketone bodies (including acetoacetate, β-hydroxybutyrate, and acetone), to make available the CoA required for the further beta-oxidation of fatty acids.

Elevated lipids and acetoacetate levels have been detected in serum samples of bladder cancer patients using H nuclear magnetic resonance (NMR)-based metabolomics analysis [77]. Similarly, a NMR-based metabolite profiling analysis of canine xenograft model (exploiting analogies between naturally-occurring canine transitional cell bladder carcinoma and human invasive bladder cancer) demonstrated high levels of acetone and β-hydroxybutyrate in invasive bladder cancer urine samples compared with healthy controls [78].

Therefore, high levels of ketone bodies could reflect increased activity of fatty-acid β-oxidation pathway instead of active glycolysis in bladder cancer cells, leading to increase in TCA activity and oxidative phosphorylation, and subsequent excess amounts of acetyl-CoA that is diverted towards ketogenesis.

Another demonstration of the increase of fatty acids beta-oxidation as an energy source in proliferating tumor cell derived from the identification of lower triglycerides levels (source of fatty acids) in patients with bladder cancer compared to healthy controls [79].

In contrast to lipolysis, when glucides exceeds immediate energy requirements, and after saturation of tissue glycogen deposits, glucose (acetyl-CoA) is converted into storage lipids (lipogenesis). Lipogenesis encompasses both fatty acid and triglyceride synthesis (where fatty acids are esterified with glycerol). Through fatty acids biogenesis and triglyceride synthesis, the surplus energy can be efficiently stored in the form of fats.

Fatty acid synthesis is catalyzed by several enzymes organized in a single multienzyme complex, termed fatty acid synthase [FASN]. FASN catalyzes the NADPH-dependent synthesis of palmitate from acetyl-CoA and malonyl-CoA, thus mediating the conversion of glucides into fatty acids. Triglyceride synthesis originates from activated fatty acids (acyl-CoA) and glycerol-3-phosphate (produced by reduction of dihydroxyacetone phosphate [DHAP] – a glycolysis intermediate - by the glycerol-3-phosphate dehydrogenase) through two acylation reactions. Therefore, the glycerol-3-phosphate dehydrogenase enzyme acts as a bridge between carbohydrates and lipids metabolism.

Tumor cells can reactivate de novo lipid synthesis, highlighting the potential role of lipogenesis in cancer pathogenesis [80]. Lipolysis, in fact, provides lipids (essential constituents of cell membranes) to proliferating cancer cells.

FASN over-expression seems to play a crucial role in BC development (being associated with tumor cell survival and migration, high histologic grade, tumor recurrence, and resistance to chemotherapy) [81-83]. Therefore, blocking FASN may represent a novel targeted-therapy strategy for BC [81,82].

Additionally, glycerol-3-phosphate dehydrogenase activity has been reported to be up-regulated in bladder cancer, supplying glycerol-3phosfate for lipid biosynthesis [84].

Various metabolites involved in lipid metabolism seem to be altered (up-regulated) in BC patients, suggesting a deregulation of physiological control mechanisms, and reinforcing a potential pro-carcinogenic action of lipid metabolism pathways.

A metabolomics analysis with liquid chromatography/mass spectrometry (LC/MS) identified increased urine concentrations of free fatty acids (oleic and palmitic acids) in BC samples [74].

Urine metabolites related to lipid metabolism seem to be robust biomarkers to differentiate bladder cancer from non-cancer controls, therefore representing potential non-invasive adjunct diagnostic to cystoscopy for early diagnosis of bladder cancer and recurrent disease management. A comprehensive global metabolomics profiling analysis of urine revealed 3 metabolites associated with lipid metabolism, palmitoyl sphingomyelin, arachidonate, and phosphocholine significantly elevated in bladder cancer patients [85].

Arachidonate is a polyunsaturated fatty acid commonly present in the phospholipids (especially phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositides) of cells membranes, particularly abundant in the brain, muscles, and liver. Increased arachidonate levels can derive from augmented release of free fatty acids from phospholipids either in the tumor or in adjacent tissue. Sphingomyelin is a key component of the outer plasma cell membranes, whose cleavage (by neutral sphingomyelinases) generates both phosphocholine and ceramide. Phosphocholine, in turn, is a constituent of both glycerophospholipids and sphingomyelin. Augmented urine levels of palmitoyl sphingomyelin and phosphocholine may suggest a higher tumor cell proliferation rate with increased lipid membrane remodeling, leading to increased urine shedding of palmitoyl sphingomyelin and subsequent upregulation of sphingomyelinases activity with augmented choline phosphate in the urine of bladder cancer patients [85].

Analogous findings came from a study using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) and gas chromatography-mass spectrometry (GC-MS) methods, which also demonstrated the up-regulation of choline-containing compounds (choline, phosphocholine and glycerophosphocholine) in bladder patients (17 cases of Ta-T1 lesions and 16 muscle-invasive tumors) compared with benign controls (26 subjects), but no significant difference was identified between superficial and muscle-invasive tumors [79].

Similarly, Lin et al. demonstrated increased by comprehensive LC-MS-based method (which utilizes both reversed phase liquid chromatography [RPLC] and hydrophilic interaction chromatography [HILIC] separations) increased serum levels of phosphatidylcholine in patients with bladder cancer [86].

Elevated serum levels of malonate were found in high-grade bladder cancer patients compared to low-grade tumors and healthy controls [87]. Malonate level depend on the amount of malonyl-CoA. Malonyl-CoA controls the metabolism of acyl-CoA, and then of fatty acids synthesis. It inhibits the rate-limiting step in beta-oxidation of fatty acids by inhibiting acyl-CoA: carnitine acyl transferase, precluding acyl groups from associating with carnitine, thereby preventing fatty acid from entering within the mitochondria (where fatty acid oxidation and degradation occur) and inhibiting the beta-oxidation and ketogenesis.

In addition, Pasikanti et al. demonstrated by urinary metabotyping using two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS) down-regulation of glycerol (end product of triglycerides hydrolysis) in bladder cancer compared with non-cancer patients [88].

In conclusion, in healthy subjects, the dynamic equilibrium of lipid metabolism results from the balance between lipid breakdown (fatty acids β-oxidation to satisfy the energy requirements) and lipid synthesis. Conversely, dysregulation of lipid metabolism (altered fatty acid transportation, β-oxidation, or synthesis) might be involved in the pathogenesis of bladder cancer.

DISCUSSION

Metastatic bladder cancer is still considered a disease orphan of effective treatments. The current standard treatment - platinum-based chemotherapy – fails to ensure long survivals, with median survival in these patients of about 12-14 months.

Many genetic and epigenetic alterations are involved in BC development, progression, and metastatization.

However, unlike other tumor types (breast, melanoma, colorectal cancer, lung adenocarcinoma), no effective molecular targets for therapy and no biomarkers with a predictive value have yet been identified in bladder cancer. Alterations in energy metabolism represent a hallmark of cancer, providing potential therapeutic targets. Recently, a complex analysis that integrated data of metabolic alterations (involving amino acid, nucleotides, lipids and glycolysis pathways) from targeted mass-spectrometry with transcriptome data from TCGA identified a molecular signature of 30 metabolic genes, whose the up-regulation was associated with tumor progression and poor prognosis [89]. This integrated metabolic/transcriptomic pathway signature linked with BCa progression demonstrates once again the crucial role of metabolic alterations in BC development and progression.

We extensively outlined the crucial role of anaerobic glycolysis in providing adequate energy supplies, but also in ensuring intermediate metabolic precursors for the biosynthesis of non-glucidic compounds (amino acids and lipids). Numerous inhibitors of glucose uptake and glycolysis are currently available, but very few have been tested in bladder cancer patients, and to date there is no evidence of a clinical benefit that supports their use in clinical practice.

Glucose transporters and several glycolytic enzymes involved in tumorigenesis can be targets for inhibition in cancer treatments. In particular, GLUT inhibitors (the natural phenol derivative Phloretin [90], or the small molecule WZB117 [91]) exert anticancer effects by blocking tumor cells growth and/or sensitizing the cancer cells to chemotherapy. The glycolytic enzyme HK can be selectively blocked by several compounds, including lonidamide, 2-deoxy-D-glucose, and 3-bromopyruvate, resulting in promising antitumor effect when combined with chemotherapy [92]. Moreover, LDH-A and PFKFB3 are promising targets for inhibition in cancer treatments [27,93,94].

Glycogen metabolism represents another deregulated pathway with a major role in BC. A potential pharmacological target of this pathway is the enzyme glycogen phosphorylase, leading to the development of several glycogen phosphorylase inhibitors. CP-320626, CP-91149, and flavopiridol

block glycogenolysis, reducing the availability of glucose to be recycled into the PPP and lipid synthesis, with subsequent arrest of proliferation and increase in apoptosis of pancreatic cancer cells [62, 95-97]. The mTOR-inhibitor everolimus has been evaluated in metastatic BC patients progressing after failure of platinum-based chemotherapy, suggesting a potential biological activity in a subgroup (yet to be determined) of patients with urothelial cancer. These studies highlight the need to preselect patients based on molecular cancer phenotype to maximize benefit from targeted agents like everolimus (i.e. PTEN loss might be associated with everolimus resistance) [98,99].

Certainly the way is still long. Effort are required to understand the metabolic abnormalities underlying bladder carcinogenesis, identify the best therapeutic targets and develop therapeutic molecules that combine their anti-tumor effect with an adequate toxicity profile.

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Figure 1. Mir-21 regulation, in brief.

TGFß1 induces the phosphorylation of SMAD2/3 by TGFßR1 and TGFßR2 receptors, which in turn, leads to up-regulation of miR-21 in human breast cancer cell lines. In particular, SMAD2/3 bind miR-21 promoter and enhance its transcription. MiR-21, which transcription is enhanced also by STAT3, represses important tumor suppressor genes including PTEN, tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), and maspin (also SERPINB5).