Metabolic Reprogramming in Cholangiocarcinoma

Chiara Raggi[1*], Maria Letizia Taddei[1], Colin Rae[2], Chiara Braconi[2,3], and Fabio Marra[1*]

[1]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

[2] Institute of Cancer Sciences, The University of Glasgow, Glasgow, United Kingdom.

[3] Beatson West of Scotland Cancer Centre, Glasgow, United Kingdom

***Address for correspondence:**

Fabio Marra, MD, PhD
Department of Experimental and Clinical Medicine, University of Florence,
Cubo Centro Polivalente 2,
Viale Pieraccini, 6
I50139 Florence, Italy
Email: fabio.marra@unifi.it

And/or
Chiara Raggi, PhD
Department of Experimental and Clinical Medicine, University of Florence,
Cubo Centro Polivalente 2,
Viale Pieraccini, 6
I50139 Florence, Italy
Email: chiara.raggi@unifi.it

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Key Points

- Metabolic processes are altered in CCA, and contribute to its progression and malignancy
- Besides glucose metabolism, many other metabolic activities involving carbohydrates, lipids and amino acids are deregulated in CCA
- Oncogenes may drive alterations in cancer metabolism, and on the other hand metabolites may control gene and protein expression
- Proteins involved in metabolism and/or metabolites should be investigated as potential innovative diagnostic and prognostic biomarkers
- A deregulation of mitochondrial-dependent metabolism contributes to stemness features of CCA
- Metabolic reprogramming in CCA may represent a potential druggable target even in a combined therapeutic setting

Summary

Metabolic reprogramming is a hallmark of cancer and allows tumor cells to meet the increased energy demands required for rapid proliferation, invasion, and metastasis. Indeed, many tumor cells acquire distinctive metabolic and bioenergetic features to survive under conditions of limited resources, mainly using alternative nutrients. Several recent studies have explored the metabolic plasticity of cancer cells with the aim to identify new druggable targets, and therapeutic strategies aimed to limit the access to nutrients have been successfully applied to the treatment of some tumors. Cholangiocarcinoma (CCA), a highly heterogeneous tumor, is the second most common form of primary liver cancer. It is characterized by resistance to chemotherapy and poor prognosis, with 5-year survival lower than 20%. Deregulation of metabolic pathways has been described during the onset and progression of CCA. Increased aerobic glycolysis and glutamine anaplerosis provide CCA cells with the ability to generate biosynthetic intermediates. Other metabolic alterations involving carbohydrates, amino acids and lipids have been shown to sustain cancer cell growth and dissemination.

In this review, we discuss the complex metabolic rewiring taking place during CCA development, leading to unique nutrient addiction. The possible role of therapeutic interventions based on metabolic changes is also thoroughly discussed.

Introduction

Cholangiocarcinoma (CCA) is the second most common primary hepatic tumor and accounts for 3% of all gastrointestinal cancers [1]. CCA belongs to a heterogeneous group of malignancies occurring at any point of the biliary tree [1] and is anatomically classified as intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) [1] CCA. pCCA and dCCA may also be collectively referred as "extrahepatic" (eCCA) [1]). pCCA accounts for approximately 50–60% of all cases, followed by dCCA (20–30%) and iCCA (10-20%) [1]. CCA is usually asymptomatic in the early stages, resulting in diagnosis only at advanced stages [1]. Although CCA is a rare cancer, its incidence and mortality rates (1-6:100000 inhabitants/year, globally) have been increasing in recent years. Moreover, despite advances in diagnosis and treatment, prognosis has not improved significantly in the past decade, with 5-year survival around 7-20% [2].

Risk factors include a variety of conditions which result in inflammation and cholestasis, such as parasitic infections, primary sclerosing cholangitis (PSC), biliary duct cysts, hepatolithiasis, toxins (including alcohol and tobacco smoking), HBV or HCV infection, cirrhosis, diabetes, obesity and genetic factors [3]. In Southern Asia, CCA has been associated with infection with liver flukes, such as *Opistorchis viverrini* and *Clonorchis sinensis* [1-3].

The main curative therapeutic option for CCA is surgical resection followed by adjuvant chemotherapy, although less than 20% of patients present with early-stage disease amenable to surgery. The results of liver transplantation are controversial and better selection criteria are needed [4]. Patients with unresectable and/or recurrent tumors are offered palliative systemic therapy, including chemotherapy (gemcitabine-cisplatin, fluorouracil-oxaliplatin), but clinical outcomes remain poor with median overall survival (OS) of 9-14 months. Recently, targeted therapies have been approved for a niche of patients with CCA harboring selected molecular alterations (FGFR2 fusions, IDH mutations, NTRK fusions). Treatment with immune checkpoint inhibitors is still under investigation and recent data seem to favor the combination of PD-L1 inhibitors and chemotherapy (https://www.astrazeneca.com/media-centre/press-releases/2021/imfinzi-improved-survival-in-biliary-tract-cancer.html). Nonetheless, therapeutic options for CCA patients remain limited.

Molecular pathogenesis of CCA is a multifactorial and complex process, in which persistent inflammation, genetic and epigenetic alterations, multicellular origin and tumor heterogeneity produce an intricate network of oncogenic mechanisms [1]. In the present review, evidence indicating the existence of a complex metabolic rewiring in CCA will be discussed, together with the opportunities that these studies may generate in the search for new treatment approaches.

General characteristics of cancer metabolism

Reprogramming energy metabolism is one of the cardinal hallmarks of cancer [5], and altered metabolism supports cancer cell survival in hostile microenvironments. A well-known example is the increase in glucose consumption through aerobic glycolysis even in the presence of oxygen [6]. More recently, cancer cells were shown to utilize different nutritional sources, including intermediates generated by the tricarboxylic acid (TCA) cycle as precursors for lipids, amino acids, or nucleotides that support tumor progression. Generally, cancer cells possess a marked flexibility in the use of energy sources, depending on the environmental availability of nutrients and on the heterogeneity of intratumoral cell populations [7].

Metabolic reprogramming may be induced by mutations of both oncogenes and tumor-suppressor genes [8]. Although oncogene-driven modifications in the metabolic network may contribute to cell transformation, only few metabolic enzymes are directly deregulated by genetic alterations. These include mutations in isocitrate dehydrogenase 1 or 2 (IDH1 or IDH2), leading to the production of the onco-metabolite D-2-hydroxyglutarate (D-2-HG) [9]. High levels of D-2-HG affect α-ketoglutarate-dependent dioxygenases, including those involved in epigenetic remodeling and DNA repair [10]. Similarly, mutations in components of the succinate dehydrogenase complex and in fumarate hydratase induce the accumulation of succinate and fumarate, which also interfere with dioxygenase function and the epigenetic profile of cancer cells [11].

Reprogrammed metabolic pathways of cancer cells support: i) bioenergetics, ii) anabolism and iii) redox homeostasis [8]. Cellular energy is primarily supplied by either glycolysis or mitochondrial oxidative phosphorylation (OXPHOS) to generate ATP. Although cells may use both pathways, one of the two frequently dominates in a given cell [7]. Based on evidence that most malignant cells are highly sensitive to glucose deprivation, the “Warburg effect” still represents an attractive therapeutic target [12]. Nevertheless, despite promising pre-clinical results, glycolysis inhibitors failed to meet the expectations in the clinic [13]. Deregulated metabolism enables cancer cells to produce macromolecules to sustain tumor growth. Increased flux through glycolysis allows cancer cells to divert glucose-derived carbons into branching pathways, to sustain the production of nucleotides, lipids and proteins. Moreover, other energy sources, like glutamine, lactate, pyruvate, β- hydroxybutyrate, acetoacetate, acetate and free fatty acids (FA), either synthesized within tumor cells or taken-up from the environment, are converted into biosynthetic intermediates for anabolic purposes [14]. Some tumor cells rely on autophagic processes to obtain an adequate amount of amino acids for energy and biosynthesis [15]. Because of this increased metabolic rate, reactive oxygen species (ROS) generation is increased in cancer cells. Although a moderate increase in ROS contributes to tumor promotion and progression, cancer cells increase antioxidant capacity [16] to

avoid the toxic effects of ROS build-up. Thus, tumor cells mainly activate the NRF2-dependent transcription program and increase flux through NADPH-producing metabolic pathways [17].

The metabolic status of cancer is also extrinsically influenced by microenvironmental cues, including the availability and composition of nutrients, different oxygen concentrations, acidity, and interaction with the extracellular matrix [18]. Indeed, cancer cells must compete for energy sources in nutritionally compromised microenvironments, and their metabolic plasticity in the use of different fuels can be of great benefit for cell survival [19].

Therapeutic approaches targeting tumor metabolism are already in clinical use. L-asparaginase, an enzyme that converts asparagine to aspartic acid and ammonia, is approved for the front-line treatment of acute lymphoblastic leukemia, where cells are typically auxotrophic for this amino acid [20]. Thus, deciphering the mechanisms responsible for metabolic reprogramming during cancer progression may disclose the susceptibility of cancer cells to novel targeted therapeutic approaches.

CCA and glucose metabolism

Like other tumors, CCA is strongly dependent on glucose metabolism, and fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) is a major diagnostic tool for staging and prognostication of CCA [21]. Although elevated FDG uptake is present in 92% of patients with iCCA [22], discordant data have been obtained in dCCA [23]. Expression of the glucose transporter GLUT-1 is correlated to ^{18}F-FDG uptake, histological differentiation [24] and poor OS [25, 26], supporting the biology behind the clinical relevance of ^{18}F-FDG PET.

The glycolytic pathway plays a central role in CCA metabolism, as shown by the deregulation of several enzymes. Hexokinase II is upregulated in CCA tissue specimens and its inhibition significantly decreases aggressiveness of CCA cells [27]. High levels of pyruvate kinase M2 (PKM2) were detected in tumor tissues of CCA patients, correlating with worse clinical outcomes, and *in vitro*, PKM2 promoted proliferation, migration and angiogenesis in CCA cells [28, 29]. Yu et al. observed that increased expression levels of PKM2 correlate with tumor progression and are an independent predictor of recurrence and survival after resection of pCCA [30]. The key role of the Warburg effect in CCA progression is further supported by the association between poor prognosis and increased expression of lactate dehydrogenase A (LDH-A), the NADH-dependent enzyme that catalyzes the conversion of pyruvate to lactate [31, 32]. Accordingly, high serum levels of LDH correlate with poor clinical outcome in patients treated with chemotherapy [33]. Moreover, it has been demonstrated that CCA cells, which avidly consume glucose, induce a cMyc-mediated increase in LDH and PKM2 levels, causing a low intracellular content of pyruvate, in favor of increased lactate levels. Pyruvate

is an inhibitor of the histone deacetylase HDAC3, which deacetylases cMyc, thus promoting its stabilization. cMyc, by decreasing pyruvate levels, removes HDAC3 inhibition and protects cancer cells from apoptosis. Hence, a positive feedback loop is maintained where, in turn, high activity of HDAC3 stabilizes cMyc, sustains low pyruvate levels and promotes CCA cell proliferation [34]. A positive effect of hyperglycemia on CCA growth has also been reported. CCA cells cultured in medium with high glucose show increased proliferation, migration and invasion, associated with activation of the oncogenic STAT3 pathway. Similarly, nuclear expression of STAT3 and p-STAT3 is higher in CCA tissues from patients with diabetes [35]. Glucose-induced activation of STAT3 is mediated by IL-1β-dependent activation of the NF-κB pathway [36]. STAT3 induces the downstream upregulation of the transcription factor FOXM1, which is responsible for the aggressive phenotype of CCA cells cultured in high glucose [37]. Other pathways participate in the promotion of CCA growth in conditions of hyperglycemia, including ROS-mediated upregulation of chromodomain helicase DNA-binding protein 8 and mannosidase alpha class 2a member 2 [38], increased expression of DPY30, a subunit of the SET1 and MLL family methyltransferase complexes [39], the long non-coding RNA FAM66 [40] and SIRT3-dependent activation of hypoxia inducible factor-α (HIF1α)/PDK1/pyruvate dehydrogenase axis [41] (Figure1). Additional evidence for the dependence of CCA on glycolysis is provided by the lower mitochondrial mass, associated with shorter survival of CCA patients [42]. Decreased mitochondrial activity, shown by high expression of uncoupling protein 2, contributes to increased proliferation and invasion through glycolysis-mediated mechanisms, and is negatively correlated with survival [43].

The glycolytic flux also provides carbon sources which contribute to anabolic biosynthesis. For instance, the glycolytic intermediate fructose-6-phosphate fuels the hexosamine biosynthetic pathway to support posttranslational modifications [44]. In CCA cell lines, the ability to disaggregate and give rise to metastasis has been related to O-GlcNAcylation of NF-κB [45, 46], activation of AKT and ERK, which decrease the expression levels of FOXO3 and α1,2-mannosidase IA, finally leading to the elevation on the cell surface of Man9, a high mannose type N-glycan [47], whose increased levels have already been correlated with breast cancer progression [48]. The glycolytic intermediate glucose-6-phosphate may be re-directed towards the pentose phosphate pathway (PPP) to produce ribose-5-phosphate and NADPH, to sustain nucleotide synthesis and to buffer increased ROS levels. Indeed, CCA cells with primary cisplatin resistance have increased activity of the PPP and antioxidant abilities [49]. Many enzymes of the PPP are under the control of the antioxidant transcription factor NRF2 [50]. In line with these findings, in CCA specimens FoxO3-Keap1 is down-regulated and NRF2 hyper-activated, leading to decreased ROS production, and protecting tumor cells against oxidative stress-induced cell death [51]. NRF2 knockdown inhibits the replicative ability of CCA

cells and increases sensitivity of these cells to the cytotoxic and anti-proliferative effects of chemotherapeutic agents [52]. In addition to PPP, the serine synthesis pathway (SSP) is another shunting of glycolysis, supporting cellular biosynthesis and antioxidant response [53]. High levels of SIRT2/cMyc inhibit OXPHOS through the phosphorylation of PDHA1 and support the SSP, contributing to redox homeostasis and to the protection of CCA cells from oxidative stress-induced apoptosis [54].

Metabolic reprogramming of CCA may also depend on mutations in *IDH1* or its paralogue *IDH2* (collectively referred to as *IDH*). These mutations are prevalent in various types of cancer, including CCA (10%), where the most common are R132C, R132G, R132S or R132L, for IDH1, and R172K, R172M or R172G for IDH2 [55]. Mutations of IDH are mainly observed in iCCA and are associated with poor differentiation of hepatic progenitor cells resulting from production of the oncometabolite D-2-HG [56]). IDH mutations promote DNA hypermethylation and increased demethylation of histone H3K79, suggesting the existence of an altered gene expression profile of IDH-mutated CCA [57]. The oncogenic potential of *IDH* mutations have been confirmed in animal models, where IDH1-R132C, loss of p53 expression, and activation of Notch signaling promote iCCA development in mice [58]. Ex-vivo, this mutation increases the growth of biliary organoids and accelerates the glycolytic flux through upregulation of phosphofructokinase-1 [59].

Despite these lines of evidence, the prognostic implications of *IDH* mutations in CCA are still controversial. While some reports showed reduced risk of relapse and longer OS for IDH-mutated CCA compared to WT, these data have not been confirmed in other studies [60]. Within the ClarIDHy phase III, second-line trial, survival of IDH mutated patients treated with placebo was not remarkably different from the others and sat around 6 months for chemo-refractory CCA patients Zhu AX [61].

Some recent reports highlight the relevance of OXPHOS in CCA progression. Overexpression of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1α), a master regulator of mitochondrial biogenesis supports CCA metastasis, upregulating PDHA1 and MPC1 expression and shifting the metabolism towards mitochondrial respiration [62]. In line with these data, pharmacological inhibition of the respiratory chain with both phenformin and metformin effectively blocks proliferation and invasion of CCA cells [63-65]. Accordingly, recent epidemiological studies showed that metformin treatment is significantly associated with a reduced risk of CCA in diabetic patients [66, 67]. Moreover, Jiang et al. showed that metformin inhibits CCA tumor growth by cell cycle arrest in vitro and in vivo [68]. Furthermore, a retrospective study by Tseng et al., demonstrated that metformin significantly decreases the risk of biliary tract cancer by 50%–60%, although does not affect overall survival in these patients [69]. In keeping, Yang et al. reported that metformin does not improve survival of CCA in patients with diabetes [70]. Thus, in light of this still controversial

evidence, inhibition of OXPHOS in cancer cell as an approach to counteract CCA progression deserves further investigation.

It has been recently demonstrated that tumor cells metabolically communicate with stromal cells predominantly in the primary tumor microenvironment [71]. Crosstalk between inflamed lymphatic endothelial cells (LECs) and CCA cells by the CXCL5-CXCR2 axis [72] induces alterations of mitochondrial respiration and glycolysis in tumor cells. Notably, CXCL5 directly induces lactate production, glucose uptake and generation of mitochondrial reactive oxygen species in CCA cells but also increases metabolic gene expression in LECs. Significant alterations in cellular bioenergetics of LECs predispose a pro-lymphangiogenic signaling that promotes lymph node metastasis [72]. These lines of evidence paved the way for strategies to target metabolic communications for improved cancer treatments (Figure1).

CCA and lipid metabolism

Altered lipid metabolism is a prominent metabolic modification in cancer. Several studies have documented the reactivation of *de novo* lipogenesis and increased FAsynthesis in various cancers, making them independent of lipid exogenous uptake [73]. Enhanced lipid synthesis or uptake contributes to cancer cell growth. FA are also involved in the synthesis of more complex lipid species (i.e. diacylglycerides (DAGs) and triacylglycerides (TAGs)) or are converted into phosphoglycerides (i.e. phosphatidic acid (PA), phosphatidylethanolamine (PE) and phosphatidylserine (PS) [73].

De novo fatty-acid synthesis involves two key enzymes, acetyl-CoA carboxylase (ACC) and fatty-acid synthase (FASN). ACC carboxylates acetyl-CoA to form malonyl-CoA, which is further converted by FASN to long-chain fatty acids [73]. *De novo* lipogenesis is largely regulated at the transcriptional level by SREBP1 and SREBP2. Furthermore, multiple oncogenic pathways, such as PI3K/AKT, control enzymes required for FA synthesis (i.e. FASN) [73]. Intriguingly, hepatocellular carcinoma (HCC) but not iCCA cells, are sensitive to FASN inhibition [74], and FASN is highly expressed in HCC tissues [75], while its expression is frequently low in iCCA [74]. Additionally, in a mouse model of AKT/NICD-driven cholangiocarcinogenesis as well as in vitro, FASN and *de novo* lipogenesis were not required [74, 76]. In contrast, CCA cells expressing low levels of FASN displayed high expression of fatty acid uptake-related proteins and robust long-chain fatty acid uptake, as a compensatory mechanism. In particular Li, Che, at al., [74] suggested the role of FATP1 (SLC27A1), a member of the FA transport protein family. Suppression of FATP1 decreased in vitro growth of iCCA cell lines and enhanced the effect of FASN inhibition [74]. In contrast, other studies showed that FASN expression was directly correlated with advanced stage CCA in a database of 155 patients and associated with shorter survival [77]. Furthermore, FASN knockdown inhibited the

growth, migration, invasion and cell cycle progression, and induced apoptosis in CCA cells. Metabolomic studies showed that purine metabolism was the most relevant pathway involved in FASN knockdown [77]. Accordingly, FASN was found to be repressed by KDM5C during iCCA progression and KDM5C downregulation was associated with poor prognosis in iCCA [78].

A possible explanation for the differential results regarding the role of FASN may be the different availability of FA in the cellular context and extracellular microenvironment. In experimental models (mostly cell lines) where there is no external source of fatty acids, FASN appears to have more importance. In contrast, FA transporters are likely to have greater relevance in vivo or in experimental models (such as co-culture with adipocytes) where exogenous fatty acids are available and a preferential source. This would explain why FASN inhibitors have been potent in cell lines, but less so in vivo, and combinations of inhibitors of FASN and FA uptake are usually more effective than either inhibitor alone.

Among the best characterized FATPs, FABP5 is highly expressed in eCCA and closely correlated with poor prognosis in eCCA compared to iCCA, suggesting differences in energy metabolism in distinct anatomic locations [79]. Along these lines, hypothesizing an adipocyte-CCA cross-talk, FABP4 was found to mediate adipocyte-induced invasion, migration and epithelial mesenchymal transition (EMT) of CCA cells [80] (Figure2).

Among their many functions, fatty acids give rise to prostaglandins, bioactive lipids regulating a number of processes relevant for cancer including CCA (reviewed in [81]). In the sphingolipid family, ceramide and sphingosine-1-phosphate (S1P) represent the most prominent bioactive lipids. S1P is a pivotal regulator of cell proliferation and survival and is produced from ceramide with subsequent phosphorylation by sphingosine kinases (SPHK), and the isoform 1 of SPHK has a major role in iCCA aggressiveness (reviewed in [81]). Cholesterol metabolism is also dysregulated in cancer [82]. In the liver, elevated levels of bile acids (BA), derived from cholesterol, may cause abnormal cell proliferation and development of CCA [83]. Notably, lack of JNK pathway modified cholesterol and bile acid synthesis resulting in enhanced proliferation of cholangiocytes and iCCA initiation. These effects are mediated by PPARα activation [84]. PPARα agonists, used for the metabolic syndrome and obesity, are associated with increased synthesis of cholesterol and bile acid, liver damage, cholangiocyte proliferation and hepatic carcinogenesis [85, 86]. Therefore, the treatment of hepatic steatosis or obesity with drugs affecting the JNK/PPARα signaling pathway and lipid metabolism should be evaluated carefully in patients at risk of developing CCA (Figure2).

Several studies indicate that dysregulation of hepatic BA synthesis in cholestatic liver injury has an impact on hepatic tumorigenesis. At this regard, the study of Lozano et al. [87] suggests that intrahepatic BA accumulation does not induce CCA directly, but facilitates a co-tumorigenic effect

stimulating proinflammatory mechanisms and impairing FXR-mediated chemoprotection against genotoxic insults. Moreover, the increased concentration of BAs in cholangiocytes during primary sclerosing cholangitis may have a role in carcinogenesis [88]. Both BAs and conjugated BAs are known as important stimulators of CCA growth and spread. In parallel, BAs also inhibit apoptosis of biliary cancer cells. These effects are obtained by BA-mediated activation of the NF-κB pathway, and stimulation of the Takeda G protein-coupled receptor 5 (TGR-5) and S1P receptor 2 (S1PR2) [89],[90]. Notably, stimulation of S1PR2 activates intracellular ERK1/2 and AKT signaling promoting CCA invasive capabilities [90], [91].

Lipids have also been investigated in metabolomic studies, in the search for biomarkers for cancer detection, monitoring, and prognostication [92, 93]. In a multicenter study, serum metabolomic profiles distinguished iCCA from healthy individuals and PSC patients [94], and phosphatidylcholines, amino acids, sphingomyelins and sterols were the families with the most abundant changes [94]. The same study showed that 102 metabolites were altered comparing iCCA versus PSC patients, mainly phosphatidylcholine and lysophosphatidylcholine species, which were lower in iCCA [94]. Moreover, low levels of N-methyl-2-pyridone-5-carboxamide and lysoPC (16:0) in serum of iCCA patients were correlated with increased recurrence-free survival after surgery, thus indicating this metabolite as a potential prognostic biomarker [95].

Very recently Padthaisong et al., [96] used high-throughput technologies integrating global metabolomic and lipidomic approaches, to demonstrate the different metabolites in CCA patients with and without recurrence. In a retrospective study on a total of 102 patients with *Opistorchis Viverrini*-associated CCA, many lipid species, especially TAGs, were upregulated in recurrent patients, suggesting that lipids may represent an important factor for cancer reappearance. Moreover, the high level of CD36, a scavenger receptor which internalizes lipids, was associated with lower recurrence-free survival, thus suggesting that high lipid levels in patients with recurrent HCC may lead to enhanced lipid uptake, which in turn leads to recurrence. These results highlight the importance of metabolomics to disclose the molecular mechanisms and potential biomarkers in CCA early-stage recurrence.

Metabolism of Amino Acids in Cholangiocarcinoma

Tumor cells have an increased demand for amino acids to support their remarkably fast proliferation [97]. Essential amino acids must be obtained from external sources, and although non-essential amino acids can be synthesized endogenously, in the case of high proliferation rates endogenous synthesis does not meet the increased demands [97]. Amino acids serve as nutrient signals to activate important pathways (i.e. mTOR or autophagy) or as neurotransmitters (glycine and D-serine).

Glutamine is a highly abundant non-essential amino acid, which participates in cell growth and proliferation, in the synthesis of other non-essential amino acids, in the modification of chromatin, in anti-oxidative defense, in the synthesis of nucleotides as nitrogen donor and in refueling the TCA cycle intermediate (anaplerosis) [98]. Moreover, reduction in extracellular glutamine concentration increases cell susceptibility to apoptosis [99]. Wappler et al. tested the impact of long-term glutamine deprivation in human eCCA cells on hypoxia-altered susceptibility to cytostatic drugs [98] showing that glutamine-depleted eCCA cells are less chemo-resistant through the inhibition of cMyc expression.

Many studies have demonstrated that nutrient transporters are upregulated in cancer cells and support their rapid growth. Knockdown of l-type amino acid transporter 1 (LAT1), an isoform of the L-amino acid transporter system [100], suppresses invasion and migration of CCA cells through the inhibition of the 4F2hc-signaling pathway, up-regulating microRNA-7 [101]. Based on its crucial role in cancer progression, a novel LAT1 inhibitor has been recently developed. Argininosuccinate synthetase, which participates in the conversion of citrulline to arginine, is an important tumor suppressor, and its deficiency has been noted in different cancers, including HCC and CCA [102]. It has been hypothesized that arginine depletion in tumor cells leads to a reduction in cell proliferation, prompting studies testing the efficacy of PEGylated arginine deiminase (ADI-PEG20) as an anticancer agent [103]. Arginine is also a substrate of the urea cycle, a metabolic process leading to safe disposal of ammonia in urea, which is less toxic for the organism. Although the mechanisms leading to suppression of the urea cycle in both HCC and CCA are still obscure, epigenetic alterations may be involved in this regulation [102, 104].

Changes in the expression and metabolism of other amino acids have been reported in CCA. Using a multi-omics approach, Murakami et al. demonstrated that lysine, proline, leucine and isoleucine were differentially expressed in iCCA versus non tumoral tissues [105].

Many studies have shown that serotonin (5-hydroxytryptamine, 5-HT), a biogenic monoamine produced from the essential amino acid tryptophan, has a stimulatory effect on cancer cell proliferation, invasion, dissemination, and tumor angiogenesis, interacting with specific receptor subtypes [106]. In CCA cells and in human CCA specimens, increased expression of tryptophan hydroxylase and decreased expression of monoamine oxidase A has been reported, together with increased synthesis of serotonin in vitro and in vivo. Human CCA lines were also found to express all 5-HT receptor subtypes, and specific inhibition of 5-HT1A, 2A, 2B, 4, and 6 receptors was associated with antiproliferative effects. Furthermore, inhibition of serotonin synthesis blocked the growth of CCA cells lines [107] (Figure3).

The mammalian target of rapamycin (mTOR) complex is another regulator of cell growth and metabolism that integrates inputs from growth factors, nutrients, amino acids and extracellular proliferative signals. mTOR is an atypical serine/threonine protein kinase which forms two distinct complexes, mTORC1 and mTORC2) [108, 109]. The PI3K-AKT-mTOR signaling pathway is frequently activated in both iCCA and eCCA [110] and has been associated with tumor progression, differentiation, and reduced OS [110, 111]. mTORC1 plays a central role in protein, lipid, and glucose metabolism as well as in proliferation, leading to the development of anti-cancer therapies targeting PI3K/mTOR signaling, based on rapamycin and its analogues, including everolimus. Everolimus binds to FKBP12, weakening the interaction between mTORC1 and Raptor, leading to inhibition of proliferation in a variety of cell lines [112].

Nutrients facilitate mTORC1 translocation from the cytoplasm to the lysosomal surface, thus leading to its activation by phosphatidylinositol 3-kinase (PI3K)/AKT signaling. The actions of mTORC1 in cellular metabolism are reviewed in [113]. Additionally, mTORC2 controls cell proliferation and survival by phosphorylating the kinases AKT, SGK and PKC. mTORC2 also functions as a downstream effector of the insulin/PI3K cascade, directly or indirectly [113].

The role of deregulated mTOR pathways in human iCCA is still unclear. Mutations that lead to mTOR activation, such as those affecting the TSC1/2 and CTNNB1 genes, occur very rarely in human iCCA [114, 115]. In contrast, FGFR fusion mutations, KRAS or BRAF mutations, which are found in iCCAs may lead to aberrant mTOR activation. Whether other mutations frequently identified in iCCA, including TP53, IDH1/2, and SMAD4, can activate the mTOR signaling cascade remains debated [115]. Gene expression profiling of invasive biliary cancer showed that downstream mediators of the mTOR pathway, such as S6K and eIF4E as well as IGF-1 may be deregulated [116]. Pan-mTOR kinase inhibitors may be beneficial for the treatment of iCCA, even in tumors resistant to standard chemotherapy, especially in the subset exhibiting activated AKT/mTOR cascade. A few studies have shown mTORC2 activation in ~70% of human iCCAs and RICTOR silencing inhibited iCCA cell growth in vitro [114]. Activated AKT cooperates with YAP, activated JAG1, or downregulated BXW7 to induce iCCA development in a mouse model [114, 117], thus supporting the tumorigenic role of mTORC2/AKT axis in iCCAs.

Methionine adenosyltransferases (MATs) produce S-adenosylmethionine, the biological methyl donor required for a plethora of cellular reactions. Mammalian systems express two genes, MAT1A and MAT2A, which encode for MATα1 and MATα2, the catalytic subunits of the MAT isoenzymes, respectively. A third gene MAT2B, encodes a regulatory subunit known as MATβ which controls the activity of MATα2. MAT1A, mainly expressed in hepatocytes, maintains the differentiated state of these cells, while MAT2A and MAT2B are expressed in extrahepatic tissues and non-parenchymal

cells of the liver. MAT1A is highly expressed also in normal bile duct epithelial cells and is repressed during chronic cholestasis and in murine and human CCA [118-120]. There are common mechanisms of MAT gene deregulation between HCC and CCA. For example, hypermethylation of the MAT1A promoter has also been observed in CCA. The transcription factors cMyc, MAFG and c-MAF, which are induced both in HCC and CCA, negatively regulate MAT1A transcription in CCA, binding to its repressor E-box promoter region [119]. Prohibitin 1 (PHB1), which is also downregulated in most human CCAs, positively regulates MAT1A while suppressing cMyc, MAFG, and c-MAF expression in mice [121, 122]. Consistently, reduced PHB1 expression predisposes to the development of cholestasis-induced CCA [119, 122] (Figure3).

Metabolic Aspects of Cholangiocarcinoma Stem Cell Compartment

Cancer stem cell (CSC) are responsible for maintenance of the tumor's malignant characteristics and resistance to treatment, including chemo- and radiotherapy as well as immune checkpoint inhibitors, in many solid tumors, including hepatic cancer [123-125] . We have recently demonstrated an intriguing role of mitochondrial-dependent metabolism in the maintenance of CCA stemness. Intriguingly, we demonstrated that the stem-subset of CCA cells, enriched by sphere culture, was more sensitive to metformin treatment than cells cultured in monolayer as shown by reduced expression of stemness marker, self-renewal, pluripotency, drug resistance, EMT and in vivo tumor growth in the sphere cultures with respect to cells grown in monolayer [123] Indeed, alteration of the integrity of the mitochondrial respiratory chain with metformin or downregulation of PGC1α (SR-18292) in the stem-subset of CCA cells severely impair tumor progression, demonstrating a crucial role of OXPHOS in CCA aggressiveness [123]. These data indicate that, besides a general increase in glucose dependency, CCA displays a marked metabolic plasticity, and different pathways may be activated in various cell subtypes within the tumor mass, due to the different availability of nutrients. OXPHOS metabolism is crucial to sustain CCA stemness and the acquisition of a phenotype prone to metastatic dissemination. We demonstrated that treatment of mice with metformin made the gene signature of tumors derived from sphere cultures more similar to that observed in tumor derived from monolayer, indicating an inhibition of the stemness features and aggressiveness of this component. These data suggest that actually the inhibitory effects of metformin treatment in patients with CCA may, at least in part, be due to the targeting of the stem component at the level of the tumor bulk. Accordingly, Di Matteo et al. [63] recently demonstrated that metformin treatment reverses EMT features in iCCA cells, both in vitro and in vivo. Support to the importance of mitochondria in CCA-CSC is provided by data showing that brain expressed X-linked gene 2 is essential for maintaining dormancy of $CD274^{low}$ CSC through mitochondrial activity [126].

Dependency of CCA CSC on glutamine been recently described, exploring the role of the cystine-glutamate transporter xCT [127]. Sulfasalazine (SSZ), a specific inhibitor of xCT-mediated cystine transport [128], increased cell sensitivity to cisplatin, killing CD44v9-positive CSC cells both in vitro and in vivo. In agreement, the CSC marker CD44v9 and the CCA proliferative marker CK-19 were reduced in the combination treatment. NMR-based metabolomic analysis of tumor tissues showed that SSZ sensitizes CCA cells to cisplatin, modifying different metabolic pathways, in particular tryptophan degradation and nucleic acid metabolism. Since CSCs play important roles in carcinogenesis, targeting CSC metabolism may represent a novel and promising therapeutic strategy in CCA (Figure 4).

Furthermore, the study by Lin Y et al. [129] reveals the potential role of fibrotic tumor stroma through recruitment of myeloid derived suppressor cells (MDSCs). Indeed IL-6 and IL-33 released by cancer-associated fibroblasts (CAFs) promotes hyperactivation of 5-lipoxygenase (5-LO) pathway in CD33+ MDSCs. Overproduction of downstream metabolite of 5-LO, the leukotriene B4 (LTB4) mediate the stemness-enhancing effects of CD33+ MDSCs by acting on its receptor leukotriene B4 receptor type 2 (BLT2) in CCA cells. By promoting CCA cell stemness through PI3K/Akt-mTOR signaling, the 5-LO/LTB4-BLT2 axis represents a promising therapeutic target for CCA aggressiveness and chemoresistance (Figure 4).

Approaches to the Metabolic Treatment of Cholangiocarcinoma

CCA treatment through metabolic reprogramming is still in its infancy (Table 1, Figure 5). Ivosidenib (AG-120), an oral small-molecule inhibitor of mutant IDH1 is the first drug in this class to have received approval for the treatment of IDH1 mutated CCA patients [61, 130, 131]. The “ClarIDHy” phase 3 trial randomized 185 chemoresistant metastatic IDH1-mutated CCA patients to ivosidenib (n=124) or placebo (n=61). Progression free survival (PFS) was improved by ivosidenib [PFS at 6 months 32% vs 0%; HR 0.37, $p<0.0001$], and a clinically meaningful stabilization of the disease reflected in increased overall survival [median OS: 10.8 vs 6.0 months after adjustment for crossover, p:0.0008). Nonetheless, two issues need to be considered: 1) more than 1500 patients had to be screened, making this therapy an option in <15% of CCA patients, 2) drug resistance due to isoform switching from IDH1 to IDH2 has been reported in AML, and may also occur in CCA [132]. Enasidenib is an inhibitor of mutant IDH2, approved for use in AML patients, which is now under investigation for use in solid tumors, including iCCA (NCT02273739). Dual inhibition of mutant IDH1 and IDH2 by vorasidenib (AG-881) was effective in a mouse model of glioma [133] and has been evaluated in a phase 1 trial, where it was found to be well tolerated and showed preliminary antitumor activity in glioma patients [134].

Newer inhibitors of mutant IDH are also being developed, and several have been tested for their potential use in CCA patients [135, 136]. IDH305 is a selective inhibitor of mutant IDH1 ($IDH1^{R132H/C}$) which inhibits tumor growth in pre-clinical xenograft models [137]. Preliminary clinical data also suggest that this agent has promising antitumor activity [138] and a basket clinical trial of IDH305 in patients harboring $IDH1^{R132}$ mutations is underway (NCT02381886). An alternative inhibitor of $IDH1^{R132}$, FT-2102, was well tolerated in hematologic malignancies in phase 1 clinical trials [134] and is currently being evaluated as a single agent and in combination with gemcitabine/cisplatin in iCCA (NCT03684811). LY3410738, an inhibitor of IDH^{R132} which differs from prior IDH inhibitors by its covalent binding mode, increased potency, and unique binding site, is currently being evaluated in a clinical trial in monotherapy and in combination with cisplatin and gemcitabine (NCT04521686). This drug has previously been shown to be well tolerated when administered alone, and some clinical activity was reported in a group of patients including CCA [139]. However, when this drug was administered in combination with novel anti-cancer therapies (i.e. inhibitors of the Hedgehog pathway, PI3K, CDK4/6), toxicity became a limiting factor [140].

The conversion of glutamine to alpha-ketoglutarate in the glutaminolysis pathway is catalyzed by glutamate dehydrogenase. This is important due to the conversion of alpha-ketoglutarate into the oncometabolite hydroxyglutarate by mutant IDH. Therefore, IDH1/2 mutated cancer cells are susceptible to treatment with drugs which inhibit glutamate dehydrogenase, such as metformin and chloroquine [141]. A phase IB/II clinical trial is evaluating the combination of these drugs for solid tumors including iCCA (NCT02496741).

ABC294640 is an inhibitor of sphingosine kinase 2, an enzyme that regulates the sphingolipid metabolic pathways, contributing to cancer development by regulating tumor proliferation, migration and angiogenesis [142]. This enzyme is overexpressed in CCA cell lines, and its inhibition inhibited proliferation and induced apoptosis in these cells [143]. A phase I trial of ABC294640 demonstrated clinical activity in CCA patients [144] and a phase IIA clinical study of ABC294640 alone and in combination with hydroxychloroquine sulfate (NCT03377179) is currently recruiting.

CPI-613 (6,8-bis[benzylthio]octanoic acid), selectively targets the mitochondrial TCA cycle in cancer cells. CPI-613 displaces lipoic acid, a co-factor for α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, thus inhibiting activity of these enzymes and the TCA cycle [145]. Furthermore, this drug induced apoptosis in pancreatic cancer cells downregulating lipid metabolism via suppression of ACC [146]. Initial reports from a phase I study evaluating the safety and efficacy of CPI-613 in combination with FOLFIRINOX in patients with metastatic pancreatic cancer indicated that this treatment was well tolerated [147]. Although it has since failed to meet the primary endpoint of increased overall survival in these patients (NCT03504423), it is now being evaluated in advanced

unresectable CCA (NCT017266219). The experimental drug genipin also targets this pathway, but has not reached clinical trials.

In a phase II clinical trial including 27 patients with advanced biliary tract cancers (NCT00973713), the mTORC1 inhibitor everolimus demonstrated clinical activity, resulting in a 12-week disease control rate (DCR) of 48% [148]. This was comparable to the 8-week DCR of 44.7% previously reported in another cohort of 39 biliary tract patients [149]. Further clinical trials evaluating everolimus as monotherapy (NCT01525719) or in combination with gemcitabine or cisplatin (NCT00949949) for advanced CCA have not reported results.

Telotristat ethyl, a tryptophan hydroxylase (TPH) inhibitor currently FDA-approved for carcinoid syndrome diarrhea, is being studied in an ongoing phase II study in combination with first-line chemotherapy in patients with advanced cholangiocarcinoma (NCT03790111). However, this study has recently been terminated due to failure to meet pre-specified PFS at month 6.

Many other drugs targeting metabolism have entered clinical trials for solid tumors, some of which may be relevant to the treatment of cholangiocarcinoma. These include the FASN inhibitor TVB-2640 and the glycolysis inhibitor 2-deoxy-glucose (2-DG). Alternative druggable targets which are under investigation but have not yet reached clinical trials include inhibitors of the glucose transporter GLUT1 (WZB117, BAY-876), FABP inhibitors (SBFI-102, SBFI-103), and blockers of fatty acid transport via FATP1 and CD36. Stearoyl-CoA desaturase (SCD) also plays a key role in lipid biosynthesis pathways involved in tumorigenesis, [150] and so pharmacological inhibitors have been developed such as MF-438, CAY10566 and A939572.

Perspectives

CCA is a highly aggressive malignancy, and its incidence seems to be increasing over the last years. Although considerable progress has been made in understanding mutational pathogenesis of CCA, with significant potential relative to disease progression and even survival (in particular FGFR and IDH inhibitors), new treatment approaches are important to achieve better outcomes in advanced iCCA patients.

Metabolic pathways are targetable by small molecule drugs (Figure 5), and several of these have progressed to clinical trials. It is proposed that the most effective ways in which to circumvent metabolic reprogramming and compensatory mechanisms in response to single agent effects is to administer combinations of drugs targeting different steps of the key metabolic pathways, including lipogenesis and glycolysis, which are dysregulated in cholangiocarcinoma. This will prevent accumulation of oncometabolites, synthesis of membrane lipids and disrupt ATP generation, thus affecting processes regulating intracellular signaling, proliferation, invasion, survival and resistance

to therapy. It is also anticipated that this approach will reduce the side-effects caused by high concentrations of the individual drugs. Importantly, targeting some of these metabolic pathways can impact cancer growth also in combination with other anticancer drugs. Better technologies at the single-cell level would achieve an even deeper understanding of the metabolic role in CCA.

Figure Legends

Figure 1. Glucose metabolism and CCA: Glycolysis and pathways shunting from glycolytic intermediates are shown. Red arrows indicate increased expression/activity of enzymes and the relative metabolic pathways. Besides enhanced glycolysis, high levels of cMyc mediate both the activation of PDHA1 and the consequent inhibition of the OXPHOS, and an increase in LDH and PKM2 expression, which reduces pyruvate levels thus removing HDAC3 inhibition and sustaining further cMyc stabilization. High expression of mitochondrial UCP2 promotes CCA proliferation and invasion supporting the glycolytic pathway. High glucose induces the expression of IL-1β and the activation of the NF-κB pathway. Nuclear translocation of NF-κB stimulates the transcription of IL-1β, which acts as positive feedback, and IL-6 which activates the STAT3 pathway. Both NF-κB and STAT3 promote the aggressiveness of CCA. PGC1α overexpression, the key regulator of mitochondrial biogenesis, drives a metabolic shift towards OXPHOS, promoting CCA stemness. OXPHOS mitochondrial oxidative phosphorylation, LDH lactate dehydrogenase, PKM2 pyruvate kinase M2, HDAC3 Histone deacetylase 3, UCP2 Uncoupling protein 2, PGC1α peroxisome proliferator-activated receptor-γ coactivator 1-α, LEC lymphatic endothelial cells.

Figure 2. Altered lipid metabolism in CCA. FA transporters are overexpressed in CCA and the consequent raise of exogenous FA up-take correlates with increased cell invasion, migration, EMT and poor prognosis. The endogenous synthesis of FA is also deregulated. increased expression of FASN sustains FA biosynthesis and correlates with progression of CCA. Both elevated S1P synthesis and high levels of cholesterol-derived bile acids have been reported, driving proliferation and aggressiveness of CCA. FASN fatty acid synthase, S1P sphingosine-1-phosphate , FA fatty acid.

Figure 3. Metabolism of amino acids in CCA. The L-amino acid transporter LAT1 is overexpressed in CCA, promoting cell invasion and migration via ERK1/2 and p70S6K phosphorylation. The activation of the PI3K/AKT/mTOR pathway is essential for tumor progression and poor prognosis. Increased synthesis of serotonin and serotonin receptors sustain CCA proliferation. Inhibition of MAT1 expression due to promoter hypermethylation or binding of cMyc, MAFG and c-MAF to the repressor E-box promoter region of MAT1 gene has been reported in CCA. Red arrows indicate increased expression/activity of enzymes and the relative metabolic pathways, blue arrow indicates decreased enzyme expression levels. LAT1 l-type amino acid transporter 1, MAT1 methionine adenosyltransferase 1

Figure 4. Altered metabolism in the CCA stem-like compartment. Compared to tumor bulk cells, cancer stem cells in CCA are characterized by a decrease of glycolytic pathway and an enhancement of OXPHOS together with an overexpression of PGC1α. The relevance of mitochondria and OXPHOS in the regulation of stemness feature in CCA is also indicated by the role of BEX2-TUFM proteins. Furthermore, high levels of xCT with a consequent increase in glutathione levels are associated with higher drug resistance. Red arrows indicate increased expression/activity of enzyme/transporters and the relative metabolic pathways, blue arrow indicates decrease enzyme expression levels. xCT cystine-glutamate transporter, OXPHOS mitochondrial oxidative phosphorylation

Figure 5. Schematic representation of potentially druggable metabolic targets in cholangiocarcinoma cells. FABP fatty acid binding protein, FASN fatty acid synthase, GLUT glucose transporter, IDH isocitrate dehydrogenase, SCD stearoyl-CoA desaturase, SPHK sphingosine kinase, TCA citric acid cycle. Experimental drugs are indicated in italics.

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Author names in bold designate shared co-first authorship

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Table 1: Ongoing clinical trials of agents targeting metabolism of cholangiocarcinoma

Study number	Molecular Target	Estimated sample size	Experimental treatment	Treatment	Primary endpoint	Secondary endpoint
NCT04088188	Mutant IDH	40	Ivosidenib	Combination with cisplatin and gemcitabine	Maximum tolerated dose	PFS, OS, toxicity and adverse effects
NCT02381886		166	IDH305	Monotherapy	Dose limiting toxicities	Adverse effects, PK, ORR
NCT04521686		180	LY3410738	Monotherapy, combination with cisplatin and gemcitabine	Recommended phase 2 dose	ORR, safety& tolerability, PK
NCT03684811		200	FT-2102	Monotherapy, combination	Dose limiting toxicity, recommended phase 2 dose	PK, ORR, PFS, OS
NCT02496741	Glutamate dehydrogenase	15	Metformin	Starting dose 500 mg/day, combination with choloroquine	Maximum tolerated dose	Pharmacokinetics, toxicity, tumor response
NCT03377179	Sphingosine kinase 2	39	ABC294640	500 mg twice per day, 28 day cycles	Response rate, overall responses	Physical and neurological exam
NCT01766219	Mitochondrial TCA cycle	17	CPI-613 (Devimistat)	1200-3000 mg/m^2 dose-escalation, 28 day cycle	Overall survival	Response rate, PFS
NCT01525719	mTOR	40	Everolimus	Monotherapy	Progression free survival	Overall survival rate
NCT00949949		38	Everolimus	Combination with cisplatin and gemcitabine	Adverse events profile, toxicxity profile, MTD	Response profile

PFS progression-free survival, OS overall survival, PK pharmacokinetics, ORR overall response rate

Figure 1

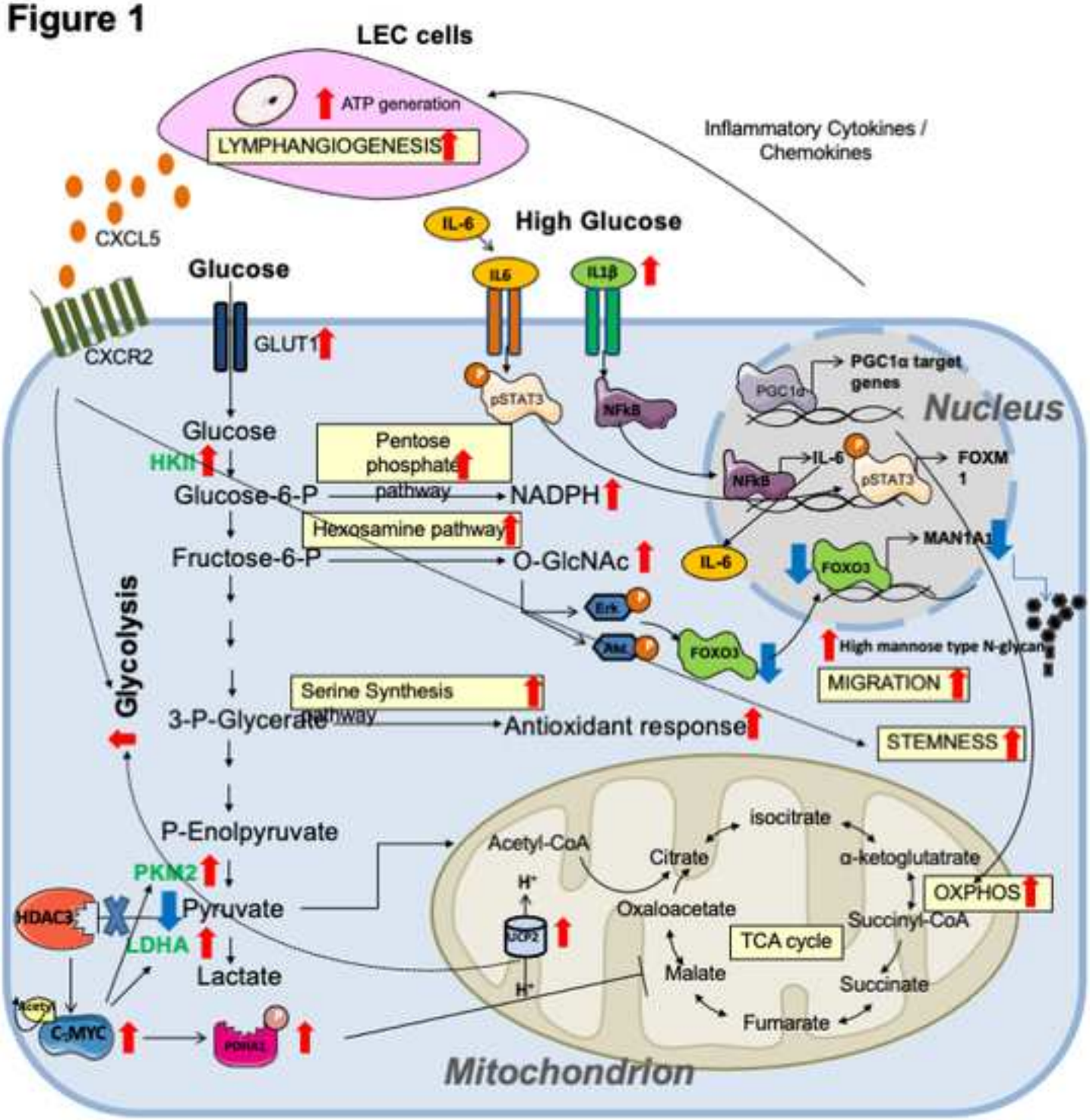

Figure 2

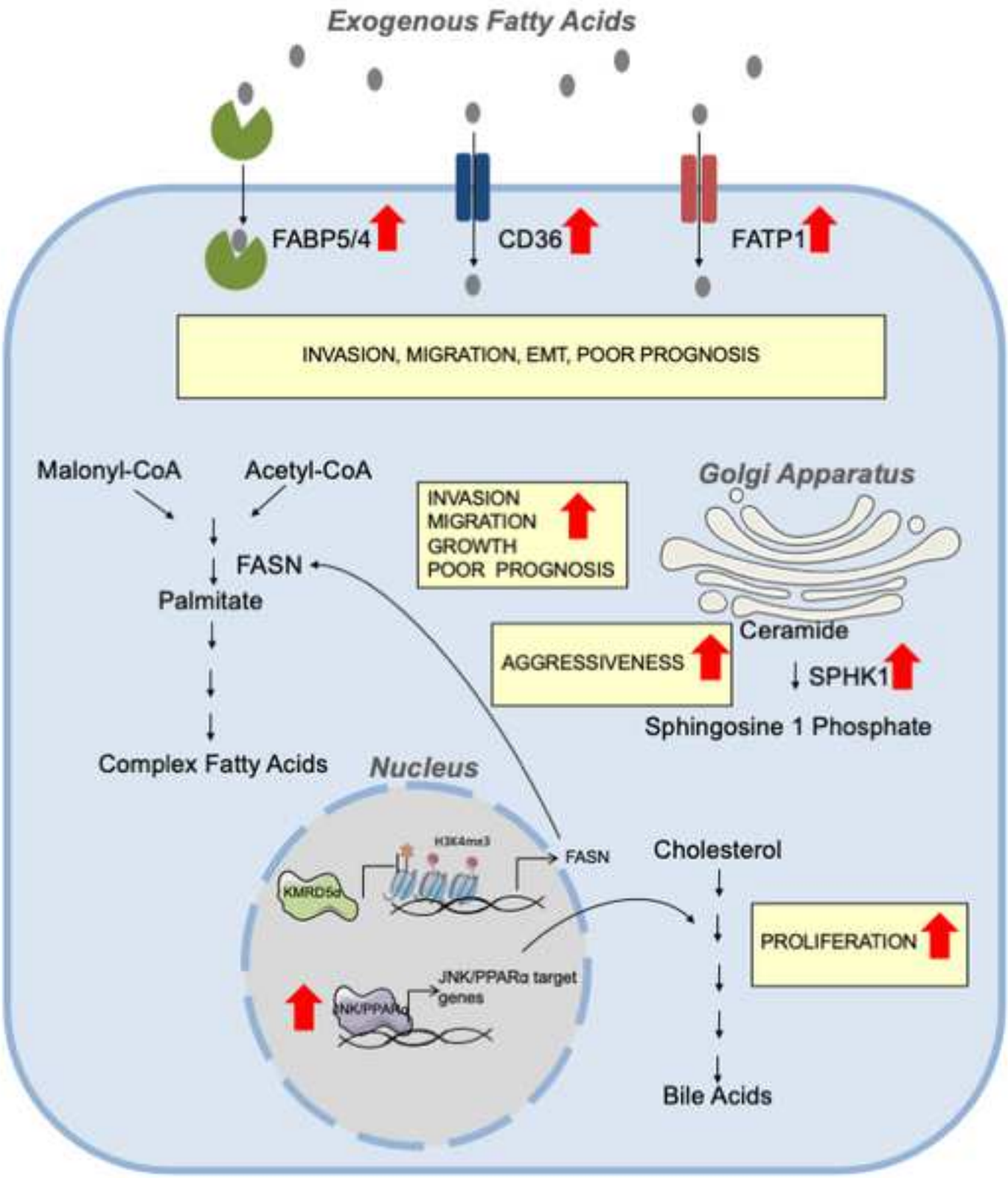

Figure 3