Stress Responses and Cellular Crosstalk in the Pathogenesis of Liver Disease Theme Issue

Endoplasmic Reticulum Stress and Metabolism in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for 85-90% of all liver cancer cases. It is a hepatocyte-derived primary tumour, causing 550,000 deaths per year, ranking it as one of the most common cancers worldwide. The liver is a highly metabolic organ with multiple functions, including digestion, detoxification, breakdown of fats, production of bile and cholesterol, in addition to storage of vitamins, glycogen and minerals, and synthesizing plasma proteins and clotting factors. Due to these fundamental and diverse functions, the malignant transformation of hepatic cells can have a severe impact on the liver’s metabolism. Furthermore, tumorigenesis is often accompanied by an activation of the endoplasmic reticulum (ER) stress pathways, which are known to be highly intertwined with several metabolic pathways. As HCC is characterized by changes in the metabolome and by an aberrant activation of the ER-stress pathways, the aim of this review is to summarize the available knowledge that links ER-stress and metabolism in HCC, thereby focusing on potential therapeutic targets.
Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for 85-90% of all liver cancer cases. It is a hepatocyte-derived primary tumour which causes 550,000 deaths per year world-wide, making it the second cause of cancer-related deaths. The incidence of HCC is currently increasing world-wide, but varies considerably per region due to differences in prevalence of underlying risk factors. Risk factors for HCC include hepatitis B or C virus (HBV or HCV) infections, metabolic disorders, such as obesity, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD), and alcohol abuse or aflatoxin B1 exposure. Most of these risk factors result in chronic liver injury, which leads to fibrosis and cirrhosis, thereby creating a favorable environment for the development of HCC. Although the main risk factor for HCC in Eastern Asia is HBV, it is currently shifting towards obesity, type-2 diabetes and NAFLD in the western societies, as their prevalence is increasing rapidly. Unfortunately, HCC remains a major health problem, with a 5-year survival of only 20% owing to late detection and limited treatment options. This highlights the urgency for a better understanding of the pathogenesis of HCC to pave the way for new treatments and improve survival of HCC patients.

Over the last years, there has been increasing evidence that endoplasmic reticulum (ER) stress plays an important role in liver cancer. Endoplasmic reticulum stress is the accumulation of misfolded or unfolded proteins in the ER-lumen, which occurs when the capacity of the ER to correctly fold proteins is exceeded. The presence of these proteins in the ER lumen activates the unfolded protein response (UPR), which aims to restore protein homeostasis by slowing down protein translation, increasing the protein folding machinery, and upregulating the degradation of unfolded proteins. However, in case of persistent or excessive ER-stress, the UPR can induce pathways leading to cell death. In cancer, it has been proposed that the key role of the UPR is to protect tumour cells from apoptosis and support their uncontrolled proliferation. Therefore, ER-stress and the UPR may have a relevant role in HCC pathogenesis which could reveal new targets for HCC treatment and diagnosis.

Metabolism has recently gained importance in the pathogenesis of HCC due to the pivotal role of the liver in different metabolic processes. Risk factors for HCC include metabolic disorders, such as NAFLD, obesity and type 2 diabetes which are characterized by an aberrant metabolism. In addition, the deregulation or reprogramming of glucose metabolism is considered a hallmark of solid tumors, since it helps to fuel tumorigenesis and allows cells to thrive under hypoxic circumstances. Lastly, most of the metabolic processes in the liver are carried out by hepatocytes, which make up to 85% of the total mass of this organ. Thus, any transformation of hepatocytes during HCC, such as mutations, increased proliferation or replicative immortality, lead to metabolic alterations. Interestingly, it has...
been established that ER-stress and metabolic deregulation are functionally intertwined and both are considered contributing factors to the pathogenesis of HCC (Figure 1). In this review, the aim is to summarize the available knowledge that links ER-stress and metabolism in HCC. First, we will provide an overview on the unfolded protein response and its involvement in HCC will be provided. Next, we will compile the role of the liver in the different metabolic pathways and the alterations found in HCC will be compiled. Finally, the connections between the disrupted metabolism and ER-stress in HCC will be disclosed.

The unfolded protein response (UPR)
Endoplasmic reticulum stress is the physiological condition characterized by an accumulation of misfolded or unfolded proteins in the lumen of the ER\(^5\). This can be triggered by several stimuli, including a high rate of cell proliferation, hypoxia, nutrient deficiency, and abnormal redox homeostasis. The presence of misfolded or unfolded proteins in the ER lumen is sensed by three transmembrane proteins, namely activating transcription factor 6 (ATF6), inositol requiring enzyme 1 alpha (IRE1\(\alpha\)), and protein kinase RNA-like endoplasmic reticulum kinase (PERK)\(^5\). These proteins each activate their own signalling cascade to induce the unfolded protein response. During protein homeostasis, binding immunoglobin protein (BiP) (also known as Grp78 or heat shock 70 kDa protein 5 (HSPA5)) is bound to ATF6, IRE1\(\alpha\) and PERK, keeping them in their inactive conformation. Upon ER-stress, BiP preferentially binds to unfolded or misfolded proteins in the ER lumen, hence dissociating from the ER-stress sensors and exposing their luminal domains\(^9\). This facilitates their activation and triggers the unfolded protein response, orchestrating a complex signalling network aiming to reduce protein translation, increase the ER’s folding capacity or, in case of severe or prolonged ER-stress, activate pro-apoptotic pathways.

Inositol-requiring transmembrane kinase endoribonuclease-alpha (IRE1\(\alpha\))
The most prominent and evolutionary conserved UPR signal transducer is IRE1\(\alpha\), which becomes activated as BiP dissociates from its binding site on the receptor. This will result in homodimerization and subsequent auto-phosphorylation of its kinase domain\(^9\). Activated IRE1\(\alpha\) also exerts an endonuclease function, which enables splicing of X-box binding protein 1 (uXBPI) messenger RNA (mRNA) into its active form, called spliced XBP1 (sXBPI). sXBPI is then able to act as a transcription factor, which induces transcription of a range of pro-adaptive genes including ER chaperones and genes involved in ER-stress associated protein degradation (ERAD). In ERAD, misfolded or unfolded proteins are transported to the cytoplasm for ubiquitination and degradation by the proteasome\(^4\). In addition, the endonuclease activity of IRE1\(\alpha\) is utilized for regulated IRE1-dependent decay (RIDD),
a process in which several ER-bound mRNAs or precursor microRNAs are spliced. These two processes reduce the amount of misfolded or unfolded proteins and mRNA, decreasing the protein folding load of the ER and hence ER-stress. Besides the main pro-adaptive roles of IRE1α, it is also suggested to induce pro-apoptotic pathways during prolonged ER-stress. This is initiated by the assembly of IRE1α with tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1). They form a signalling complex together, which further activates downstream signalling pathways leading to apoptosis. Furthermore, it is suggested that RIDD can contribute to apoptosis in case of severe ER-stress. Therefore, IRE1α can activate both pro-adaptive as pro-apoptotic pathways, depending on the level and duration of ER-stress⁶.

**Protein kinase RNA-like endoplasmic reticulum kinase (PERK)**

The second transmembrane ER-stress protein is PERK, which gets activated by dimerization and subsequent auto-phosphorylation upon dissociation of BiP during ER-stress⁹. After activation, PERK phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), resulting in a general suppression of protein translation. The cell cycle regulator cyclin D1 is one of the many proteins that is downregulated, which causes the cell cycle to arrest and offers the cell some time to restore protein homeostasis¹⁰. Simultaneously, phosphorylated eIF2α enhances translation of a specific set of mRNAs, including activating transcription factor 4 (ATF4)¹¹. This protein can induce transcription of various genes involved in the adaptation to ER-stress, such as genes involved in autophagy and ROS protection¹¹. In contrast, ATF4 can induce transcription of CCAAT-enhancer-binding protein homologous protein (CHOP) (also known as growth arrest and DNA damage inducible gene (GADD153)), which stimulates expression of pro-apoptotic genes¹². Hence, like IRE1α, PERK is known to induce both pro-adaptive and pro-apoptotic pathways.

**Activating transcription factor (ATF6)**

The last transmembrane ER-stress protein is ATF6. It is translocated to the Golgi apparatus upon dissociation of BiP, where it is cleaved into its active form by Site-1 and Site-2 proteases¹³. Active ATF6 then acts as a transcription factor and stimulates the production of ER-chaperones including BiP, which aid in the folding of proteins and hence reduce ER-stress¹⁴. In addition, it induces the production of XBPI mRNA which can subsequently be spliced by IRE1α, leading to the initiation of downstream signalling pathways¹⁴. It has been suggested that ATF6 is one of the first branches that is activated, creating a timeframe for the cell to restore protein homeostasis solely by an increase in ER-chaperones before activation of other pathways¹⁵. This highlights the importance of ATF6 in the initial stages of ER-stress.
The UPR in Hepatocellular Carcinoma

Several characteristics of solid tumours are known inducers of ER-stress; therefore ER-stress has been suggested to be a novel hallmark of cancer\(^7\). For example, a high rate of cell proliferation, hypoxia, nutrient deficiency, and abnormal redox homeostasis can all trigger the unfolded protein response\(^{16}\). In addition, tumour cells possess numerous mutations that can induce the production of misfolded proteins\(^{17}\). Furthermore, ER-stress can also be induced by viral infections, such as HBV or HCV\(^{18,19}\), which are both known risk factors for HCC. As a result, an increased expression of ATF6, BiP and spliced XBP1 has been seen in HCC-patients with different aetiologies, thus indicating an upregulation of the ATF6 and IRE1α branches of the UPR in HCC\(^2,20\).

The upregulation of the UPR can contribute to the survival of tumour cells, leading to the development and progression of cancer. Studies have shown that the PERK branch of the UPR is crucial for inducing tumour cell proliferation and growth by altering redox homeostasis\(^{21}\). This prevents the activation of oxidative DNA checkpoints, allowing tumour cells to survive in hostile tumour microenvironments. Studies have therefore shown that pharmacologically targeting different arms of the UPR can form a promising target to slow down tumour growth\(^{22,23}\). For instance, inhibiting IRE1α reduced the development of HCC in a diet induced model for obesity in vivo\(^{20}\). Likewise, a decrease in tumour cell proliferation and metastasis was seen in a chemically induced HCC mouse model when IRE1α endonuclease activity was pharmacologically inhibited\(^{24}\). Similarly, a study by Vandewynckel et al. (2015) attempted to investigate pharmacological inhibition of PERK by GSK2656157, which suggested to reduce the expression of ER chaperones and tumour growth in an HCC mouse model\(^{25}\). However, a more recent study showed that GSK2656157 and GSK2606414, which are commonly used with the purpose to inhibit PERK, are in fact not specific for PERK, thereby questioning the accuracy of previous studies using these inhibitors\(^{26}\). Lastly, not much is known about the effect of inhibition of the ATF6 branch on HCC pathogenesis, which could be interesting for further research due to its suggested upregulation and/or missense polymorphisms in HCC\(^{27,28}\). For instance, there have been cases where a missense single-nucleotide polymorphism (SNP) can significantly increase the expression levels of ATF6 mRNA and/or ATF6 regulated genes, such as GRP78, CHOP and XBP1, contributing to HCC susceptibility. This missense mutation is strongly associated with HBV-related HCC, with a reported frequency of 28% of HCC-patients carrying this mutation\(^{28}\).

Carbohydrate metabolism

The liver is responsible for maintaining glucose homeostasis by continuously regulating glucose production and storage, and ER-stress pathways are known to interfere with these different processes.
In order to maintain glucose homeostasis, hepatocytes perform four metabolic reactions: glycolysis, glycogenesis, gluconeogenesis and glycogenolysis. Glycolysis is a catabolic pathway of enzyme-catalysed reactions that break down glucose in order to generate energy. In normal conditions, cells catabolize glucose to pyruvate and later to acetyl-CoA through the tricarboxylic acid (TCA) cycle to finally produce large amounts of adenosine triphosphate (ATP) via oxidative phosphorylation. However, in hypoxic or anaerobic conditions, cells follow glycolysis instead and metabolize glucose to lactate producing smaller amounts of energy per glucose molecule. Otto Warburg reported back in the 1920s that tumorigenic cells tend to shift their main energy source from oxidative phosphorylation to glycolysis, even under normal oxygen conditions. During this metabolic change, known as the Warburg effect, tumour cells consume larger amounts of glucose and produce energy in a faster way, thus stimulating proliferation. Therefore, inhibition of enzymes involved in these pathways could potentially limit tumour proliferation. It has been reported that downregulation of triosephosphate isomerase 1 (TPI1), which is involved in the glycolytic and gluconeogenic pathway, inhibits HCC-cell growth, migration, invasion and survival. Moreover, TPI1 expression is significantly decreased in HCC tissues and is correlated with HCC progression and survival, suggesting that TPI1 might serve as tumor suppressor. It has also been shown that ATF4 knockdown reduces the expression of TPI in Drosophila, which might suggest a role of the UPR in the regulation of this enzyme. Another glycolytic key enzyme involved in tumor proliferation is hexokinase and more specifically, one its four isoenzymes, hexokinase 2 (HXK2). HCC exhibits aberrant expression of HXK2, contributing to early recurrence and poorer prognosis of surgically resected HCC. In another study, DeWall et al. observed that HCC-cells express a different isoform of the hexokinase enzyme, the hexokinase 2 (HXK2). Inhibition of HXK2 with 3-bromopyruvate promotes tumour cell death by inducing ER-stress in human HCC cell lines (Huh-7 and SNU-761) and improves efficacy of sorafenib in in vivo models of HCC. Similarly, PERK-silencing blocks the translocation of HXK2 to mitochondria via inhibition of p-Akt in glioma cells, which inhibits tumour growth. This suggests PERK-dependent regulation of HXK2 in tumour cells, linking ER-stress to glycolysis. In addition, depletion of HXK2 results in inhibition of glycolysis and induction of oxidative phosphorylation, opposite to what tumour cells usually experience during the Warburg effect. This also sensitizes HCC cells to metformin, a compound broadly used in HCC-patients, which seems to regulate ER-stress response in HCC-cells, endothelial cells and T2D patients. More specifically, metformin has been reported to reduce activation of the PERK-pathway, thus preventing activation of the pro-apoptotic arm of ER-stress signalling.
When there is a need for increased blood glucose levels, the liver can produce glucose from glycogen (glycogenolysis) or from other molecules such as amino acids, lactate, pyruvate and glycerol (gluconeogenesis). Phosphoenolpyruvate carboxykinase 1 (PCK1), the main control point of gluconeogenesis, seems to have a pro-survival role during the ER-stress response and is involved in tumour cell adaptation to nutrient availability. Downregulation of PCK1 is shown to reduce tumour growth and promote oxidative stress and apoptosis in the malignant cells. On the other hand, elevated blood glucose levels (hyperglycaemia) activate glucose conversion and storage into glycogen in the liver via the process of glycogenesis. Guang-Zhi Jin et al. reported in 2018 that HCC progression can be reduced by targeting the phosphorglucomutase-1 enzyme (PGM-1). This enzyme seems to promote glucose flow to glycogenesis instead of glycolysis in several in vivo and in vitro models for HCC. Therefore, up-regulation of PGM-1 could potentially slowdown the cellular ATP-production and HCC progression by regulating glucose trafficking. Expression of this gene has also been shown to be part of a hypoxia-related gene signature that could serve as an independent prognostic factor for HCC.

Other proteins and processes related to glucose metabolism might also be involved in the pathological process of HCC. For instance, glucose metabolism is highly intertwined with ER-stress and the UPR (figure 2). It has been seen that ER-stress impairs the systemic glucose metabolism in chronic metabolic diseases such as obesity, insulin resistance and type 2 diabetes. In fact, a reduction of ER-stress attenuates glucose metabolism disorders in livers of mice with type-2 diabetes mellitus. Additionally, ER-stress has been shown to mediate the expression of glucose transporters (GLUT1, GLUT2) in diabetes mellitus and insulin resistance, specifically through the PERK/ATF4 branch, yet its role in tumorigenesis and in HCC remains largely unknown. However, GLUT1 and GLUT2 are upregulated in liver cancers to favour glucose uptake as energy source, and inhibitors for these transporters have been described as potential anti-tumoral drugs.

In addition, ER-stress is suggested to suppress insulin receptor signalling through the IRE1α-pathway. This UPR pathway activates c-Jun N-terminal kinase (JNK) which phosphorylates the serine residues of insulin receptor substrate-1, leading to inhibition of insulin signalling and impairment of glucose metabolism. The UPR mediator sXBP1 is suggested to interact with Forkhead box O1 (FoxO1), a transcription factor that enhances the expression of genes involved gluconeogenesis like phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. The interaction between sXBP1 and FoxO1 results in proteasomal degradation of FoxO1, and thus downregulation of the gluconeogenesis pathway, which is known to drive HCC. Furthermore, other studies have found
that IRE1α can act as a sensor of glucose metabolism and regulate glucagon signalling via activation by the protein kinase A. The PERK/ATF4 branch of UPR is also suggested to control glucose levels, since inhibition of PERK-eIF2α-ATF4 pathway enhances glucose metabolism in diabetic livers. Lastly, the ATF6-arm of UPR seems to be associated with impaired glucose homeostasis in type 2 diabetes. Together, these studies implicate a main role of ER-stress and the UPR in the regulation of carbohydrate metabolism and clearly show their importance in the context of diabetes. However, how and if this is affecting the development of HCC remains largely unknown and warrants further research.

**Lipid metabolism**

Deregulation of hepatic lipid metabolism, including synthesis, storage and break down of lipids, is considered a driving force of liver cancer. During synthesis of lipids (lipogenesis), fatty acids are generated from acetyl coenzyme A (Acetyl-CoA) molecules, due to excess of carbohydrates and proteins. Gene expression of enzymes and transporters involved in lipogenesis are upregulated in HCC, including fatty-acid synthase, ATP-citrate lyase or the sterol regulatory element-binding protein 1 (SREBP-1). On the contrary, the degradation of lipids (lipolysis) allows to produce energy or ketone bodies via the TCA-cycle or β-oxidation, respectively. Lipolytic enzymes, such as adipose triglyceride lipase (ATGL), are also highly expressed in HCC, increasing lipid degradation and energy production. One of the genes responsible for altered lipid metabolism is liver kinase B1 (LKB1), which is mutated in 22% of HCC-cases and its allelic loss leads to abnormal expression and activation of multiple molecules related to lipid metabolism. Furthermore, mutations in feedback-regulators of the Wnt-pathways, such as Ring Finger Protein (RNF43) and Zinc And Ring Finger 3 (ZNRF3), have been shown to increase hepatic lipid accumulation and enhance tumorigenesis after liver injury, which could imply that RNF43/ZNRF3-mutated individuals might be at higher risk to develop HCC in a background of fatty liver disease. Nearly 20% of HCC-patients carry an activating mutation in the CTNNB1-gene, which is responsible for encoding beta-catenin in Wnt-pathways. It has been shown that beta-catenin-activated HCCs are characterized by enhanced fatty acid oxidation, reduced glycolysis and upregulated expression of peroxisome-related genes. Various peroxisome-related genes are also associated with aberrant tumoral metabolic signalling and act as key regulators in tumorigenesis and tumour progression, by affecting lipid synthesis and utilization. Recent studies showed that alterations in lipid metabolism contribute to an increased energy production, thereby facilitating epithelial-mesenchymal transition and proliferation in HCC cells. Therefore, regulation of pathways involved in lipid metabolism might improve HCC prognosis.
It is known that lipid metabolism is strongly intertwined with ER-stress and the UPR. Firstly, the ER is the main site of lipid production, and many enzymes involved in lipid metabolism are produced in the ER\(^6^4\). In addition, the three UPR branches regulate lipid homeostasis by activating transcription factors and pathways which modulate lipid metabolism\(^6^4\). For instance, IRE1\(\alpha\) controls lipid metabolism by inducing the degradation of mRNAs encoding for lipid metabolism regulators through RIDD\(^6^5\), which has been specifically shown to be relevant in cancer\(^6^6\). Moreover, the IRE1\(\alpha\)/XBP1 branch is involved in the regulation of hepatic VLDL-production\(^6^7\), and upregulates Peroxisome proliferator-activated receptor alpha (PPAR\(\alpha\)) expression during starvation hence stimulating \(\beta\)-oxidation and ketogenesis\(^6^8\). Studies on Drosophila have shown that IRE1\(\alpha\) deficiency leads to an increased mobilization of lipids and sensitizes flies to starvation, thus demonstrating that IRE1\(\alpha\) is a catabolic sensor acting through the XBP1s-FoxO axis\(^6^9\). Regarding PERK/eIF2\(\alpha\), different studies have reported that this arm of the UPR could promote lipogenesis \textit{via} different downstream effectors. As an example, PERK seems to activate SREBP-1 and -2 pathways, thereby increasing cholesterol, fatty acid, triacylglycerol and phospholipid synthesis, thus leading to increased lipid accumulation in HCC-cells\(^7^0\). Lastly, ATF6 antagonizes SREBP2 to regulate the homeostasis of lipids and glucose, but also increases fatty acid oxidation by controlling the activity of PPAR\(\alpha\)\(^7^0\). As the different arms of the UPR are suggested to participate in several processes involved in lipid metabolism, the relation between ER-stress and lipid metabolism is suggested to be bidirectional. For example, triglycerides are shown to induce the expression of ER-stress markers such as BiP, IRE1\(\alpha\), XBP1, p-eIF2\(\alpha\), CHOP and p-JNK\(^7^1\). Some studies suggest that ER-stress mediates lipotoxicity by inducing apoptosis or modulating the membrane composition of phospholipids, but it is still not completely understood how this happens \(^2^5^,\ ^7^2\). A widely believed hallmark of the unfolded protein response is an increase of \textit{de novo} lipogenesis to allow the expansion of lipids in the ER. However, this phenomenon has mostly been studied on cell cultures and a very recent \textit{in vivo} study by Ward et al shows that hepatic \textit{de novo} lipogenesis and cholesterogenesis might actually reduce after tunicamycin-induced ER-stress\(^7^3\). Concerning pharmacological intervention, several chemical molecules, such as cinnamaldehyde\(^7^4\), Asiatic acid\(^7^5\) or Schisandra chinensis extract\(^7^6\) have been shown to improve chronic liver disease \textit{in vivo} by regulating ER-stress and lipid accumulation and metabolism. Targeting this interaction between ER-stress and lipid metabolism could therefore contribute to new potential therapies to treat liver diseases, including HCC.

\textbf{Metabolism of proteins}

The excess of amino acids obtained from diet are also catabolized in the liver. In liver cancer, tumour cells can use the degradation of certain amino acids like glutamine or leucine as an alternative energy
source. Dysregulation of glutaminases and glutamin synthase is a hallmark of several cancers, including HCC, leading to several glutamin analogs and glutaminase-inhibitors to have been tested in preclinical and clinical studies, with variable success. Zhou et al. found that HCC-cells can survive and maintain tumour progression in low glucose concentrations by activating glutaminolysis through the upregulation of glutamate dehydrogenase 1, a key enzyme in the degradation pathway of glutamine. In this condition, glutaminase inhibitors could be used as and adjuvant treatment in cancer reducing glutamine conversion to glutamate. Specifically, since glucose deprivation is a strong inducer of ER-stress and glutamine treatment can reduce ER-stress-signalling in these glucose-deprived conditions. However, it is well established that glutamine synthetase (GS) is also increased in the majority of HCC-tissues. Because GS can synthesize glutamine from glutamate, it allows cells to survive in glutamine-depleted conditions, thus supporting an anaplerotic flux for glutamine, which feeds TCA-cycle. In a zebrafish model for HCC, it has been shown that GS can increase glutamine levels for nucleotide biosynthesis and support growth of liver cancer cells in a Yes-associated protein1-dependent manner. Furthermore, clinical data has shown that patients with CTNNB1-mutations show a 6 to 45-fold increase in mRNA-expression of Glutamate-Ammonia Ligase, the gene encoding for GS. As all these tumors with increased GS expression also showed an increase in mammalian target of rapamycin (mTOR)-signalling, this led to testing the combination treatment of mTOR inhibition with rapamycin in combination with a GS-β-catenin inhibitor CG1 (sobetirome). Successfully, this combined treatment synergistically reduced HCC burden. These results warrant the intricate complexity and need for further studies to further unravel the role of glutamine and GS in the pathophysiology of HCC.

Protein metabolism generates ammonia, a potentially toxic amino group, which is eliminated in the liver via the urea cycle. Genes of the urea cycle (carbamoyl phosphate synthetase-1 (CPS1), ornithine carbamoyl-l transferase, arginase, argininosuccinate synthase and lyase) and its derived metabolites (citrulline, arginine and ornithine) are decreased in HCC. A large-scale gene expression data analysis study has revealed that dysregulation of genes involved in the urea cycle and specifically downregulation of CPS1, are characteristic for HCC-patients. Furthermore, CPS1-deficient HCC-cells have a distinctive metabolic phenotype characterized by a deceleration of the TCA-cycle, increased ATP-levels, and higher dependency on fatty acid oxidation, rather than glucose or glutamine.

Besides amino acid and protein metabolism, the liver is responsible of more than 80% of the protein synthesis. Albumin, growth factors, and many other functionally important peptides and non-essential amino acids are produced by hepatocytes. Tumour cells usually increase their protein production in
order to promote tumour growth and proliferation\textsuperscript{83}. The PERK-branch induces biosynthesis of specific amino acids and upregulation of their corresponding tRNA-synthetases, suggesting that amino acid metabolism might be governed by protein synthesis demands during ER-stress\textsuperscript{84}. Moreover, Envelop Protein E2 of HCV, a risk factor for HCC, can modulate protein synthesis and ER-stress through PERK-activation\textsuperscript{85}. Furthermore, deprivation of different amino acids, like glutamine, arginine and leucin, seems to induce ER-stress\textsuperscript{86}. Particularly in the liver, intake of L-leucine can reduce ER-stress in the fatty liver by downregulating splicing of XBP1-mRNA\textsuperscript{87}. All these findings highlight once more the close relation between ER-stress and protein metabolism in HCC and the possibilities for finding new therapeutic targets.

**Vitamin metabolism**

The synthesis and storage of many vitamins, such as vitamin D and K, is another major function of the liver. Previous studies have reported a reduced production of vitamins in several types of cancers, including HCC\textsuperscript{88}. In the case of vitamin D, antitumor, anti-proliferative, anti-angiogenic, and pro-apoptotic activities have been found for this molecule, suggesting potential therapeutic benefits in HCC via the use of complementary vitamin D-based treatments\textsuperscript{88}. This has been further supported once anti-inflammatory and anti-fibrotic activities in HCC cell models and mice were reported\textsuperscript{89}. Moreover, Riek et al. demonstrated that vitamin D is a natural macrophage ER-stress reliever\textsuperscript{90}, which would potentially further improve the anti-cancer treatment, as it was previously shown that ER-stress can promote a pro-tumoral phenotype in macrophages\textsuperscript{3}. Vitamin K mainly participates in bone metabolism, but it is also responsible for the regulation of clotting factors production. Like vitamin D, supplementation with vitamin K seems to reduce the HCC proliferation and invasion in HCC cells\textsuperscript{91}, improving the current treatments. The role of this regarding ER-stress is still unclear, yet a recent study has shown that vitamin K can modulate organelle damage and decrease the expression of ER-stress markers such as CHOP and IRE1\alpha/XBP1\textsuperscript{92}. However, more research is necessary to establish whether this is relevant in the context of HCC.

**Nucleic acids biosynthesis and the pentose-phosphate pathway**

Nucleic acid metabolism is the final and most critical process for tumour cell replication and fast proliferation\textsuperscript{93}. Consequently, the synthesis of nucleic acids is also upregulated in HCC. Genes involved in the pyrimidine biosynthesis, like carbamoyl phosphate synthetase 2, aspartate transcarbamylase or dihydroorotase, are increased in HCC\textsuperscript{94}. Furthermore, this upregulation has been associated with tumour cell stemness and poor prognosis\textsuperscript{91}. Likewise, the pentose-phosphate pathway (PPP), which is a source of glycolytic metabolites for nucleotide metabolism, is highly active in HCC.
tumour cells\textsuperscript{95}. However, metabolite levels from this pathway are often reduced, suggesting a fast usage of intermediates for nucleotide biosynthesis. In addition, some upstream signalling pathways that regulate PPP-enzymes contribute to cancer initiation and progression, opening the possibility of using them as targets in cancer therapy\textsuperscript{96}. Lastly, the nucleic acid metabolism is also related to ER-stress as inhibition of the pyrimidine metabolic pathway and inhibition of PPP induces ER-stress\textsuperscript{97}.

**Detoxification and drug metabolism**

Elimination and detoxification of drugs from the blood is mainly conducted by the liver. As a result, liver function is crucial for the response to anticancer therapies and drug response. Particularly, this activity is developed by some cytochromes in the ER lumen. Downregulation of cytochromes like Cytochrome P450 3A4 (CYP3A4) and UDP-glucuronosyltransferase 1A9 has been found in microsomes of HCC-patients which seems to decrease the metabolism of sorafenib, a drug widely used in HCC\textsuperscript{98}. Besides, several drugs, including anti-cancer compounds like tunicamycin, doxorubicin or erlotinib, can induce ER-stress and drug-induced liver injury\textsuperscript{99}. These lead to hepatic lesions, steatosis, steatohepatitis, hepatocellular adenoma and cirrhosis among others, which are previous steps of HCC and contribute to the development of an already existent tumour\textsuperscript{100}. In addition, hepatotoxicity commonly leads to reduction of drug dosing or even early termination of treatment, thus drastically decreasing the patient’s survival rates\textsuperscript{101}. Moreover, Doxorubicin is known to cause heart failure in some cancer patients through activation of the ATF6, PERK and IRE1-pathways, which has been shown to be reduced by combinational treatment with ER-stress inhibitors, including alginate oligosaccharide, sodium hydrosulfide and sacubitril\textsuperscript{102, 103}. Therefore, combination treatment of ER-stress inhibitors with chemotherapeutics could potentially decrease adverse effects and improve therapeutic outcomes in several types of cancer (table 1).

During the corona virus disease-19 (COVID19) pandemic several drugs, including dexamethasone, hydroxychloroquine and remdesivir, were repurposed for treating COVID19-patients (table 1). A recent study showed that both dexamethasone and remdesivir induced ER-stress through IRE1\(\alpha\) and CHOP in HCC-cell lines, as well as in healthy hepatocyte cell cultures, suggesting a possible hepatotoxic effect\textsuperscript{104}. The effect was exacerbated by pre-treatment with alcohol, thus further emphasizing the importance of factoring in drug-drug or alcohol-drug combinations on cellular stress responses, particularly in the context of liver injury.

Moreover, the liver is crucial for the metabolism and detoxification of alcohol. When alcohol reaches the liver through the bloodstream, it is metabolized by alcohol dehydrogenase, catalase, or CYP2E1 into acetaldehyde. This highly reactive metabolite is known to cause protein adducts, which results in
mutations, conformational changes of proteins, and abnormal protein degradation\textsuperscript{105}. These alterations can cause ER-stress and contribute to the development of alcoholic liver disease, thereby increasing the risk for HCC. Both acute and chronic alcohol exposure increases the expression of \textit{ATF6}, \textit{CHOP}, and \textit{BiP}, and enhances the phosphorylation of PERK, eIF2\textalpha{}, and IRE1\textalpha{}\textsuperscript{105, 106}. In mice subjected to chronic and binge ethanol feeding, levels of ERdj5, an ER-resident chaperone protein, are markedly increased, correlating with XBP1s mRNA-levels, further supporting the direct effect of alcohol on ER-stress\textsuperscript{105}. However, loss of ERdj5 can aggravate alcoholic liver disease through inhibition of Nrf2, thus suggesting a protective role of ERdj5 in this pathology\textsuperscript{105}. In addition, \textit{CYP2E1}-overexpressing primary human hepatocytes and HepG2-cells that were treated with alcohol showed an upregulation in \textit{ATF4}\textsuperscript{106}, suggesting that excessive alcohol intake also increases the expression of \textit{ATF4}. Together, this suggests that alcohol induces ER-stress, resulting in activation of all arms of the UPR. Several pharmacological approaches to reduce ER-stress during alcoholic liver injury have been tested preclinically, including magnesium isoglycyrrhizinate\textsuperscript{106}, kinsenoside\textsuperscript{107} and geniposide\textsuperscript{108} (table 1). Whether these drugs are also effective to prevent HCC-development in a background of alcoholic liver disease, remains to be elucidated.

\textbf{Concluding remarks}

Hepatocellular carcinoma is associated with increased ER-stress and upregulation of the UPR, which contributes to its development and progression. Besides ER-stress, metabolism is also highly altered in tumour cells to facilitate proliferation and tumorigenesis. This includes up- or downregulation of enzymes in metabolic pathways, different enzymatic isoforms, and altered metabolite flow. However, the causes of the metabolic alterations in HCC and their regulation at distinct levels (genes, RNA expression, protein expression etc) are partially unclear. In addition, ER-stress seems to be involved in the regulation of different metabolic processes, but the exact mechanisms that link these processes together are largely unknown. Therefore, more research is needed in order to increase the knowledge in ER-stress, liver metabolism and their interaction in HCC in order to target these processes and improve therapeutic outcomes.
References


[99] Malik A, Bagchi AK, Jassal DS, Singal PK: Interleukin-10 Mitigates Doxorubicin-Induced Endoplasmic Reticulum Stress as Well as Cardiomyopathy. Biomedicines 2022, 10:890.


Figure Legends

**Figure 1.** A human protein-protein interaction (PPI) network of UPR-associated and metabolism-associated genes (STRING) ([https://string-db.org](https://string-db.org), last day of accession: 2022/08/08). In the above network, the nodes correspond to the proteins and the edges represent the interactions. Colored nodes represent the query proteins and first shell of interactors. The blue edges represent interactions from curated databases, while the pink edges represent experimentally determined interactions. The green, red and blue edges correspond to predicted interactions based on gene neighbourhood, gene fusions and gene co-occurrence, respectively.

**Figure 2.** Functional connection between ER-stress and glucose metabolism. The three major UPR signaling pathways, ATF6, PERK and IRE1a, attenuate proteostatic burden, either by endorsing the transcription of folding chaperones or by inhibiting protein translation. The UPR interacts with key transcription factors that regulate glucose homeostasis by controlling metabolic reactions, such as glycogenesis, glycogenolysis, glycolysis and gluconeogenesis. Although the catabolism of glucose into pyruvate is the main pathway of ATP-generation, cytosolic pyruvate can be incorporated into the mitochondrial matrix, converted to acetyl-CoA by pyruvate dehydrogenase complex, and enter the tricarboxylic acid cycle along with oxaloacetate, generating NADH and FADH2. FoxO1 is a key transcription factor in energy metabolism that controls hepatic gluconeogenesis via the transcriptional regulation of key genes, such as Glucose-6-Phosphatase. Loss of glucose homeostasis is a hallmark of cancer cells and it remains unknown whether inhibition of UPR-associated and metabolism-associated modulators could be employed to limit tumor proliferation. Figure created with BioRender.com, Toronto, Canada.
Table 1: Overview of drugs and toxins influencing ER-stress pathways and potential combinational therapies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Affected ER-stress pathways</th>
<th>Combinational treatments</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tunicamycin</td>
<td>ATF6, PERK and IRE1</td>
<td>Chemotherapeutics</td>
<td>Cancer</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>ATF6, PERK and IRE1</td>
<td>Alginate oligosaccharide (an antioxidant), sodium hydrosulfide (a hydrogen sulfide donor), sacubitril (anti-hypertensive).</td>
<td>Cardiomyopathy, Cancer</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>PERK and XBP1</td>
<td></td>
<td>Cancer</td>
</tr>
<tr>
<td>Remdesivir</td>
<td>IRE1 and CHOP</td>
<td></td>
<td>Covid19</td>
</tr>
<tr>
<td>Alcohol</td>
<td>ATF6, PERK and IRE1</td>
<td>magnesium isoglycyrrhizinate (anti-inflammatory), kinsenoside (anti-oxidant) and geniposide (anti-oxidant)</td>
<td>Alcoholic liver disease, HCC</td>
</tr>
</tbody>
</table>