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RUNNING HEAD: ER stress and NAFLD

ABBREVIATIONS:

ATF, activating transcription factor;
ASK1, apoptosis signal-regulating kinase 1;
BI-1, bax-inhibitor 1;
CHOP, C/EBP homologous protein;
CRP, C-reactive protein; SAP, serum amyloid P component;
ER, endoplasmic reticulum (rough RER or smooth SER);
ERAD, ER-associated degradation;
FFAs, free fatty acids;
GADD34, growth arrest and DNA damage-inducible 34;
GRP78, glucose-regulated protein 78;
HCC, hepatocellular carcinoma;
HFD, high-fat diet;
HSP47, heat shock protein 47;
IP3R, inositol trisphosphate receptor;
IRE1α, inositol-requiring enzyme 1α;
JNK, c-Jun amino terminal kinase;
LPS, lipopolysaccharide (endotoxin);
MAM, mitochondria-associated ER membranes;
MCDD, methionine-choline-deficient diet;
MOMP, mitochondrial outer membrane permeabilization;
NAFLD, non-alcoholic fatty liver disease;
NASH, non-alcoholic steatohepatitis;
NLRP3, NOD-like receptor family, pyrin domain containing 3
NRF2, nuclear factor erythroid 2-related factor 2;
PERK, PKR-like ER kinase;
PP1c, type 1 protein serine/threonine phosphatase;
PTP, permeability transition pore;
RIDD, Regulated IRE1-dependent decay of RNA;
RNase, endoribonuclease;
ROS, reactive oxygen species;
SERCA, sarco-ER Ca^{2+}-ATPase;
UPR, unfolded protein response;
VLDL, very low density lipoproteins;
XBP1, X-box binding protein 1;

Abstract

The obesity epidemic is accompanied by a worldwide burden of non-alcoholic fatty liver disease (NAFLD) that manifests from simple steatosis to non-alcoholic steatohepatitis (NASH), possibly evolving into hepatocellular carcinoma (HCC). Although much attention has been focused on NAFLD, its pathogenesis remains largely obscure. The hallmark of NAFLD is the hepatic accumulation of lipids subsequently leading to cellular stress and hepatic injury, which eventually results in chronic liver disease. Abnormal lipid accumulation often coincides with insulin resistance in steatotic livers and is associated with perturbed endoplasmic reticulum (ER) proteostasis in hepatocytes. In response to chronic ER stress, an adaptive signaling pathway known as the Unfolded Protein Response (UPR) is triggered initially to restore ER proteostasis. The UPR can be accountable for inflammation, inflammasome activation and, in the case of non-resolvable ER stress, for the death of hepatocytes. Experimental data suggest that the UPR influences hepatic tumor development, aggressiveness and response to treatment, offering novel therapeutic avenues. Herein, we provide an overview of the evidence linking ER stress to NAFLD and discuss possible points of intervention.

Keywords: Steatosis, Steatohepatitis, Liver Cancer, Endoplasmic Reticulum Stress, Unfolded Protein Response, Inflammation, Cell death, Therapeutic Targets

Introduction

The global epidemic of obesity poses drastic threats to public health systems because of the increasing incidence of its related comorbidities. Lifestyle changes in developed countries have led to caloric excess, lack of physical activity and increased life expectancy. Faced with these new challenges, physiological responses are no longer able to cope, leading to an imbalance between the homeostatic processes governing energy expenditure and energy uptake that largely affects liver function. Non-alcoholic fatty liver disease (NAFLD) is the most common etiology of chronic liver disease affecting 25% of the general population worldwide(1) and 85-98% of morbidly obese patients(2). NAFLD is often considered as the hepatic manifestation of the metabolic syndrome (insulin resistance, obesity and hyperlipidemia), comprising hepatic steatosis and non-alcoholic steatohepatitis (NASH). NASH is a combination of lipid accumulation, hepatocyte death, inflammation and fibrosis, making this pathology a significant risk factor for hepatic cirrhosis, hepatocellular carcinoma (HCC) and ultimately liver-related mortality(3). Dysfunction of the Endoplasmic Reticulum (ER), the main cellular compartment involved in secretory and transmembrane protein productive folding, calcium homeostasis and lipid biogenesis, has been involved in metabolically-driven NAFLD pathologies(4) through the activation of ER stress signaling. This review compiles the recent findings supporting the functional impact of ER stress signaling on chronic liver disease from steatosis to NASH and HCC and discusses possible therapeutic strategies.

The physiology of the liver and the adaptive UPR

The liver assures vital metabolic, secretory and excretory functions to preserve whole-body homeostasis. Hepatocytes represent up to 70% of the total liver cells(5). They are highly secretory cells responsible for the assembly and secretion of very low-density lipoprotein (VLDL) and for the synthesis of the plasma proteins including albumin, α-1 antitrypsin, apolipoproteins and coagulation factors. Hepatocytes are responsible for lipogenesis, cholesterol biosynthesis, glucose and xenobiotic

metabolism. To fulfil their myriad of metabolic functions, hepatocytes are enriched in both smooth and rough ER(6).

The ER in hepatocytes has a remarkable capacity to adapt to extracellular and intracellular changes to ensure that vital hepatic metabolic functions are preserved. However, in humans, numerous disturbances (e.g. hyperlipidemia, inflammation, viruses, drugs) can perturb hepatocyte ER homeostasis thereby contributing to the dysregulation of hepatic lipid metabolism and liver disease. As a consequence, the ER engages an evolutionarily conserved pathway termed the unfolded protein response (UPR)(7) to control hepatic protein and lipid homesotasis. The UPR reduces the secretory protein load, enhances ER protein folding (transcription of chaperones and foldases) and increases clearance capacity by promoting autophagy and ER-associated degradation (ERAD) **(Figure 1)**.

Induction of the UPR involves the activation of three transmembrane ER-resident stress sensors: (i) inositol-requiring enzyme 1 (IRE1), (ii) PKR-like ER kinase (PERK) and (iii) activating transcription factor (ATF6). Under unstressed conditions, IRE1, PERK and ATF6 are maintained inactive upon binding to GRP78/BiP. Above a critical threshold of misfolded protein accumulation, GRP78 dissociates from the ER stress sensors, thereby priming all branches for activation. PDIs may also regulate UPR stress sensors(8-10), suggesting that a combinatorial of factors control UPR stress sensor activation.

IRE1 is the most conserved ER stress transducer, possessing two isoforms in mammals: IRE1α and IRE1β[11]. IRE1α is a ubiquitously expressed transmembrane protein that possesses dual enzymatic activities due to its serine/threonine kinase and endoribonuclease (RNase) domains on its cytosolic tail. Sensing ER stress through its luminal N-terminal domain, IRE1α dimerizes and oligomerizes while stimulating trans-autophosphorylation, inducing a conformational change that activates the RNase domain(12). Recently, the ER chaperone Hsp47, notably involved in the folding and secretion of collagen in the liver, was identified as an instigator of IRE1α signaling by binding to its luminal domain, thus reducing the association of GRP78 and facilitating IRE1α's oligomerization(13).

Unfolded proteins may directly bind to IRE1α, inducing allosteric changes that trigger its activation(14). As a direct target of IRE1α RNase activity, the mRNA encoding the X-box binding protein 1 (XBP1) undergoes unconventional splicing through the concerted action of IRE1α and the tRNA ligase RTCB(15) at a stem-loop structure that results in the translation of an active transcription factor, termed spliced XBP1 (XBP1s)(6). XBP1s enhances ER protein folding, secretion, ERAD and lipid synthesis(16). In mice, the genetic ablation of either *IRE1α* or *XBP1* results in embryonic lethality with major liver defects(17, 18). In sustained conditions of ER stress, IRE1α RNase broadens its specificity to degrade many other ER-bound mRNAs, including itself, at both stem-loop and non-stem-loop sites, through regulated IRE1α-dependent decay (RIDD)(19-21). Enhanced RIDD leads to pro-death signaling by reducing the levels of select microRNAs that normally repress pro-apoptotic targets(22). While the pro-apoptotic BCL2 members BAX and BAK activate IRE1α signaling via its cytosolic C-terminal domain(23, 24), Bax inhibitor-1 (BI-1, or transmembrane BAX inhibitor motif containing 6 (TMBIM6)) prevents this activation(25-28). These findings lead to the proposition of the UPRosome, where IRE1α is envisioned as a scaffold where many components assemble to control its activity and also connect the UPR with other stress pathways through signaling crosstalk(29). In the last years, a series of novel regulators and posttranslational modifications have been proposed to selectively tune the activity of UPR stress sensors (30).

PERK is a transmembrane protein with an N-terminal stress-sensing domain and a cytosolic kinase domain. The major PERK substrate is the eukaryotic translation initiation factor eIF2α(31). To alleviate the protein overload in the ER, phosphorylated eIF2α halts mRNA translation by preventing 80s ribosome assembly while paradoxically increasing the translation of several mRNAs that have upstream open reading frames in their 5´ region, such as *ATF4* (32, 33). ATF4, a transcription factor, transactivates UPR target genes involved in protein folding, autophagy, redox homeostasis, amino acid metabolism and apoptosis (34, 35). ATF4 regulates the expression of DNA damage-inducible transcript 3 (best known as C/EBP homologous protein, CHOP) (36) and subsequent growth arrest and DNA

damage-inducible 34 (GADD34)(37). PERK activation results in an antioxidant response (34, 38).

ATF6 is a transmembrane protein with a cytosolic bZip transcription factor domain and two mammalian isoforms: ATF6α and ATF6β (39, 40). When activated, ATF6α translocates to the Golgi apparatus where it is cleaved by the resident proteases S1P and S2P, releasing a cytosolic fragment that migrates to the nucleus to regulate gene transcription (41-43). Cleaved ATF6α not only regulates genes involved in protein folding and ERAD, but can also activate the transcription of XBP1u (44, 45). Although PERK and ATF6α are dispensable for liver development, studies conducted on global knockouts have shown that both are required to facilitate recovery from dietary or pharmacological challenges (40, 46, 47).

If the adaptive UPR is overwhelmed by chronic or acute ER stress, it may become unable to preserve ER function, switching to a "terminal UPR" that induces cell death(48) through pathways involving ROS production, upregulation of pro-apoptotic BCL-2 family members, microRNA regulation, sustained ER-calcium release among other mechanisms (reviewed in(49) **(Figure 2)**).

The UPR in NAFLD: two faces of Janus

The UPR initially aims to maintain liver physiology by protecting hepatocytes from nutrient and xenobiotic exposure via the portal vein or from cellular stress that arises from increased secretory demand or cellular differentiation. Concerning the physiological regulation of hepatic metabolism, transient UPR activation has been reported in the postprandial switch from glucose production to glucose storage (50). A transient RIDD activity reduces the mRNA of two cytochrome P450 enzymes that metabolize the drug acetaminophen into a cytotoxic metabolite (51), protecting the liver from hepatotoxicity. Normal RIDD function was found to represses levels miR-34 and miR-200 in hepatocytes, protecting against steatosis (52). Although the liver experiences transient ER stress in physiological conditions, this stress could become chronic in NAFLD, then contributing to its progression towards more severe stages by inducing inflammatoy responses and cell death.

A link between chronic ER stress and obesity was established by the presence of ER stress markers in steatotic livers of genetically obese and high-fat diet (HFD)-fed mice (53) as well as in steatotic livers of obese patients. Normalization of ER stress markers correlated with weight loss and reduced hepatic lipid content after bariatric surgery (54). Furthermore, activation of the UPR was observed in the livers of obese patients with NASH as well as in mice fed a methionine-choline-deficient diet (MCDD) exhibiting NASH features without obesity (55-58). A direct activation of the IRE1α-XBP1 branch was observed *in vivo* during the development of insulin resistance in the liver (59). Furthermore, hepatic overexpression of the ER-resident chaperones, such as ORP150/HYOU1 and GRP78/BiP improved insulin sensitivity and resolved steatosis in obese mice (60, 61), providing a direct evidence that altered ER protein folding in the liver contributes to steatosis associated with obesity.

The role of ER stress in the initiation of NAFLD

Adding to its processing of secreted and membrane proteins; the ER is the major site of lipid synthesis in hepatocytes. Hepatocytes contain all the major lipid metabolic pathways, including lipogenesis, triglyceride synthesis and storage, apolipoprotein assembly and secretion, and fatty acid oxidation. First, *de novo lipogenesis* is regulated by ER membrane-localized transcription factors, the sterol regulatory element-binding proteins (SREBP1c for fatty acid synthesis and SREBP2 for sterol synthesis). Second, hepatocytes store lipids in the form of triglycerides (TG) that are synthesized from fatty acids (FA) and glycerol by ER-localized acyltransferase enzymes including diacylglycerol acyltransferase (DGAT). Third, hepatocytes can secrete VLDL, which are assembled in the ER prior to trafficking to the Golgi. Hepatic ER homeostasis is crucial in maintaining membrane lipid composition and in controlling both intrahepatic and plasma lipid homeostasis.

Steatosis is the first stage of NAFLD, characterized by ectopic triglyceride accumulation in hepatocytes. In patients with steatosis, 59% of triglycerides found in the liver were derived from serum

nonesterified fatty acids stored in adipose tissue and 26% from increased *de novo* lipogenesis, while only 15% from diet(62). Since the bulk of lipid synthesis takes place in the smooth ER, an important role for ER stress responses has emerged in steatosis pathogenesis. Studies using transgenic mice with liver specific or whole-body deletions in specific UPR mediators have been performed to understand how the UPR regulate hepatic lipid homeostasis. In this section, we will describe how chronic ER stress could have causative role in steatosis. Chronic ER stress can directly affect hepatic lipid metabolism by inducing *de novo* lipogenesis and indirectly through the alteration of VLDL secretion, insulin signaling and autophagy. We will emphasize how lipid homeostasis is intrinsically embedded to ER homeostasis and vice versa, how enhanced lipid content in hepatocytes can lead to the genesis of chronic ER stress.

ER stress regulates lipostasis **-** The UPR is extensively studied in terms of proteostasis control but mostly ignored in the context of lipostatic control. The UPR regulates hepatic lipid homeostasis. While each master regulator pathway of the UPR is required to protect from steatosis under pharmacologically-induced severe ER stress(46), each may contribute to hepatic steatosis development by promoting *de novo* lipogenesis and lipolysis, reducing fatty acid oxidation and disturbing lipoprotein and VLDL secretion.

First the IRE1α-XBP1 arm was shown to be a crucial player in hepatic lipid metabolism through regulation of VLDL secretion and lipogenesis (17, 18, 63). Mice with a specific hepatocyte deletion of *IRE1α* and challenged with tunicamycin displayed increased steatosis due to the inhibition of lipid synthesis regulators (C/*EBPβ*, C/*EBPβγ*, *PPARγ*) and halted VLDL secretion (64). Similarly, the overexpression of BI-1 leading to the inhibition of the IRE1α pathway resulted in the downregulation of key enzymes of lipid homeostasis pathways (*C/EBPα, SREBP1, PCG1α*) (25). Unlike hepatocyte-specific *IRE1α* deletion, livers of mice with conditional *XBP1* deletion had limited hepatic *de novo* lipogenesis, lower levels of serum triglycerides and cholesterol, and decreased steatosis in response to

lipogenic diets (65). In hepatocytes, XBP1s binds to promoters of lipogenic genes (*DGAT2*, *SCD1*, *ACC2*) (65). The discrepancy in the metabolic phenotype between *IRE1α*-deficient and *XBP1*-deficient mice is still under investigation. Furthermore, *XBP1* deficiency results in a feedback activation of IRE1α, inducing the mRNA degradation of lipid metabolism genes and lipoprotein catabolism genes (66).

Second, the PERK-eIF2α-ATF4 arm was reported to regulate lipogenesis and steatosis. Sustained dephosphorylation of eIF2α due to the hepatic overexpression of GADD34 in the liver protects mice from HFD-induced obesity and NAFLD(67). However, a basal level of eIF2α phosphorylation prevents lipid accumulation in response to a direct challenge of ER stress as inactive eIF2α in mice led to tunicamycin-induced fatty liver (46). *ATF4* deficiency protected mice from steatosis in response to high fructose feeding through attenuated *de novo* lipogenesis(68). *ATF4*-null mice were protected from age-related and diet-induced obesity and steatosis(69). In addition, CD154, ligand of CD40 and key mediator of inflammation, may play a protective role in hepatic steatosis through a possible connection to UPR signaling. Livers of CD154 knockout mice fed an olive-oil rich diet presented reduced ApoB100 expression and increased lipogenic enzyme gene expression associated with reduced p-eIF2α(70).

Third, the ATF6α arm may be protective in steatosis. *ATF6α* knockout mice presented sustained CHOP expression, C/EBPα inhibition and hepatic steatosis when challenged with tunicamycin (46, 47) (71). In hepatocytes, ATF6α interacted with PPARα to enhance its transcriptional activity towards hepatic fatty acid oxidation(72). ATF6α suppresses SREBP2 transactivation in hepatocytes, downregulating lipogenesis(73).

Overall, studies suggest that the UPR has a relevant role in lipostasis in addition to proteostasis control. The lipid gene expression networks are directly responsive to chronic ER stress. While a transient UPR protects from steatosis, chronic ER stress in NAFLD aggravates hepatic steatosis, with each arm contributing to detrimental effects on hepatic lipid pathways. The crosstalk between these

pathways remains to be explored. The challenge remains the identification of which UPR mediators contribute to adaptation versus those that induce metabolic dysfunction.

ER modulates hepatic insulin sensitivity - ER stress can trigger insulin resistance through excessive fat accumulation. In addition, ER stress can disrupt insulin action through the activation of each arm of the UPR (53, 74-76). IRE1α's kinase function phosphorylates JNKs and IkB kinase, inhibiting insulin signaling. However, IRE1α-mediated JNK activation alone was found to be insufficient to induce hepatic insulin resistance, thus advocating a causal relationship in which hepatic insulin resistance is secondary to ER stress-activated lipogenesis(77). Similarly, the PERK arm contributes to insulin resistance. The selective dephosphorylation of eIF2α through enforced expression of GADD34 led to enhanced insulin sensitivity(67). Interestingly, hepatic levels of p-eIF2α increased with worsening insulin resistance in obese, nondiabetic patients(78). The role of ATF6α in hepatic insulin signaling has not been fully explored. The knockdown of *ATF6α* led to upregulated hepatic glucose output (79). In support of a beneficial role for ATF6α, genetic overexpression of hepatic *ATF6α* in mice with diet-induced obesity led to improved insulin signaling and metabolic health(80).

ER regulates hepatic autophagic flux - ER stress and autophagy are delicately balanced. Impaired autophagy in livers of lean mice augments ER stress, while the rescuing of autophagy alleviates obesity-induced hepatic ER stress as seen from the downregulation of LC3, Beclin, Atg5 and Atg7 in *ob/ob* and HFD murine models of obesity(81). Interestingly, PERK and IRE1α were upregulated in livers of *ob/ob* models(81). Furthermore, increased expression of LC3 in NASH murine models (82, 83) and p62 in NASH patients has been shown (84). In addition, ER stress increased parallel to elevated p62, LC3-II and autophagosomes in livers of murine models of NASH (84). BI-1 expression was also shown to modulate IRE1 signaling and autophagy in the liver of animals undergoing experimental ER stress (85). Although it is clear that ER stress-driven autophagy leads to cell death in

NASH, it may still be too speculative to advocate a mitigative or augmentative role of autophagy in NAFLD.

The role of lipids and Ca^{2+} homeostasis in triggering chronic ER stress - Although triglycerides are the most abundant lipid category, lipidomic analyses from human liver biopsies revealed diglycerides and ceramides in steatotic livers(86). These lipids inhibit hepatic insulin signaling pathways, contributing to the onset of hepatic insulin resistance and the genesis of ER stress. Interestingly, the expression of their transporter -fatty acid translocase CD36- was upregulated in patients with NAFLD (87). While it remains unclear whether hepatic insulin resistance or steatosis develops first, it is well established that insulin resistance is a risk factor in NAFLD progression (88-90). Nevertheless, the common denominator between hepatic insulin resistance and steatosis is the unusually elevated amount of circulating and intracellular lipids.

The aberrant lipid changes in hepatocytes could directly trigger chronic ER stress in the liver by disrupting Ca^{2+} homeostasis. In obese mice, the hepatic lipid burden leads to an imbalance in ER membrane lipid composition with a higher ratio of phosphatidylcholine versus phosphatidylethanolamine compared to lean mice, thus altering membrane fluidity. Accumulation of phosphatidylcholine leads to the inhibition of *SERCA* activity and reduction of ER [Ca^{2+}], which induces ER stress as the majority of ER chaperones are Ca^{2+}-dependent(91). SERCA overexpression or ER phospholipid composition correction reduced ER stress and steatosis in obese animals(91), thus advocating that lipid perturbations and Ca^{2+} homeostasis (and protein-misfolding due to Ca^{2+} homeostasis disturbance in ER) are main proponents of hepatic ER stress in obesity(91). It remains unclear how obesity alters SERCA expression. The disruption of Ca^{2+} homeostasis may trigger ER stress and affect lipid metabolism as well. The ER makes close physical contacts with the mitochondria via mitochondrial-associated-membranes (MAMs) allowing Ca^{2+} and lipid transfer between these 2 organelles. During obesity, the number of MAMs increases (92), which could overload mitochondria

with Ca^{2+} and alter oxidative phosphorylation capacity. Suboptimal β-oxidation in NAFLD accompanied by dysfunctional tricarboxylic acid cycle activity results in the accumulation of toxic lipid intermediates, like ceramides and diacylglycerols, inevitably leading to chronic levels of ROS with inflammation and fibrosis during NASH(93). Recent evidence indicates that steatosis is concurrent with fastened accretion of triglycerides in the liver, upregulating mitochondrial oxidative function and suggesting that mitochondrial remodelling is present in NAFLD (94, 95). Higher diacylglyceride, phospholipid, free cholesterol and FFA levels activate cellular ER stress. Hager *et al.* showed that 2% cholesterol-rich diet leads to its accumulation in hepatic ER, resulting in the perturbed free cholesterol/phospholipid ratio of ER membranes (96). Also, higher free cholesterol in the ER results in enhanced esterification due to acyl-CoA cholesterol acyl transferase (ACAT), leading to elevated free cholesterol/esterified cholesterol levels, further aggravating ER stress (97).

Lipids were suggested to directly induce ER stress through its sensors, particularly IRE1α and PERK. A study using yeast genetics established that the luminal unfolded protein stress-sensing domain of IRE1α is dispensable to ER stress caused by lipid-induced membrane aberrancy, suggesting an additional sensing mechanism specific to ER stress sensors(98). Furthermore, WT and luminal domain-mutated IRE1α proteins could equally sense ER stress due to lipid- or membrane-related aberrations caused by inositol depletion. Myriocin, a sphingolipid biosynthesis inhibitor, prevented IRE1α activation induced by inositol depletion, but not by DTT exposure. Saturated fatty acid overload via palmitate or pharmacological inhibition of stearoyl CoA desaturase 1 increases XBP1 mRNA splicing 8- and 4-fold respectively, both in IRE1α WT and IRE1α luminal domain-deleted cells(99). Moreover, the sensitivity of PERK activation towards membrane lipid perturbation was also revealed(98-100) This clearly suggests that the transmembrane domains of IRE1α and PERK could act as lipid sensors, discerning the biophysical properties of lipid membranes, dependent on the ratio of unsaturated/saturated acyl chains that affect membrane fluidity and thickness(99). Halbleib *et al*, utilizing tools like *in vitro* reconstitution and molecular dynamics, elaborated on the molecular

mechanism of UPR activation by lipid bilayer stress. Changing the amphipathic character of the amphipathic helix of IRE1α reduced cellular resistance to DTT, eventually imposing functional defects. This amphipathic helix region was required to sense aberrant physical membrane properties and for normal IRE1α functionality (101). Overall these observations suggest a novel sensing mechanism of ER stress sensors in response to lipid bilayer stress, which forms a vicious circle that disturbs lipid homeostasis and cell fate (see Section "*ER stress-induced cell death in NASH").* Several mechanistic questions are raised from these observations: What is the role of each class of lipids in activating the UPR sensors? Which class of lipids will activate cell death due to an unresolved ER stress driving steatosis-NASH transition?

The role of chronic ER stress in NASH

Under chronic conditions of ER stress, rather than alleviate the current stress the UPR can become counterproductive and drive key features of progressive NASH including inflammation and cell death. It is well established that patients with NASH exhibit increased inflammation that correlates with their histological severity (102). Liver biopsies from NASH patients also present a significantly greater number of TUNEL-positive hepatocytes, correlating with active Caspases-3 and -7, activation of proapoptotic BCL2-family members, and death receptor Fas when compared to livers from normal or steatotic patients(103).

ER stress-dependent inflammatory responses in NASH

Consequences of ER stress-driven NF-kB and JNK in NASH **-** Upon ER stress, activated IRE1α activates protein IkB and JNKs implicated in transcriptional activation of pro-inflammatory and pro-apoptotic pathways. The activation of NF-kB is pivotal in the induction of inflammatory responses to primarily promote survival. In NASH, NF-kB has been qualified as a two-edged sword, acting as a central link between hepatic injury, fibrosis and even favoring progression to HCC. Activation of the

PERK branch reduces translation of IkB, increasing NF-kB activity (104, 105). Although ATF6α can also trigger NF-kB activation via phosphorylation of Akt(106), the IRE1α and PERK arms appear crucial for ER stress-induced NF-kB activation. A vicious cycle of injury and inflammation exists between JNK-dependent hepatocyte death and NF-kB activation in Kupffer cells releasing mediators like IL-1β and TNFα(107). Gut-derived pathogens such as lipopolysaccharide (LPS) and subsequent LPS-induced cytokines may be toxic to hepatocytes, initially activating an NF-kB-mediated response that possess a pro-inflammatory yet anti-apoptotic dual function in hepatocytes. This suggests that normal or slightly upregulated NF-kB activity in hepatocytes protects against NASH by preventing hepatocyte death, but above a certain threshold, the fact that NF-kB promotes the secretion of inflammatory and chemotactic factors in hepatocytes leads to a worsening of hepatic inflammation and initiates fibrosis(107). In the livers of mice challenged with MCDD, NF-kB was a pro-inflammatory mediator of lesion development in NASH (108). A potential mechanism involved in steatosis progression to NASH was proposed through CHOP-mediated activation of NF-kB signaling in human primary hepatocytes exposed to saturated fatty acids(109).

Consequences of ER stress on ROS production in NASH - Oxidative stress due to overproduction of reactive oxygen species (ROS) and free radicals in NAFLD contributes to its pathogenesis (110, 111). During steatosis, the fatty acids and Ca^{2+} overload alters mitochondria function (oxidation and respiration) leading to an increased ROS production (as state previously). Alternative oxidative pathways are then activated in the peroxisomes (β-oxidation) and microsomes (ω-oxidation) which results in more and more free radical production and reduced cellular state(112-118). This reduced cellular state impairs FA oxidation and enhances glycerol-3-phosphate production, lipogenesis and contributes to aggravate steatosis (118, 119). Similarly, saturated FA and cholesterol accumulation also contribute to the altered cellular redox state and subsequent sequential changes in lipid

metabolism(120, 121). Excessive toxic lipid peroxidation/oxidative stress could then acts as a trigger in steatosis-NASH transition.

Studies have reported connections between the UPR and oxidative stress. Among them, NRF2 plays a central role orchestrating the antioxidant response. NRF2 is highly expressed in the liver. In response to an increased protein-folding load in the ER, PERK can phosphorylate and stabilize NRF2 to compensate for high ROS levels, which are important mediators of inflammation (34, 38, 122). NRF2 therefore plays a cytoprotective role in response to ER stress-dependent inflammation in animal models with NASH. *NRF2*-deficient mice fed an MCDD presented aggravated NASH features when compared with control mice due to significantly increased oxidative stress and iron accumulation. Conversely, sustained NRF2 activation protects mice against NASH progression by inhibiting oxidative stress and the release of inflammatory cytokines and fibrosis stimulation factors (123). Pharmacological activators of NRF2 signaling significantly reduced fibrosis in rats with diet-induced NASH, demonstrating a potential strategy to treat NASH patients with hepatic fibrosis (124). NRF2 can also repress the transcription of genes encoding pro-oxidant machinery, such as TXNIP(125), a protein known to link oxidative stress to inflammasome activation in a ROS-dependent manner(126). IRE1α and PERK branch activation can increase TXNIP, ROS production, inflammasome activation and β-cell death(127, 128). Overwhelmed IRE1a RNase activity in BI-1 deficient mice with NASH is associated with enhanced hepatic TXNIP expression(27). Collectively, ER stress activates oxidative stress markers such as TXNIP that strengths inflammation and cell death. ER stress tightly cooperates with oxidative stress in NASH. Further studies are needed to address the role of this crosstalk.

ER stress and acute-phase proteins in NASH **-** Acute-phase proteins secreted by hepatocytes are indicators of hepatic inflammation and can be associated with ER stress. CREBH is a UPR-mediated transcription factor expressed only in the liver and implicated in the acute-phase response. Upon ER stress in hepatocytes, full-length and latent CREBH on the ER membrane undergoes regulated

intramembrane proteolysis that transactivates genes of the hepatic acute-phase response including C-reactive protein (CRP) and serum amyloid P component (SAP)(129). Compared to patients with steatosis, patients with NASH displayed significantly elevated serum CRP levels (130). However, while plasma CRP levels were elevated in another cohort of severely obese patients, they were not predictive of the diagnosis of NASH (131). An additional acute-phase protein induced by CREBH is hepcidin, a peptide hormone that regulates iron homeostasis and is secreted from the liver in response to ER stress and inflammation(132). Iron overload was identified in patients with NASH with an increased risk of worsened histological severity and advanced fibrosis (133) and found to modulate ER stress-associated pathways in a murine model of iron- and HFD-induced liver injury(134).

ER stress-dependent NLRP3 inflammasome activation in NASH - Much evidence has linked ER stress to the NLRP3 inflammasome, found to be particularly active in livers of mice and humans with NASH(135-137). Mice expressing a constitutively active form of global-specific versus myeloid-cell-derived NLRP3 developed more extensive liver inflammation, hepatocyte pyroptosis, stellate cell activation and liver fibrosis (138). A stronger NASH phenotype would need to be directly confirmed in hepatocyte-specific NLRP3 active mice. Livers from *caspase-1*-deficient mice were protected from ER stress-induced inflammasome activation (139). More specifically, endotoxemia provoked in genetically obese mice overwhelmed hepatic IRE1α and PERK activities. This led to the overexpression of CHOP found to regulate caspase-1, -11 and PUMA expression triggering hepatocyte pyroptosis and apoptosis(136). Inflammation and cell death (pyroptosis, apoptosis) in NASH may thus drive a feed forward cycle in liver disease progression.

ER stress-induced cell death in NASH

Apoptosis induced by ER stress - The notion that enhanced ER stress-induced apoptosis within liver cells may be relevant in the progression from steatosis to NASH in humans was supported by the

display of elevated ER stress markers, namely *CHOP* and *GRP78,* in liver biopsies from NASH patients (84). The deleterious *CHOP/GRP78* ratio was positively correlated with both NAFLD activity score and liver injury in humans(136). This study suggested that both ER-apoptosis and pyroptosis contributed to NASH transition in humans. Since, a central role for IRE1α RNase activity has been reported in NASH patient liver biopsies and NASH animal models(27).

A previous report on NASH patient liver biopsies found inconsistencies in the downstream pathways of PERK, displaying activated eIF2α but unchanged ATF4-GADD34-CHOP signaling, and IRE1α, presenting activated JNK but unmodified XBP1(55). Supporting a pro-apoptotic role for JNK in NASH pathogenesis, *JNK1*-deficient mice fed an MCDD were protected from steatohepatitis development due to the cutback in apoptosis (140).

Lipotoxicity and cell death - The dangerous build-up of lipids in steatotic livers increases hepatic exposure to lipotoxicity that can aggravate the symptoms leading to NASH. Lipidomic analyses revealed significantly elevated serum FFA levels in NASH patients(141). Data indicate that FFAs may contribute more to lipotoxicity-induced liver damage than their esterified product (triglycerides). The inhibition of triglyceride synthesis at DGAT2 in MCDD-fed mice led to a FFA build-up accompanied by higher levels of cytochrome P4502E1 (CYP2E1), markers of lipid peroxidation/oxidant stress, lobular necroinflammation and fibrosis than in MCDD-fed mice with uninhibited DGAT2(142). Saturated FFA, such as palmitic acid or stearic acid, as opposed to unsaturated FFA, such as oleic acid, can become toxic to hepatocytes, activating ER stress-induced apoptosis present in NASH pathogenesis(143). Rats fed a HFD rich in saturated fats showed increased expression of CHOP and active caspase-3(144). In hepatocyte cell lines, palmitic acid activated PERK and IRE1α, increasing CHOP expression (145), which induces expression of DR5 leading to caspase-8 generation of truncated BID (tBID)(146). Importantly, deletion of BAX and BAK in the liver fully blocks apoptosis induction under ER stress, correlating with a modification of UPR signaling (23). Palmitic acid-induced

activation of the IRE1α pathway through sustained JNK and/or CHOP activities promotes the expression of BH3-only proteins, PUMA and BIM, that directly activate BAX(147) and hepatocyte death. Thus, lipotoxicity triggers PERK- and IRE1α-mediated cell death via the mitochondrial pathway of apoptosis (148, 149). However, in models of liver damage induced by CCL4, deletion of CHOP did not protect animals against tissue damage despite observing clear signs of ER stress(150).

Ca^2 and cell death **-** The inability of cells to restore normal ER proteostasis due to aberrant Ca^{2+} signaling can lead to Ca^{2+} efflux from the ER to the mitochondria through MAMs, triggering ER stress-induced apoptosis that contributes to the development of NAFLD (92, 151). A myriad of downstream effectors of Ca^{2+} release from the ER include the opening of the permeability transition pore leading to mitochondrial swelling and outer membrane permeabilization, and the activation of cathepsins, proteases implicated in pathological hepatocyte death that can be seen in humans with NASH(152).

Finally, ER stress is an important trigger of apoptotic, necrotic and other forms of hepatocyte death and hence a potential accelerator of inflammation leading to NASH, fibrosis and HCC.

The role of chronic ER stress in HCC

The high prevalence of NAFLD worldwide may contribute to the rising incidence of HCC. HCC occurs within an established background of chronic liver disease and cirrhosis, with risk factors including obesity and diabetes leading to NASH, excessive alcoholic intake or viral hepatitis(153). In the United States, the increasing incidence of HCC coupled with an overall 5-year survival rate of less than 12% makes HCC the fastest rising cause of cancer-related mortality (154).

The role of ER stress signaling in the development of HCC remains to be defined. The tumor microenvironment in HCC may activate ER stress via oxygen and nutrient deprivation and acidic waste accumulation. While chronic UPR initiates steatosis development and activates hepatocyte death in steatohepatitis, the HCC microenvironment adapts to alter the integrated signaling of the UPR and

prevent ER stress-induced apoptosis, thereby safe-guarding the cancerous cells and increasing their aggressiveness and resistance towards chemotherapeutic agents.

The roles of ER stress proximal sensors IRE1a, PERK and ATF6 in HCC - The monitoring of UPR kinetics in HCC revealed the IRE1α pathway to be the first activated during tumor initiation, the PERK pathway during tumor progression and the ATF6α pathway to be modestly activated in developed tumors(155).

IRE1α exhibits one somatic mutation in human HCC tumors (156, 157), which could account for a modulation of its kinase and/or RNase function. HCC biopsies from human patients revealed elevated XBP1s expression levels (158, 159). A specific target gene of XBP1 in the liver is the alpha-fetoprotein (AFP)(17), the most widely used biomarker for HCC surveillance(154). Cancerous cells deficient for *XBP1* are less prone to developing solid tumors in nude mice(160). Similarly, *IRE1α* or *XBP1* deficiency compromises the mircoenvironment support for cancerous cell growth (161). Among the 37 RIDD substrates of IRE1α identified in metazoans, 68% are associated with cancer(162). The RIDD function targets cancer-relevant mRNAs like SPARC and microRNAs such as miR-17 in the liver. SPARC inhibits tumorigenic capacity of HCC cells and improves survival of HepG2-injected nude mice (163).

PERK signaling contributes to adaptive rather than terminal pathways, promoting tumor growth and angiogenesis. Under hypoxic stress, PERK regulates proangiogenic genes involved in cell-cell adhesion, matrix remodeling and extracellular matrix proteolysis (164).

The role of ATF6α in cancer still remains unclear. Data suggested a role for ATF6α in hepatocarcinogenesis (165) without clear mechanistic information. However, ATF6α is essential for the adaptation of dormant cells to their environment including chemotherapy, nutrient starvation and in vivo microenvironmental challenges (166).

The roles of UPR targets GRP78 and CHOP in HCC- GRP78 is constitutively overexpressed in HCCs(158). Both ATF6α- and IRE1α-XBP1-dependent UPR systems are essential for the

transformation-associated expression of the GRP78 gene in HCC(158). Cleaved GRP78 have been reported in the sera of HCC patients (167). Elevated GRP78 level correlates with higher pathologic grade, recurrence, and poor patient survival(168). Autoantibodies against GRP78 were identified as promising biomarkers for HCC (169).

Due to the pro-apoptotic function of CHOP under ER stress, a strategy aimed at inducing CHOP to kill cancerous cells would be expected; however, CHOP may contribute to HCC pathogenesis. *CHOP*-deficient mice exhibit both reduced apoptosis and cellular proliferation and are resistant to hepatocarcinogenesis(170). Nevertheless, we can question the cell autonomy of CHOP's oncogenic effects in the liver. The proinflammatory genes differentially expressed in *CHOP*-deficient mice were suggested to arise from a hepatocyte-independent function. In that respect, *CHOP*-deficient mice with carcinogen-induced HCC presented reduced inflammation (171). Despite robust expression of CHOP in HCC tumors, no evidence of cell death was observed, advocating a potential switch of CHOP function from pro-apoptotic to pro-tumorigenic (171). Finally, the ATF6α branch was specifically tumor-activated in DEN-induced HCC and described as a putative inducer of CHOP (171). In liver biopsies from human patients, CHOP protein levels increased parallel to the evolution of the stages from steatosis, NASH, liver cirrhosis to HCC (172).

Lastly, ER stress and HFD feeding were found to synergistically induce HCC through an inflammatory mechanism dependent on the TNF-TNFR1-IKKβ-NF-kB pathway, and independent of CHOP, perpetuating NASH pathogenesis to HCC (173). This study demonstrated that ER stress and steatosis trigger oxidative stress leading to genomic instability, oncogenic mutations and/or gene-copy-number variations.

Current and potential therapies for the treatment of NAFL/NASH

In the early stages, NAFLD can be successfully managed with lifestyle changes. Only an improved diet and/or increased physical activity can slow the progression of NAFLD(174).

Nevertheless, due to the long-term challenge, many patients are unable maintain their weight loss. A more drastic option includes bariatric surgery for the morbidly obese, but a recent retrospective study on a 10-year follow-up showed that 41% of patients having underwent Roux-en-Y gastric bypass had weight regain(175). As no established treatment yet exists for NAFLD, it is urgent to develop effective therapies.

The development of animal models of NAFLD that meet the pathophysiological criteria of the disease and its evolution is necessary to test promising compounds before initiating clinical trials. Experimental models of NAFLD reported in the literature mainly cover dietary, genetic and/or chemical interventions in mice **(Table 1)**. An international consensus on the ideal murine model is needed to improve the translatability into human NAFLD pathogenesis and treatment.

Over the past three years, 74% of published articles studying NASH pathogenesis have used dietary models (176). MCDD presents the most common used diet to produce steatohepatitis with liver fibrosis. While this diet is adequate for intrahepatic study, it may be inadequate for multisystemic study as mice fed an MCDD lose considerable weight and do not develop systemic insulin resistance. Other diets have sought to mimic the development of NAFLD by diet-induced obesity, which is the major risk factor in humans. Obesogenic diets are characterized by high-fat and/or high-sucrose and/or high-cholesterol, which may often exceed the dietary amounts found in western culture diets. Genetically and diet-induced obesity as well as MCDD have been extensively used to study ER stress signaling in NAFLD pathogenesis (**Table 1**). C57BL/6J mice fed with MCD showed robust activation of eIF2α and Chop, therefore a slight preferential indication of PERK arm(177). Reversibly, MCD-diet-induced steatohepatitis was reduced in Chop knockout mice. In a similar context, we evinced that the *ob/ob* mice challenged with LPS mimicked the extent of steatohepatitis recapitulating the human NASH progression with concurrent activation of IRE1α and PERK resulting in synergic CHOP activation(136). In addition to this, C57BL/6J WT mice fed with high fat high sucrose diet significantly perturbed the ER FC/PL ratio (Free cholesterol to phospholipid) which precisely correlated with

upregulated CHOP and XBP1s levels (178). Similarly, a study confirmed the upregulation of Chop with atherogenic diet-induced steatohepatitis mice model(179). Furthermore, C57BL/6J WT mice fed with high cholesterol-containing fast food diet (24 weeks) resulted in disrupted ER and significantly elevated p-PERK levels(180).

Genetic models are frequently used in combination with modified diets to induce a robust steatosis to NASH transition. For example, *foz/foz* mice are an overnutrition model featuring obesity, diabetes and hypercholesterolemia. When challenged with an atherogenic diet *foz/foz* mice developed NASH with fibrosis, which enabled the testing of an anti-inflammatory approach to NASH treatment (181). It was reported that ER stress does not contribute to NASH in HFD-foz/foz mice (182). The diet-induced animal model of NAFLD (DIAMOND) was recently developed to recapitulate human NAFLD pathophysiology by crossing two mouse strains (C57BL6/J and 129S1/SvImJ) and feeding the mice a high-fat and high-carbohydrate diet with a high fructose-glucose solution (183). Moreover, DIAMOND fed with HFD developed fastened NASH, mild fibrosis (16-22 weeks), bridging fibrosis (around 52 weeks) and imminently HCC (32-52 weeks). Interestingly, they showed similar transcriptional, metabolic, histological, physiological and cell-signaling patterns that of the human progressive NASH(184). The pathological progression of steatosis to NASH with fibrosis and eventually HCC in STAM mice is very similar to the human disease (185). The study of the lipidomic status of STAM mice recapitulated that of human NASH (8 weeks) and fibrosis (12 weeks) (186). They found elevated sphingolipids including ceramides in STAM mice which at NASH stage might trigger inflammation, apoptosis and insulin resistance eventually culminating into the fibrosis (186). Studies are needed to study the contribution of ER stress in DIAMOND and STAM mice. In any case, all animal models should be limited to clearly defined and liver-specific research goals for the development of therapeutics.

Potential promising strategies with drugs reducing ER stress levels, pharmacological inhibitors of the UPR or genetic probes are listed in **Table 2**. Some of these molecules have been tested in mice with NAFLD (summarized below).

Broad-spectrum ER stress-reducing drugs - Compounds have been identified to alleviate ER stress by increasing protein folding capacity. These include the 4-phenyl butyric acid (PBA), a short-chain fatty acid also described as a potent histone deacetylase inhibitor, and the taurine-conjugated ursodeoxycholic acid (TUDCA), a derivative of an endogenous bile acid that has been safely used as a hepatoprotective agent in humans(187). Due to their poor selectivity, these chaperones usually require high concentrations to take effect, making them largely neglected as therapeutic agents. Treatment with either 4-PBA (1g/kg/day; orally) or TUDCA (500mg/kg/day; i.p.) for up to 30 days resolved hepatic steatosis in *ob/ob* mice(76). 4-PBA (100-200mg/kg) and TUDCA (50-100mg/kg) administered in rats with both normal and steatotic livers before partial hepatectomy and ischemia-reperfusion protected against liver injury and regeneration failure by reducing inflammation, apoptosis and necrosis (188). TUDCA (500mg/kg/day; i.p.) administered as a protective 5-day pretreatment or as a potential 6h treatment protected against NASH by limiting ER stress-dependent NLRP3 inflammasome activation in *ob/ob* mice with endotoxemia induced by a single LPS injection (2mg/kg; i.p.)(136). In HFD-fed *MUP-uPA* mice, daily TUDCA (250mg/kg; i.p.) injections over four weeks limited hepatic steatosis and liver injury (173). TUDCA and PBA have been approved by the Food and Drug Administration for the treatment of primary biliary cirrhosis and urea-cycle disorders respectively. TUDCA is currently in a phase-2 clinical trial for type 1 diabetes. While the direct mechanisms of action of 4-PBA and TUDCA have yet to be clearly defined, they have proven their potency in reducing ER stress in metabolic disorders and may represent a therapeutic option for the treatment of NAFLD.

Pharmacological inhibitors of the UPR - Screening studies have identified multiple compounds targeted against distinct UPR signaling members (reviewed in(189)). However, the efficacy and safety of most of these compounds remain to be investigated in murine models of NAFLD to confirm their therapeutic potential.

The dual functions of IRE1α, which structurally possesses a catalytic core on its RNase domain and an ATP-binding pocket on its kinase domain, have the potential to be manipulated therapeutically. Small molecules inhibit IRE1α RNase activity, specifically blocking RIDD and XBP1 splicing, without affecting its kinase activity or ability to dimerize/oligomerize. Such compounds include salicylaldehyde-based RNase inhibitors exemplified by 4μ8c (27, 190), STF-083010 (27, 127, 190, 191) and MKC-6688(192). Treatment of hepatic mouse macrophages with STF-083010 (30μM) resulted in specific inhibition of tunicamycin (10 μg/ml)-induced XBP1 splicing and TNFα transcription (191). This study emphasized the importance of targeting ER stress-induced IRE1α RNase activation in the production of inflammatory cytokines, which could be applied to NASH. Pretreatment of both mouse and human macrophages with either STF-083010 (150μM) or 4μ8c (100μM) prevented the synergistic effects of palmitate (1mM)-induced ER stress coupled with LPS (200 ng/ml)-induced inflammasome activation (190). *In vivo*, administration of either IRE1α RNase inhibitors STF-083010 or 4μ8c (10 mg/kg/day; i.p.; 4-6 weeks) in *foz/foz* mice fed a western-type diet counteracted atherosclerotic plaque formation and metaflammation without inducing hepatic toxicity (190). Similarly, administration of STF-083010 or 4μ8c (30 mg/kg/biweekly; i.p.) in the last two weeks of a 3-month HFD rescued *BI-1*-deficient mice, vulnerable to IRE1α RNase hyperactivation, from NASH development(27).

It was further shown that IRE1α RNase function could be modulated through its kinase function by ATP-competitive ligands, forming a new pharmacological class of inhibitors called Kinase-Inhibiting RNase Attenuators (KIRAs)(193) with therapeutic potential in diabetes(194, 195). ASK1 is an intermediary of the IRE1α-JNK1 signaling pathway that is associated with hepatic inflammation, apoptosis and fibrosis, but can be targeted by the selective inhibitor selonsertib. In a multicenter phase-2 trial, 72 patients with NASH and liver fibrosis were randomized to receive 24 weeks of treatment with either selonsertib (GS-4997; 6 or 18mg/day; orally), with or without simtuzumab (125mg/week; subcutaneous injection) a monoclonal antibody directed a collagen and elastin cross-linking enzyme, or

simtuzumab alone(196). NASH patients treated with a combination of selonsertib and simtuzumab exhibited improved collagen content and lobular inflammation as well as reduced apoptosis and necrosis serum biomarkers (196).

Compounds have also sought to modulate the PERK pathway primarily through eIF2α. Salubrinal prevents eIF2α dephosphorylation to maintain protein translation inhibition, resulting in a reduced protein load on the ER. In HepG2 cells incubated with tunicamcyin (1.25 μg/ml) for 48h, co-treatment with salubrinal (50 μM) significantly improved hepatocyte viability(155). Similarly, Guanabenz promotes persistent eIF2α phosphorylation in ER stress conditions by selectively binding to GADD34(197). However, excessive inhibition of global translation may have undesirable effects in the long-term. On the contrary, integrated stress response inhibitor (ISRIB) was identified to inhibit the effects of eIF2α phosphorylation (198).

Compounds that directly modulate ATF6α expression or activity are scarce. Recently, high-throughput screening has identified small molecule inhibitors of ATF6α called Ceapins that trap the full-length protein in ER-resident foci, preventing ATF6α from ER stress-induced trafficking to the Golgi and subsequent proteolytic activation(199). The testing of these compounds inhibiting IRE1α, PERK and/or ATF6 is still limited and has yet to be explored in the context of NAFL/NASH.

Gene therapy - The UPR has central roles in the maintenance of the physiology of various organs and specialized secretory cells (200). This is why serious side effects are predicted for the long-term administration of UPR targeting drugs (189). Gene therapy is emerging as an alternative approach to target the UPR on a tissue specific manner (201). Promising results have featured gene therapy as a novel approach to traditional therapies in order to combat chronic liver disease. Hepatic overexpression of GRP78 improves the UPR dynamics, reducing steatosis associated with obesity (60, 173). Recently, the hepatoprotective effects of GRP78 in obese mice were proposed to act through a negative feedback loop that suppresses UPR activity (by suppressing ATF6α) but stimulates the degradation of its own

mRNA (by perpetuating IRE1α's RIDD function) (202). Interestingly, chronic ER stress may be marked by cycles of activation and deactivation that suppress UPR-dependent transcription but possibly maintain RIDD. The inhibition of IRE1α by adenoviral gene transfer of BI-1 in genetically and diet-induced obese mice significantly improved hepatic glucose metabolism and insulin sensitivity (25). BI-1 could prevent ER stress-associated CYP2E1 activation which leads to oxidative stress(203). BI-1 could also inhibit hepatic lipid accumulation through the regulation of ApoB secretion(204), although this remains controversial. In livers of *ob/ob* mice, ectopic overexpression of XBP1s improved glucose metabolism and insulin sensitivity by binding FoxO1 (205). While XBP1s overexpression may protect against hepatic insulin resistance, it cannot be excluded that it may increase hepatic steatosis due to its function in activating lipogenic machinery.

Current and potential therapies for the treatment of HCC

The oral multitargeted tyrosine kinase inhibitor sorafenib is the only Food and Drug Administration-approved medication for patients with advanced HCC. Due to genetic heterogeneity, some HCC cells are initially resistant to sorafenib, increasing the interest in a combination therapy strategy with the small molecules mentioned previously. Of course, the intention of such treatment in this case would be to override the adaptive response of cancerous cells to lead to their eradication. In that sense, the use of chemical chaperones (TUDCA, 4-PBA) in HCC may be questionable as the targeted increase in the adaptive versus terminal UPR may actually promote cancer cell survival.

Specific targeting of the UPR branches is still under investigation. The IRE1α-ASK1-JNK pathway is at the crossroads of ER stress and cell death. ASK1-deficient mice had increased susceptibility to DEN-induced HCC, and the reintroduction of ASK1 by adenoviral vector restored the JNK-BIM pathways of hepatocyte death (206). ASK1 overexpression was further confirmed to eliminate the tumorigenicity of subcutaneous HCC xenografts in nude mice(207). Concerning a potential strategy aimed at PERK inhibition in the the liver, 25 weeks following DEN injection, mice

treated with GSK2656157 (100 mg/kg/bidaily; i.p.) for 4 weeks presented greater ER-stress-driven cell death and HCC regression via proteotoxicity(155). However, the selectivity profile of GSK2656157 has been questioned due to the discovered potency of GSK2656157 as RIPK1 inhibitors. No studies have been reported targeting the ATF6 branch in HCC, probably due to the lack of availability of specific inhibitors working in vivo.

Another promising therapeutic target involves the ER protein PDI. Aggressive pathological features of HCC and poor clinical outcome are associated with upregulated PDI expression, as seen in HCC tissues from patients (208, 209). A combinatorial daily treatment of sorafenib (30mg/kg; i.p.) and a PDI inhibitor (PACMA 31; 20mg/kg; i.p.) in a Hep3B xenograft model reduced HCC tumor volume by improving the efficiency of sorafenib in inducing proteotoxic stress, leading to apoptosis through JNK and CHOP induction (209). Another PDI inhibitor, bacitracin (10, 50 or 100mg/kg/day; i.m.; 12 days, enhanced HK II inhibitor-induced anti-tumor efficacy synergistically by activating JNK, leading to apoptosis and limiting angiogenesis in mouse models of HCC (210).

Pharmacological agents may conditionally enhance oncolytic viral efficacy in destroying malignancies without harming normal tissues. Small-molecule valosin-containing protein inhibitors cooperate with M1 virus by suppressing the IRE1α-XBP1 pathway and promoting irresolvable ER stress to kill HCC cells(211). This study also presented the favorable outcomes of this combinatorial therapeutic strategy in nonhuman primates, encouraging the rapid clinical translation of such therapies.

Certain natural compounds may modulate the ER stress-related pro-apoptotic pathway in HCC. Melatonin and resveratrol have been shown to act as pro-apoptotic agents by initiating a terminal ER stress response in HCC cells through CHOP (212, 213). Further study of ER stress signaling will hopefully allow for the development of innovative therapeutic strategies.

Conclusion & perspectives

From an experimental point of view, the implication of ER stress in NAFLD has become a subject of considerable interest. This review is the first to incorporate such literature from NAFL to NASH and HCC and highlight the therapeutic potential of recent compounds and methods that target ER stress-related pathways in these diseases. While the role of the ER in hepatocytes has been extensively studied, its function in the non-parenchymal fraction of the liver has not been explored. It would thus be relevant to study ER stress in Kupffer cells that may influence liver inflammation seen in NASH. The UPR in surrounding cells of the HCC microenvironment (immune cells, stellate cells) may also participate in tumor survival and aggressiveness. By restoring normal proteostasis, we may protect the liver from pathogenic stresses involved in NAFLD. Certain therapeutic options could transcriptionally reprogram the ER stress network to continue to protect organisms from exposure to improperly folded secretory proteins instead of causing an excessive ER stress-induced inflammation and cell death loop. However, given the delicate balance between physiological and pathological responses, targeting the ER for therapeutic purposes still remains challenging. Will the pharmacological prevention of terminal ER stress protect from NASH but promote HCC development? This must be tested in animal models that regroup both the metabolic context and intrahepatic features of NAFLD and its evolution. Furthermore, as the UPR may involve unsynchronized responses to ER stress from the three branches in terms of initial activation and duration, the temporal aspects of UPR activation must be considered through more targeted manipulation of each UPR pathway. UPR activation may be synchroniszed by the circadian clock under physiological conditions. Chronic liver disease may disrupt the hepatic rhythmicity of ER stress and predispose to disease progression. Aging represents another obstacle to the proteostasis network due to the degenerating function of protein quality control mechanisms combined with increasing damage to proteins.

In response to obesity, it still remains to be clarified whether ER stress is the cause or a result of dysregulated lipid signaling, as well as activated cell death and inflammatory pathways present in

patients with progressive NAFLD. What "hits" drive the transition of the UPR from adaptive to terminal to resistant as the stages from NAFL to HCC progress? In any case, the globally high prevalence of NAFLD may soon become the leading cause of end-stage liver disease, HCC and indication for liver transplantation. We can always question how the triggering of ER stress should be interpreted, but continuing to study ER stress and the UPR should enable us to take on novel therapeutic avenues to combat ER stress-related disorders, including NAFLD, diabetes, neurodegeneration and cancer.

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Figure legends

See text for definitions of abbreviations.

Figure 1: The UPR signaling cascade.

Multiple endogenous and exogenous stimuli can create ER stress leading to the accumulation of unfolded proteins and subsequent activation of the UPR. GRP78 dissociates from the three ER transmembrane sensors PERK, IRE1α and ATF6 that activate their respective signaling cascades to first re-establish proteostasis and promote cell survival through the transcription of UPR target genes of the mechanisms listed at the bottom. Oligomerized PERK phosphorylates eif2α that inhibits translation to reduce the protein load on the ER, but concomitantly increases the expression of the transcription factor ATF4. In a negative feedback mechanism, ATF4 induces CHOP that induces GADD34, a regulatory subunit of PP1C, thus inhibiting eif2α phosphorylation and reinitiating translation. PERK phosphorylation of NRF2 is involved in the regulation of redox homeostasis. Oligomerized IRE1α possesses both RNase and kinase activities. HSP47, a selective regulator of IRE1α, displaces GRP78 from IRE1α to facilitate its oligomerization. Activation of the RNase function of IRE1α leads to RIDD-mediated degradation of mRNAs and microRNAs, such as miR17 leading to uninhibited TXNIP

expression and NLRP3 inflammasome activation, and the splicing of XBP1 resulting in the activation of the potent transcription factor XBP1s. Activation of IRE1α can also lead to the recruitment of stress kinases involved in NFkB-mediated inflammation or JNK-mediated apoptosis and insulin resistance. ATF6 (p90) is transported from the ER to the Golgi to be cleaved by the proteases S1P and S2P, thereby releasing the cytosolic transcription factor ATF6 (p50). Similarly, SREBP1 is also proteolytically cleaved to activate the transcription of genes involved in lipid synthesis and uptake.

Figure 2: Irremediable ER stress promotes hepatocyte apoptosis.

Terminal ER stress andCa^{2+} dysregulation leads to the mitochondrial release of apoptogenic factors, such as cytochrome c, that activate the caspase cascade of apoptosis. Regulating Ca^{2+} balance in the ER, SERCA actively pumps Ca^{2+} into the ER opposite IP3-gated channels (IP3R) that passively release Ca^{2+} into the cytosol. Massive efflux of Ca^{2+} by IP3R that overcomes the mitochondria results in the opening of the mitochondrial PTP leading to ionic imbalances, mitochondrial transemembrane potential disruption, matrix swelling and permeabilization of the mitochondrial outer membrane (MOMP), resulting in a liberation of reactive oxygen species and cytochrome c. While anti-apoptotic proteins BCL2 and BI-1 aim to inhibit abnormal Ca^{2+} release, pro-apoptotic BAX/BAK promote Ca^{2+} release at the ER and mitochondria. When hyperactivated, PERK- and IRE1α-mediated signaling pathways detailed in Fig. 1 promote proteotoxicity, inflammatory responses with associated pyropotosis and apoptosis. Finally, terminal ER stress signaling converges onto the pro-apoptotic transcription factor CHOP that upregulates pro-apoptotic BH3-only proteins (e.g. PUMA, BIM, BID) while suppressing anti-apoptotic BCL2/BCLXL expression.BH3-only proteins directly activate BAX and BAK to form a pore, release cytochrome c and induce apoptosis.

Figure 3: ER stress drives NAFLD progression.

The three main actors of the UPR play a key role in chronic liver disease progression. Chronic UPR can drive steatosis through its involvement in hepatic insulin resistance and fat accumulation. The transition from a chronic to a terminal UPR may be responsible for steatohepatitis development through its involvement in cell death, inflammation and oxidative stress. With HCC development, a resistant UPR promotes cancerous cell survival and proliferation and stimulates angiogenesis.

Impaired post-translational processing

Genetic mutations

Oxidative stress

Ca^{2+} imbalance

ER STRESS

Free fatty acids

Hyperglycemia

High secretory demands

Environmental pathogens

ER lumen

GRP78

Unfolded protein

HSP47

PERK

IRE1α

ATF6α
p90

SREBP
p125

P

RNase

Kinase

Translation

eIF2α

eIF2α

RIDD

XBP1u

JNK

Golgi

ATF6α
p50

S1P
S2P

Splicing

miR17

Apoptosis
Insulin resistance

NRF2

GADD34
PP1C

TXNIP

NFκB

SREBP
p60

Antioxidant
response

Inflammasome

Inflammation

Lipid synthesis
and uptake

CHOP

ATF4

XBP1s

ATF6

UPR target genes

UPR target genes

UPR target genes

Chaperones	Foldases	ERAD machinery
Redox homeostasis	Amino acid metabolism	Lipid metabolism
Autophagy	Inflammatory response	Apoptosis

FIGURE 1

ER lumen

ER STRESS

Ca++

Ca++

IP3

IP3R

BI-1

SERCA

ATP

BCL2

BAX/BAK

BAX/BAK

IRE1α

PERK

TRAF2

RIDD

miRNA

TXNIP

ASK1

eIF2α

eIF2α

Translation

GADD34

PP1C

NLRP3 inflammasome

ROS

Caspase 1

Pyroptosis

Proteotoxicity

Ca++

Ca++

PTP

MOMP

Mitochondria

BAX/BAK

Cytochrome c

Caspases 3/7

Apoptosis substrates

Apoptosis

JNK

BCL2/BCLXL

BH3-only proteins

tBID

BID

Caspase 8

DR5

Nucleus

ATF4

CHOP

XBP1s

ATF6

FIGURE 2

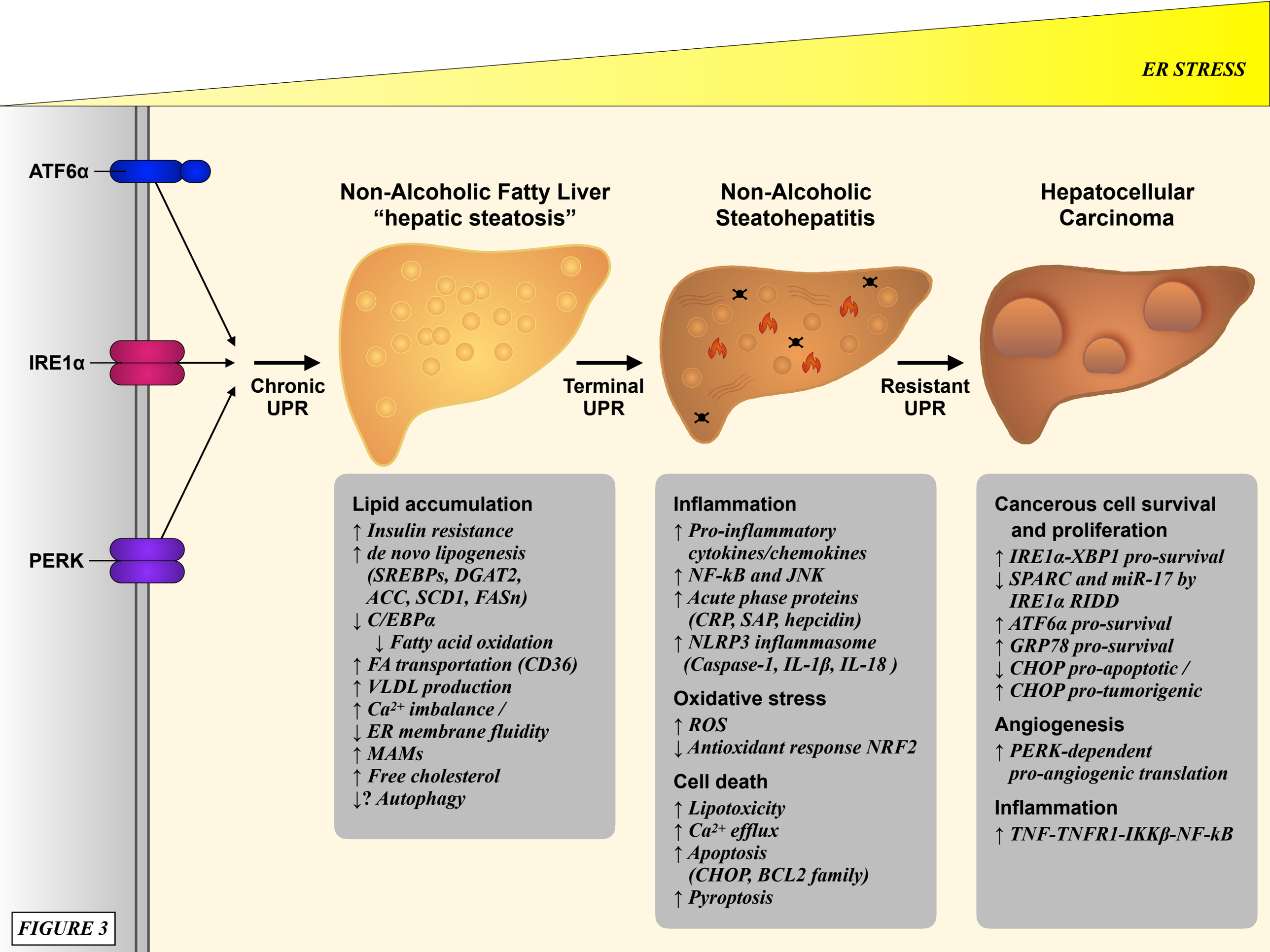
ER STRESS
ATF6α
IRE1α
PERK
Chronic UPR
Non-Alcoholic Fatty Liver "hepatic steatosis"
Terminal UPR
Non-Alcoholic Steatohepatitis
Resistant UPR
Hepatocellular Carcinoma
Lipid accumulation
↑ Insulin resistance
↑ de novo lipogenesis (SREBPs, DGAT2, ACC, SCD1, FASn)
↓ C/EBPα
↓ Fatty acid oxidation
↑ FA transportation (CD36)
↑ VLDL production
↑ Ca2+ imbalance /
↓ ER membrane fluidity
↑ MAMs
↑ Free cholesterol
↓? Autophagy
Inflammation
↑ Pro-inflammatory cytokines/chemokines
↑ NF-kB and JNK
↑ Acute phase proteins (CRP, SAP, hepcidin)
↑ NLRP3 inflammasome (Caspase-1, IL-1β, IL-18)
Oxidative stress
↑ ROS
↓ Antioxidant response NRF2
Cell death
↑ Lipotoxicity
↑ Ca2+ efflux
↑ Apoptosis (CHOP, BCL2 family)
↑ Pyroptosis
Cancerous cell survival and proliferation
↑ IRE1α-XBP1 pro-survival
↓ SPARC and miR-17 by IRE1α RIDD
↑ ATF6α pro-survival
↑ GRP78 pro-survival
↓ CHOP pro-apoptotic /
↑ CHOP pro-tumorigenic
Angiogenesis
↑ PERK-dependent pro-angiogenic translation
Inflammation
↑ TNF-TNFR1-IKKβ-NF-kB

FIGURE 3

Table 1. Overview of mouse models of NAFLD with ER stress markers

Intervention	Model	Characteristics	Obesity	Steatosis	NASH	Fibrosis	HCC	Increased ER stress markers?
Dietary* (*primary models used to study NAFLD, but composition usually exceeds western culture diets in fat and cholesterol content)	High-fat	Usually 40-75% fat to bring about triglyceride accumulation in the liver.	Yes	Yes	Yes (mild)	Yes (mild)	No	Yes (IRE1α, PERK and ATF6 signaling pathways, GRP78)
	High-fat high-sugar	Usually 30-55% fructose and/or sucrose.	Yes	Yes	Yes (mild)	Yes (mild)	No	Yes (IRE1α-XBP1, PERK, CHOP, GRP78)
	High-cholesterol	Usually 1-2% cholesterol.	Yes	Yes	Yes	Yes	No	?
	High-cholesterol high-cholate	Atherogenic diet. 1.25% cholesterol and 0.5% cholate added to diet, promoting cholesterol and fat absorption and suppressing the conversion of cholesterol to bile acids, thus promoting atherosclerosis.	No	Yes	Yes	Yes	No	Yes (CHOP)
	High-fat high-sugar high-cholesterol	Fast-food diet.	Yes	Yes	Yes	Yes	Yes	Yes (p-PERK, p-ATF2, p-ELF2)
	Methionine- and choline-deficient	Deficiency of essential nutrients, resulting in impaired β-oxidation and production/secretion of very low-density lipoprotein (VLDL) particles. Usually contains high sucrose (40%) and moderate fat (10%).	No (significant weight loss)	Yes	Yes	Yes	Yes (if animals survive)	Yes (p-PERK, p-eIF2α, XBP1s, p-JNK, p-NF-kB, CHOP, GRP78)
	Choline-deficient L-amino acid-defined	Diet proteins are substituted with an equivalent and corresponding mixture of L-amino acids. Sometimes iron-supplemented.	No	Yes	Yes	Yes	Yes	Yes (CHOP)
Dietary and Lifestyle	ALIOS (American lifestyle-induced obesity syndrome)	High-fat (45%, rich in trans fat (30% of fat content)) high-fructose diet and promoted sedentary lifestyle (removal of cage racks).	Yes	Yes	Yes (without hepatocyte ballooning)	Yes	Yes	Yes (CHOP)
Genetic* (*dietary interventionis frequently required to induce NAFL to NASH transition)	*ob/ob*	Leptin-deficient (spontaneous mutation). Hyperphagic and inactive.	Yes	Yes	No (not spontaneous)	No (resistant)	No	Yes (IRE1α, PERK and ATF6 signaling pathways, GRP78)
	db/db	Leptin receptor-deficient (spontaneous mutation). Hyperleptinemic, hyperphagic and inactive.	Yes	Yes	No (not spontaneous)	No	No	Yes (IRE1α, PERK and ATF6 signaling pathways, GRP78)
	foz/foz	Alstrom syndrome 1 (Alms1)-deficient (more severe on C57BL6/J than BALB/c strain).	Yes	Yes	No (not spontaneous)	No	No	?
Dietary and Genetic	*ob/ob*+ MCD		Yes	Yes	Yes	No	No	?
	db/db+ MCD		Yes	Yes	Yes	Yes	?	Yes (IRE1α, PERK and ATF6 signaling

								pathways)
	foz/foz + Atherogenic diet	Mice fed a diet composed of 23% fat, 45% carbohydrate, 20% protein, 0.2% cholesterol.	Yes	Yes	Yes	Yes	?	?
	MUP-uPA + HFD	Urokinase-type plasminogen activator (uPA) transgenic mice under the control of the mature hepatocyte-specific promoter for major urinary protein (MUP). Mice fed a diet composed of 60% fat.	Yes	Yes	Yes	Yes	Yes	Yes (p-eIF2α, p-JNK, CHOP)
	DIAMOND	Inbred isogenic strain of C57BL6/J and 129S1/SvImJ mice fed a high-fat, high-carbohydrate diet with sugar water.	Yes	Yes	Yes	Yes	Yes	Yes (p-JNK, CHOP)
Chemical* (*not very physiological, but rapid disease evolution)	Tunicamycin	Single injection (1mg/kg).	No	Yes (transient)	No (unless transgenic model vulnerable to ER stress)	No (mild if transgenic model vulnerable to ER stress)	No	Yes (all markers significantly increased)
	CCL_4	Repeat dosing (0.2-0.5ml/kg) induces oxidative stress leading to toxic lipid and protein peroxidation product accumulation and a strong necrotic response.	No (weight loss)	Yes	Yes (without hepatocyte ballooning)	Yes	Yes	Yes (CHOP)
	DEN	Single DEN injection (1mg/kg). Has been associated with repeat dosing of CCL_4(0.2ml/kg).	No	?	?	Yes	Yes	?
Dietary and Chemical	DEN+HFD or DEN+MCD		HFD yes MCD no	Yes	Yes	Yes	Yes	Yes (CHOP)
	STAM	Neonatal streptozotocin (200µg) type 1 diabetes model + HFD feeding.	No	Yes	Yes	Yes	Yes	?

Table 2. Notable compounds that target ER stress-related pathways in NAFLD.

Family	Name	ER Targets	Effects	Therapeutic potential in liver Disease	Reference
Chemical chaperones	4-PBA	Unfolded proteins	Promotes protein folding capacity and ERAD efficiency. Stabilizes the adaptive UPR.	(NAFL/NASH) Urea-cycle disorders	(Özcan et al., 2006) (Ben Mosbah et al., 2010)
	TUDCA			(NAFL/NASH) Cholestatic liver disease	(Lebeaupin et al., 2015) (Nakagawa et al., 2014)
Chemical inhibitors	4μ8c	IRE1α RNase	Inhibits XBP1 mRNA splicing. Inhibits RIDD function.	(NASH) (HCC)	(Tufanli et al., 2017) (Lebeaupin et al., 2018)
	STF-083010				(Lerner et al., 2012) (Kim et al., 2015) (Tufanli et al., 2017) (Lebeaupin et al., 2018)
	MKC-3946				(Mimura et al., 2012)
	KIRA6/KIRA8	IRE1α kinase and RNase	Promotes cell survival under ER stress.	(Insulin resistance) (NAFL/NASH)	(Wang et al., 2012) (Ghosh et al., 2014) (Morita et al., 2017)
	APY29	IRE1α kinase	ATP-competitive inhibitor thatinhibits IRE1α kinase, but increases dimerization/oligomerization of IRE1α, enhancingRNase activity.	?	(Korennykh et al., 2009)
	Selonsertib	IRE1α-ASK1	Reduces collagen content and inflammation.	(NASH with liver fibrosis)	(Loomba et al., 2017)
	Salubrinal	PERK-eIF2α	Prevents eIF2α dephosphorylation, maintaining protein translation inhibition to limit the ER protein load.	(NAFL/NASH)	(Vandewynckel et al., 2015)
	Guanabenz				(Tsaytler et al., 2011)
	ISRIB	PERK-p-eIF2α	Inhibits p-eIF2α to resume global protein translation.	?	(Sidrauski et al., 2015)
	Ceapins	ATF6α	Prevents ATF6α cleavage and activation.	?	(Gallagher et al., 2016)
	GSK2656157	PERK	Inhibits PERK autophosphorylation. Reduces viability and proliferation of HCC cells, reduces HCC tumor growth *in vivo*	(HCC)	(Axten et al., 2013) (Vandewynckel et al., 2015)
	LBY135	Agonistic DR5 antibody	Sensitizes resistant HCC cells to apoptosis.	(HCC)	(Chen et al., 2010)
	PACMA 31	PDI inhibitor	Increases ER stress to reduce cell viability.	(HCC)	(Won et al., 2017)
	Bacitracin	PDI inhibitor	Increases ER stress to reduce cell viability.	(HCC)	(Yu et al., 2012)
Gene therapy	Adenovirus-GRP78	Overexpression of GRP78	Reduces hepatic steatosis and improved insulin sensitivity in obese mice.	(Insulin resistance) (NAFL/NASH)	(Kammoun et al., 2009) (Nakagawa et al., 2014) (Gomez and Rutkowski, 2016)
	Adenovirus-BI-1	Overexpression/reintroduction of BI-1	Improves glucose metabolism and insulin sensitivity. Reduces steatosis.	(Insulin resistance) (NAFL/NASH)	(Bailly-Maitre et al., 2010) (Lee et al., 2016)
	Adenovirus-XBP1s	Overexpression of XBP1s	Improves glucose metabolism and insulin sensitivity.	(Insulin resistance) (NAFL/NASH)	Zhou (Zhou et al., 2011)
	Adenovirus-ASK1	Overexpression/reintroduction of ASK1	Restores the JNK-BIM and TNFα pro-apoptotic pathways.	(HCC)	(Nakagawa et al., 2011)
Oncolyticviro-therapy	M1 virus with valosin-	IRE1α-XBP1	Suppresses adaptive and promotes terminal UPR	(HCC)	(Zhang et al., 2017)

	containing protein inhibitors		pathway.		
Natural compounds	Melatonin	Induction of CHOP	Increases ER stress-induced apoptosis.	(HCC)	(Moreira et al., 2015)
	Resveratrol	IRE1α	Reduces DNA-binding capacity of XBP1s to its pro-survival target genes.	(HCC)	(Rojas et al., 2014)

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