



Endoplasmic Reticulum Stress and Autophagy in the Pathogenesis of Non-alcoholic Fatty Liver Disease (NAFLD): Current Evidence and Perspectives

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Abstract

Purpose of Review Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease with rising prevalence worldwide. Herein, we provide a comprehensive overview of the current knowledge supporting the role of ER stress and autophagy processes in NAFLD pathogenesis and progression. We also highlight the interrelation between these two pathways and the impact of ER stress and autophagy modulators on NAFLD treatment.

Recent Findings The pathophysiological mechanisms involved in NAFLD progression are currently under investigation. The endoplasmic reticulum (ER) stress and the concomitant unfolded protein response (UPR) seem to contribute to its pathogenesis mainly due to high ER content in the liver which exerts significant metabolic functions and can be dysregulated. Furthermore, disruption of autophagy processes has also been identified in NAFLD. The crucial role of these two pathways in NAFLD is underlined by the fact that they have recently emerged as promising targets of therapeutic interventions.

Summary There is a greater need for finding the natural/chemical compounds and drugs which can modulate the ER stress pathway and autophagy for the treatment of NAFLD. Clarifying the inter-relation between these two pathways and their interaction with inflammatory and apoptotic mechanisms will allow the development of additional therapeutic options which can better target and reprogram the underlying pathophysiological pathways, aiming to attenuate NAFLD progression.

Keywords Non-alcoholic fatty liver disease · NAFLD · NASH · HCC · ER stress · Autophagy

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by accumulation of fat inside hepatocytes in the absence of excessive alcohol consumption [1]. NAFLD is strongly associated with metabolic syndrome features, such as obesity (particularly central), with increasing prevalence rates in both developed and developing countries which follow the corresponding rising prevalence of obesity and type 2 diabetes (T2DM) [1]. Currently, NAFLD constitutes the second most common cause for liver transplantation in the USA and is predicted to take the first place soon [1, 2]. Recently, a panel of international experts proposed that NAFLD should be renamed to metabolic-dysfunction-associated fatty liver disease (MAFLD) in order to better reflect the underlying pathophysiology [3]. NAFLD begins with simple steatosis, where triacylglycerol (TAG) accumulation is present in more than 5% of hepatocytes, while it evolves to fatty infiltration with inflammation, leading to the more severe form of NAFLD, namely nonalcoholic steatohepatitis (NASH) which can further progress into liver fibrosis and ultimately cirrhosis and hepatocellular carcinoma (HCC) [4].

The liver is a highly secretory organ and one of the major targets of insulin and glucagon [5•] and plays a fundamental role in the lipid and carbohydrate metabolism. Of note, the liver is characterized by the remarkable feature to proliferate upon damage [5•].

Hepatocytes have large amounts of endoplasmic reticulum (ER) that exert important liver metabolic functions such as protein and lipid synthesis, transmembrane protein folding and calcium homeostasis [6, 7]. Dysfunction of hepatic ER is implicated in the spectrum of the NAFLD pathology via activation of ER stress signaling [7, 8•].

Autophagy is another crucial process implicated in cellular homeostasis delivering cytoplasmic content to the lysosomes in order to degrade and/or recycle components to form macromolecules [9]. The potential role of autophagy in hepatic lipid metabolism has been recently recognized, while dysregulated autophagy has also been found to contribute to the pathogenesis of NAFLD [10•].

ER stress and autophagy co-operate to stimulate the degradation of intracellular lipid droplets (lipophagy) in liver. However, under increased and sustained ER stress, an aberrant inflammation, along with reduced autophagic process can lead to hepatocyte death [11, 12].

This review presents the current knowledge supporting the impact of ER stress signaling and autophagy and their interrelation on NAFLD, ranging from simple steatosis to NASH and HCC, while summarizing potential therapeutic interventions targeting these two processes.

Obesity and NAFLD

Obesity has been linked to the development of metabolic syndrome and its comorbidities, such as T2DM and NAFLD, and increases the risk of mortality in these individuals [13, 14]. Interestingly, obesity has emerged as an independent risk factor, increasing the risk of NAFLD incidence by 3.5-fold [15]. The global prevalence of NAFLD is estimated to be about 25.24% (over 2 billion people over the world) [16, 17], although prevalence can vary according to the diagnostic method used. Characteristically, when magnetic resonance spectroscopy—which is a highly sensitive method—was used to measure hepatic triglyceride content in a US study population, the prevalence of NAFLD was estimated to be 33.6% [18]. Of note, there is a higher prevalence of NAFLD among individuals with obesity compared to general population, although the exact percentages vary due to the different diagnostic methods and the characteristics of each studied population, such as nationality, age and predisposing factors, including T2DM [13]. In Italy, the Dionysos study identified that the prevalence of NAFLD was 75.8% in persons with obesity [19], while NAFLD prevalence was 27.1% among overweight Dutch and 81.7% among Dutch people with obesity [20], and 67.5% in US persons with obesity [21]. A study in a French population identified NAFLD with mild steatosis (5–33%) in 53% and moderate steatosis (33–66%) in 20% of patients with metabolically healthy obesity [22]. NAFLD has a prevalence between 15% and 80% in people with obesity in the Asia-Pacific region, with a prevalence ranged from 50 to 80% in Japanese people with obesity, between 70% and 80% in Chinese with obesity, from 10 to 50% in Korean individuals with obesity, between 15% and 20% in Indians with obesity and a prevalence of about 47% in Indonesian people with obesity [23]. Furthermore, the prevalence of obesity among global NAFLD patients is 51.3% [17]. Obesity can cause energy imbalance, insulin insensitivity and lipolysis, and alter metabolic functions such as insulin resistance and dyslipidemia, entities that can contribute to NAFLD occurrence [15, 16, 24]. Obesity can disturb the metabolism of the adipose tissue and when the excess energy cannot be stored in this tissue, then this role is taken over by other tissues, with the hepatic being one of them [13, 25]. In this case, circulating free fatty acids (FFAs) derived from increased hepatic de novo lipogenesis or from diminished uptake in subcutaneous adipose tissue can result in ectopic fat accumulation, in our case in the liver, and multiorgan insulin resistance, which is considered a major contributor towards NAFLD development [26, 27]. The high levels of lipids and carbohydrates that hepatocytes are exposed to due to obesity can lead to hepatocellular injury and consequently NAFLD due to ER stress, oxidative stress and mitochondrial dysfunction [11]. The accumulation of lipids in hepatocytes, if not appropriately managed, can provoke the infiltration of immune cells in the liver

and the initiation of an inflammatory process [13, 28]. During this process the immune cells secrete inflammatory cytokines and immunomodulatory mediators that can worsen the hepatocyte dysfunction and lead to necrosis, hepatic steatosis and liver fibrosis, leading to NAFLD and NASH [15, 29]. Obesity can also affect the liver through hormones produced by the adipose tissue, the so called adipokines, which are implicated in the pathogenesis of NAFLD and its progression to NASH and HCC through their contribution to the aforementioned inflammation [30]. In particular, the adipokines are separated in pro- and anti-inflammatory ones and while in normal metabolic status there is a balance between the two categories, in obesity when the adipose tissue enlarges, the secreted adipokines shift towards a pro-inflammatory, steatogenic and fibrogenic profile [13, 31]. In addition, the interaction of the adipokines with the immune cell-derived cytokines can affect NAFLD pathogenesis and progression [13, 32].

ER Stress

The ER is responsible for proper folding of secreted and transmembrane proteins, and the accumulation of misfolded or unfolded proteins leads to ER stress and the activation of the unfolded protein response (UPR) to restore homeostasis [33, 34]. Activation of the UPR relies on the activation of three transmembrane ER stress sensors: the inositol-requiring enzyme 1 (IRE1), the PKR [double-stranded RNA-activated protein kinase]-like ER kinase (PERK) and the activating transcription factor 6 (ATF6) [33, 34]. These three sensors are maintained inactive through the binding of the ER chaperone GRP78/Binding immunoglobulin protein (BiP) on their cytosolic domains [8, 35].

IRE1 α , which catalyzes the first and most conserved branch of the ER stress response, is a transmembrane kinase/endoribonuclease (RNase) [8, 34]. Its cytosolic domain senses stress by binding unfolded proteins when GRP78/BiP is dislocated from the chaperone Hsp47, leading to autophosphorylation and dimerization of IRE1 α and activation of its RNase domain [8, 34]. This domain cleaves the mRNA encoding the X-box binding protein 1 (XBP1), creating the spliced form XBP1s, that reinforces ER protein folding, secretion and degradation (ER-associated degradation-ERAD) [36]. Furthermore, the RNase domain of IRE1 α targets other ER-related mRNAs as well, through regulated IRE1 α -dependent decay (RIDD) [37], which leads to pro-apoptotic signaling through destruction of specific microRNAs that block pro-apoptotic caspase-2 translation [38].

PERK is also transmembrane with an external domain that senses stress and a cytosolic kinase domain that preferentially phosphorylates the translation elongation factor eIF2 α which halts translation while at the same time it increases the amount of specific mRNAs, such as ATF4 [8, 39]. ATF4 activates

downstream genes, is implicated in restoring homeostasis and regulates the expression of the DNA damage-inducible transcript 3 (DDIT3, or growth arrest and DNA damage 153 (GADD153) or best known as CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) [39], and subsequent growth arrest and DNA damage-inducible 34 (GADD34) [40].

ATF6 is a basic leucine zipper protein (bZIP) functioning as an ER stress response element, which promotes transcription of ER-resident chaperones [41]. Upon ER stress, ATF6 α is translocated from the ER to the Golgi apparatus, where it is cleaved from the 90-kDa to the active 50-kDa protein by Site-1 protease (S1P) and Site-2 protease (S2P) [41, 42]. The active form is then transported to the nucleus [42] where it activates the transcription of ER chaperone genes, as well as of the transcription factors CHOP and XBP-1 [43].

Of note, apart from its role in protein processing, ER is also critically implicated in lipid synthesis in hepatocytes, thus it is implicated in lipid disorders, including hepatic steatosis [44]. Indeed, 59% of accumulated hepatic TAG causing NAFLD comes from non-esterified fatty acids, 26% from de novo lipogenesis, and only 15% from the diet [45], underlying the important role of lipogenesis in NAFLD pathophysiology.

ER Stress and NAFLD

The IRE1 α /XBP1 Axis

The significance of IRE1 α /XBP1 axis in the pathogenesis of NAFLD has been proven mostly by genetic ablation studies. When molecules of the three sensing pathways are deleted, the response to ER stress is compromised leading to hepatic steatosis [46]. Hepatocyte-specific deletion of IRE1 α in mice led to defective protein transport from the ER to the Golgi apparatus, defective oxidative protein folding, and ER-associated degradation of misfolded proteins, factors that induce ER stress and activate the UPR [47]. These mice developed modest hepatosteatosis in the absence of ER stress, which was worsened after its induction [47]. IRE1 α represses the expression of metabolic transcriptional regulators, including CCAAT/enhancer-binding protein (C/EBP) β , C/EBP δ , peroxisome proliferator-activated receptor γ (PPAR γ) and enzymes involved in triglyceride biosynthesis, while is also necessary for apolipoprotein secretion when ER homeostasis is disrupted [47]. Furthermore, liver-specific XBP1 disruption is related to decreased hepatic lipogenesis [48]. Overexpression of Bax inhibitor-1 (BI-1) and concomitant inhibition of IRE1 α endonuclease activity leads to downregulation of lipogenesis genes, such as *stearoyl-CoA desaturase 1 (Scd-1)* [48]. XBP-1 deficiency leads to a feedback activation of IRE1 α and mRNA degradation of lipid metabolism genes, such as *Dgat2*, *Acacb*, *Pcsk9*, *Angptl3*, and *Ces1* [49].

Apart from sensing the unfolded proteins, studies in yeast and mammalian cells have shown that the ER stress cytosolic (or transmembrane, in mammalian cells) sensing domain of IRE1 α is sensitive to membrane lipid composition, suggesting a lipid sensing mechanism as well which can activate the UPR [50, 51]. Changes in membrane lipid composition can lead to IRE1 α and PERK activation by enhanced dimerization via their transmembrane domain which proved to be the important domain for both—albeit especially for IRE1 α —in terms of XBP1 splicing [51]. On the other hand, small heterodimer partner (SHP; also known as nuclear receptor subfamily 0, group B, member 2, NR0B2) deficiency downregulates XBP1s protein level and transcriptional activity, compromising the ER stress response [52]. Apart from the role of splicing in the formation of XBP1s which mediates key functions of the ER stress response, its post-translational modification plays an equally important role [53•]. In particular, deacetylation of XBP1s by sirtuin 6 (Sirt6) protects against hepatic steatosis, since in Sirt6 knockout and obese mice the acetylated XBP1s levels and hepatic steatosis were increased, while these phenomena were decreased after genetic overexpression and pharmacological activation of Sirt6 [53•]. The results linking acetylated XBP1s positively with NAFLD activity score (NAS) and negatively with Sirt6 level were also confirmed in liver biopsies from NAFLD patients [53•].

Upon ER stress and concomitant UPR activation, activated IRE1 α can lead to subsequent activation of several downstream molecules, such as the pro-inflammatory and pro-apoptotic c-Jun-NH2-terminal kinases (JNKs) which lead to inflammation and cell death, as well as the nuclear factor- κ B (NF- κ B) [8•]. NF- κ B in the liver acts as a link between hepatic injury and fibrosis—and even progress to hepatocellular carcinoma—with a double role, mediating both pro-inflammatory and anti-apoptotic responses to protect cells from death while inflammatory or immune responses are initiated [54]. This is necessary since these responses are responsible for protecting against pathogens/infections, but should also be tightly regulated to prevent liver injury from the toxic pathogen products [54]. Indeed, NF- κ B can act as a “two-edged sword” in NAFLD, since balance between its two roles is needed: normal or slightly elevated NF- κ B activity can protect hepatocytes from death, but exaggerated activity can lead to fibrosis and NASH through facilitating excessive inflammation [8•, 54]. Data from cell culture experiments suggest that free fatty acids induce the UPR which when prolonged leads to cell death through CHOP-mediated activation of NF- κ B signaling [55]. Furthermore, in mice deficient for BI-1, the unrestrained action of IRE1 α RNase leads to NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation, increased thioredoxin-interacting protein (TXNIP) levels and enhanced NASH [56]. Indeed, hyper-activated IRE1 α increases TNXIP mRNA stability and protein expression leading to

programmed cell death through NLRP3 inflammasome induction [57], and possibly to NASH occurrence. However, it seems that, not only IRE1 α hyper-activation, but also its downregulation can exacerbate hepatic steatosis progression. Data from cultured hepatocytes, which were also confirmed in high-fat diet (HFD) fed mice and human liver biopsies, suggest that the inactivation of the RNase activity of IRE1 α caused by a HFD disrupts the RIDD-mediated degradation of its microRNA targets [58]. The latter includes the miR-200 and miR-34 families, and, in turn, was shown to lead to decreased abundance of their targets, peroxisome proliferator-activated receptor α (PPAR α) and deacetylase sirtuin 1 (SIRT1), that are implicated in lipid homeostasis [58]. As a result, lipids accumulate and progression towards hepatic steatosis is noted [58]. Furthermore, studies on liver biopsies from patients with metabolic syndrome with or without NAFLD or NASH found that NASH is associated with failure to splice XBP-1 and generate XBP-1s [59]. Palmitate which contains palmitic acid -a saturated fatty acid- has been found to activate the IRE1 α pathway, which activates CHOP and JNK proteins leading to enhanced expression of the BH3-only proteins PUMA and Bim which activate the apoptosis regulator BAX and lead to hepatocyte death [60].

In HCC, IRE1 α signaling has been associated with tumor initiation [61]. XBP-1s expression levels have been found elevated in HCC human biopsies [62]. Studies in mice revealed that primary tumors display increased XBP-1 expression which correlated with heightened aggressiveness, namely faster doubling times of the tumor [63].

The PERK-eIF2 α -ATF4 Axis

The PERK-eIF2 α -ATF4 axis has been found to be primarily implicated in lipogenesis and steatosis regulation [8•]. Enforced overexpression of the GADD34 phosphatase in the liver, which selectively dephosphorylates eIF2 α , leads to downregulation of phosphorylated eIF2 α and subsequent attenuation of the adipogenic nuclear receptor PPAR γ and the metabolic transcriptional regulators C/EBP α and C/EBP β [64]. This sequence of events has been linked to enhanced glucose tolerance and insulin sensitivity, as well as with lower incidence of hepatic steatosis in HFD fed mice compared to normal diet fed counterparts [64]. However, complete dephosphorylation of eIF2 α leads to hepatic steatosis in mice, underpinning the importance of a basal eIF2 α phosphorylation level in order to avoid fat accumulation when ER stress is challenged [46]. Liver biopsies from nondiabetic patients with obesity revealed that eIF2 α phosphorylation increased with worsening insulin resistance [65]. Similar to IRE1 α , PERK lacking its luminal unfolded protein sensing domain, can sense aberrations in lipid composition through its transmembrane domain and activate the UPR [51]. Moreover, ATF4

was found to play a significant role in regulating hepatic lipid metabolism since its deficiency protected mice from hypertriglyceridemia and steatosis in response to high fructose diet, by attenuating hepatic lipogenesis [66]. *ATF4*-null mice are lean, protected from age-induced and diet-induced obesity and they are also protected from diet-induced diabetes, hyperlipidemia and hepatosteatosis [67]. CD154 (CD40 ligand) is a key player in inflammatory processes and CD154 knock-out mice fed a diet rich in olive oil were found to develop hepatic steatosis [68]. These mice also displayed reduced apolipoprotein B100 (apoB100) expression and secretion of very low-density lipoproteins, associated with modified UPR, reduced XBP-1s and phosphorylated eIF2 α , thereby providing another link between inflammation and the UPR [68]. ATF4 was also found to functionally promote lipid biosynthesis, while overexpressed ATF4 stimulated the UPR and led to liver steatosis in zebrafish model system as well [69]. Interestingly, ATF4 is suppressed by microRNA-214 (miR-214) and this suppression is reversed by the long noncoding RNA maternally expressed gene 3 (MEG3) [70]. Studies in primary hepatocytes, as well as in HFD fed mice and ob/ob mice, revealed this interaction and highlighted the role of MEG3 in hepatic insulin resistance promotion [70], a role that may be also implicated in NAFLD. As mentioned above, excessive NF- κ B activation can cause NASH development. Indeed, data from cultured mouse fibroblasts show that the phosphorylation of eIF2 α by PERK inhibits the synthesis of I κ B, an inhibitor of NF- κ B, leading consequently to NF- κ B activation [71]. PERK activation phosphorylates and, in turn, activates the transcription factor NF-E2-related factor-2 (Nrf2), which controls the amount of reactive oxygen species (ROS) through regulating glutathione levels during UPR [72]. Thus, PERK-mediated Nrf2 activation during ER stress alleviates the oxidative stress, thereby increasing cell survival [72]. The significant protective role of Nrf2 against steatohepatitis has also been proven in animal models. Specifically, *Nrf2* null mice fed a methionine- and choline-deficient diet for 13 weeks displayed deteriorating NASH compared to wild-type mice, in terms of increased oxidative stress, iron accumulation, fatty change, inflammation and fibrosis [73]. On the other hand, although activation of Nrf2 had no effect in fat accumulation, oxidative stress and iron deposition, it significantly decreased inflammation and fibrosis [73]. Moreover, Nrf2 activation via PERK can lead to downregulation of TNXIP transcription, which links oxidative stress to inflammasome activation, and, thereby, protects hepatic cells from pyroptosis [74]. In addition, the farnesoid X receptor (FXR) inhibits the ER stress-induced NLRP3 inflammasome activation that leads to hepatocyte death, inflammation and progression of liver fibrosis by inhibiting TNXIP activity [75]. FXR exerts these protective effects against NASH progression through the PERK pathway, and specifically through the downstream transcriptional regulator CHOP [75].

On the contrary, PERK activation as well as IRE1 α activation induced CHOP overexpression that leads to hepatocyte death through pyroptosis and apoptosis [76]. The PERK-CHOP branch of ER stress, in particular, has been found to upregulate dual-specificity phosphatase 5 (DUSP5) expression in liver fibrosis—both in human and animal models—which in turn contributes to hepatocyte death [77]. Interestingly, liver biopsies from patients with metabolic syndrome with or without NAFLD or NASH revealed that phosphorylated eIF2 α was increased in NAFLD and NASH patients with metabolic syndrome compared to those with normal liver histology, although levels of the downstream effectors ATF4, CHOP and GADD34 remained unchanged [59].

Regarding the role of PERK-eIF2 α -ATF4 axis in HCC, the PERK pathway is activated during tumor progression [61]. Experiments with transformed mouse embryonic fibroblasts and their tumorigenic effects in mice revealed that PERK plays a role in tumor adaptation to hypoxic conditions by regulating the translation of proangiogenic genes implicated in cell-cell matrix remodeling, adhesion and extracellular matrix proteolysis [78].

The ATF6 α Axis

There are data indicative of a protective role of ATF6 α pathway in hepatic steatosis [8•]. *ATF6 α* knockout mice when challenged with tunicamycin, an ER stress inducing agent, display CHOP upregulation, liver dysfunction and steatosis due to lipid accumulation [46, 79]. ATF6 has been found to bind and suppress the sterol regulatory element-binding protein 2 (SREBP2) and diminishes its lipogenic effect [80]. Additionally, ATF6 physically interacts with PPAR α in hepatocytes, upregulates its transcriptional activity, promotes fatty acid oxidation and protects against hepatic steatosis [81]. ATF6 α reduces hepatic glucose output [82] and improves insulin signaling, reducing hyperglycemia and hyperinsulinemia when overexpressed in livers of obese mice [83], pointing towards a positive effect of ATF6 α in regulating insulin signaling. However, more recent data have implicated the activation of ATF6 signaling pathway in NAFLD progression [84]. Indeed, the downregulation of ATF6 pathway attenuated NAFLD progression by reducing the ER stress-induced inflammation and apoptosis in liver cells of HFD-fed NAFLD mice [84]. Regarding NASH, empagliflozin found to attenuate steatosis, hepatocellular ballooning and lobular inflammation by—among others—reducing ATF6 expression, in HFD-fed Apo^(-/-) mice [85••].

Moreover, the ATF6 α pathway seems to be the last to operate in HCC compared to IRE1 α and PERK pathways, and it is modestly activated only after tumor initiation [61]. ATF6 α has been also identified as a regulator in HCC. Indeed, ATF6 mRNA levels have been found increased and the activated ATF6 product has displayed high nuclear localization in

HCC biopsies [62]. Furthermore, ATF6 α upregulated 18 genes specifically expressed in an ATF6 α -transfected HCC cell line and HCC tissues in comparison to a vector transfected cell line and non-cancerous liver tissues [86]. Finally, ATF6 α is necessary for the adaptation of dormant cancer cells to chemotherapy, nutrient starvation, and changes in the in vivo microenvironment, promoting their survival [87]. ATF6 has been recognized as a potential activator of CHOP in the tumor environment with both molecules being elevated in HCC induced by the carcinogen N-diethylnitrosamine (DEN) [88]. CHOP overexpression in HCC tumors did not lead to apoptosis, suggesting a change in CHOP function from pro-apoptotic to tumorigenic [88]. CHOP's role as a promoting factor for HCC has been identified from other studies as well. More specifically, CHOP null mice display reduced apoptosis, cellular proliferation and fibrosis, key factors for HCC development [89]. In human liver biopsies, CHOP expression increased in parallel to NAFLD progression, from steatosis to NASH to HCC [89]. The ATF6 and IRE1/XBP-1 pathways lead to the transformation-associated expression of the GRP78 gene in HCC [62]. Interestingly, autoantibodies against GRP78 were significantly higher in the serum of patients with HCC compared to control groups and could possibly serve as potential diagnostic markers [90].

Autophagy

Autophagy is an evolutionary conserved process in which cellular components are enclosed in membrane vesicles and transported to lysosomes for degradation [91]. However, the purpose of autophagy is not only the degradation of unnecessary material, but mainly the recycling of simple components, like amino acids or monosaccharides, which can then be reused in the formation of their more complex macromolecules, and thereby maintain cellular homeostasis and survival in periods of starvation [91]. Macro-autophagy, the major type of autophagy, involves the enclosure of cytosolic material, including proteins and degraded organelles, into an isolation membrane named phagophore, which forms the autophagosome [92]. The latter then fuses with lysosomes (with or without previously fusing with endosomes) to form the autolysosome and degrade its contents [92]. The mammalian target of rapamycin (mTOR) functions as a nutrient sensor that can activate or not the autophagy mechanism through regulation of the ULK1 complex consisting of unc-51-like kinase 1/2 (ULK1/2), Atg13 and focal adhesion kinase family interacting protein of 200 kDa (FIP200) [92, 93]. The ULK1 complex is suppressed under nutrient-rich conditions and is activated translocating to the ER under starvation and autophagy-inducing conditions [92, 93]. Apart from the mTOR complex which is responsible mainly for short-term regulation of autophagy [94], long-term regulation can be accomplished by the transcription factors class O of forkhead box

transcription factors (FoxO) and transcription factor EB (TFEB), which under starvation and in the absence of insulin signaling translocate to the nucleus and transactivate their target autophagic genes [95]. Beclin 1 is also a central player in autophagy functioning more downstream, and is starvation-sensitive (interacting with Bcl-2 under nutrient-rich conditions and dissociating from Bcl-2 under nutrient limitations) [92, 93]. Light chain 3 (LC3), an autophagy-related protein 8 (Atg8) ortholog, is another major player in autophagy, with its membrane-bound LC3II form being required for autophagosome formation, in contrast to the cytosolic LC3I form [93, 96]. Finally, important players in autophagosome formation are the autophagy-related (Atg) proteins. Although 31 *Atg* genes have been identified, only 15 have been characterized as core *Atg* genes necessary for the biogenesis of autophagy-related membranes, namely Atg1-10, 12-14, 16 and 18 [97]. A characteristic autophagy substrate whose levels are commonly used as an indicator of autophagy activation and autophagic flux is p62/sequestosome 1 (SQSTM1), an autophagy adaptor protein which controls the formation of protein aggregates and inclusion bodies, links ubiquitinated proteins to LC3 and transfers them to autophagosomes for autophagic turnover [98]. Autophagy is necessary for the degradation of p62/SQSTM1, which is an activator of NF- κ B and of the oxidative-stress responsive transcription factor Nrf2, which as previously mentioned is implicated in UPR as well [93, 99, 100].

Autophagy and NAFLD

In the context of NAFLD, autophagy is related to lipid metabolism, insulin resistance, hepatocellular injury and inflammation [93]. Overall, autophagy has been generally found reduced in NAFLD [101]. Lipid droplets, where excess free fatty acids and glucose are converted to triglycerides and stored, need a conjugation system of LC3 and Atg7 for their formation [102]. Atg7 deficiency, which leads to suppression of Atg5, results in ER stress induction and severe insulin resistance [103]. Furthermore, pharmacological induction of autophagy by carbamazepine and rapamycin mitigated hepatic steatosis and improved insulin sensitivity in a HFD-induced mouse model of NAFLD [104]. Aggregation of the autophagic substrate p62 (SQSTM1) in hepatocytes was found in 68% or 88% of patients with NAFLD according to two clinical studies, while absence of p62 expression was identified in the control groups consisting of patients with normal liver function or with liver metastatic tumors without markers of infection for hepatitis or HIV [105, 106]. Moreover, liver-specific overexpression of p62 induced steatosis in regular chow fed mice [107]. Hepatic autophagy was suppressed in HFD-fed mice which displayed insulin resistance and hyperinsulinemia, while it has been shown that insulin inhibits the expression of key autophagy genes in a FoxO1-dependent

manner [108]. Furthermore, mTOR mRNA and protein expression levels were significantly increased in rats fed a HFD which exhibited mild and severe steatosis compared to rats fed a standard diet, indicating that mTOR contributes to NAFLD development and progression [109]. Wang et al. exploring the underlying mechanisms found that mTOR regulates insulin resistance and chronic liver inflammation [109], while in vitro and animal studies showed that mTOR activation accompanied with inflammation aggravated NAFLD through disrupting low density lipoprotein receptor (LDLR) expression [110].

Autophagy impairment has also been linked to NASH development. The expression levels of Beclin-1 were found significantly higher in patients with NASH compared to patients with simple steatosis [111]. Protein levels of p62 were also elevated in patients with NASH compared to patients with steatosis and individuals with normal liver, along with the ratio of LC3II/LC3I proteins which was significantly increased in patients with NASH and steatosis compared to normal liver subjects [111]. These data indicate decreased autophagy in patients with hepatic steatosis and NASH [111]. However, a study performing Western blot analysis in liver tissues identified no difference in p62 protein levels between NAFLD, NASH patients and normal controls [112]. In line with the clinical data, increased phosphorylated (activated) mTOR and its downstream target S6K1 proteins were observed in mice with steatosis and NASH compared to control mice fed a regular chow diet, indicating autophagy suppression [111]. Indeed, p62 protein and LC3II/LC3I ratio were increased in steatosis and NASH-model mice compared to control, and correlated with prolonged and terminal ER stress [111]. Liver-specific overexpression of p62 in mice exacerbated NASH-induced fibrosis in the methionine-choline deficient diet model of NASH [107]. LC3B has also been found elevated in another NASH mouse model [113]. However, as mentioned elsewhere, evaluation of autophagic flux via p62 levels and LC3II/LC3I ratio, although initially interpreted as a reliable index of macro-autophagy [114], should always be conducted carefully, since the LC3I levels can display naturally occurring fluctuations and p62 levels can be elevated due to other factors, independently of autophagy [94].

Regarding HCC, the role of autophagy remains controversial and seems to be dual [115]. As such, autophagy can limit chromosomal instability and suppress tumor initiation by eliminating senescent and “defected” cells, whereas can also promote cancer cell survival by providing energy and milestones for them through recycling damaged organelles, DNA and proteins [94]. Emerging evidence points towards a tumor suppressive role of autophagy. Mice with heterozygous disruption of Beclin-1 gene display reduced autophagy and increased cellular proliferation, and develop HCC among other types of tumors [116]. In terms of mRNA expression [117] and protein expression assessed by immunohistochemistry [118, 119] and Western blot analysis

[119], Beclin-1 was also found lower in human HCC tissues in comparison to non-cancerous tissues. Interestingly, lower Beclin-1 protein expression was associated with poorer survival in patients with HCC [118], and could serve as a marker for HCC prognosis [119]. However, a discrepancy exists here, since an older study had identified increased Beclin-1 mRNA and protein expression in HCC [120]. LC3 protein can also serve as a prognostic marker [121] for predicting survival and recurrence after HCC surgical removal; low levels or absence of LC3 from adjacent non-tumor tissues were found to be predictive of immediate mortality [121, 122]. Furthermore, low LC3 expression in tumor and adjacent non-tumor tissues was associated with repeated recurrence after surgical resection of HCC [121, 123]. By contrast, divergent findings come from other studies indicating that high expression of LC3B was correlated with vascular invasion, metastasis and poor prognosis and survival in HCC patients [124]. In line with the aforementioned, LC3 was highly expressed in HCC compared to non-cancerous tissues, while, in correlation to hypoxia-induced factor 1 α (HIF1 α), it was significantly associated with tumor size, serving as a predictor of HCC recurrence after surgery, albeit only in large tumors [125]. Several tumor suppressors, such as protein tyrosine phosphatase receptor type O (PTPRO) [126], and TGF β -activated kinase 1 (TAK1) [127], induce autophagy by increasing LC3 expression [126] and decreasing p62 expression [126] and mTOR complex 1 (mTORC1) formation [127] and thus suppressing proliferation of human HCC cell lines [126] or hepatic carcinogenesis in mice [127]. According to the role of mTORC1 as a consistent autophagy inhibitor [128], activation of mTOR signaling has been identified in patients with HCC, while inhibition of mTOR decreased tumor growth and extended survival in experimental HCC xenograft models [129]. Interestingly, mTOR activation has been found to occur selectively in human and mice HCCs with a background of metabolic syndrome and NASH, unlike tumors derived from other etiologies, such as those related to hepatitis viral infection [130]. Additionally, activation of mTORC1 specifically in mice livers leads to spontaneous HCC development [131]. Deletion of Atg5 and Atg7 in the liver leads to development of benign liver adenomas, originating from autophagy-deficient hepatocytes as shown by p62 autophagy substrate accumulation [132]. Expression levels of Atg5, Beclin 1, and Atg7 mRNA were downregulated in HCC cell lines compared to normal cell lines, while Beclin 1 gene expression was also decreased in HCC tissue samples compared to non-tumor samples [133]. Accumulation of the autophagy substrate p62 is also linked to hepatic carcinogenesis, since p62 was originally discovered in HCC cell inclusions, while it was undetectable in non-neoplastic liver tissue [134–136]. Further studies revealed that high p62 expression predisposes to rapid recurrence after surgical treatment of HCC [137]. Moreover, p62 is needed for activation of NRF2 and mTORC1 and protects HCC-initiating cells from cell death caused by oxidative stress [137]. Overexpression of p62 in

human hepatoma cell lines caused anti-apoptotic effects [135]. In addition, young transgenic mice that overexpress p62 display a steatotic phenotype in the absence of inflammation or liver damage [138], suggesting that p62 promotes the progression of NAFLD towards HCC [139].

Notably, there is also evidence that autophagy promotes cancer survival. In a study by Lage et al., immunohistochemical analysis of p62 revealed reduced content in HCC specimens compared to non-cancerous liver tissue, with p62 levels being decreased as HCC cells were differentiated further [140]. Contradicting data exist also for mTOR, since pharmacological mTOR inhibition promoted HCC development, at least in mice with hepatocyte-specific ablation of the specific mTORC1 subunit Raptor [141]. It should be highlighted that, autophagy seems to inhibit tumor suppressors further promoting HCC development once hepatic carcinogenesis has initiated. Indeed, it has been demonstrated that autophagy inhibition by Atg5 deletion in mice led to the appearance of only benign tumors instead of hepatic carcinogenesis, even after treatment with HCC-inducing agents [142]. Of interest, inhibition of autophagy by pharmacological inhibitors or siRNA against Atg5 and Atg7 was found to sensitize HCC cells to the multikinase inhibitor linifanib [143]. Furthermore, reduced Atg7 expression by the micro-RNA miR-375 was shown to reduce viability of cancer cells under hypoxic conditions [144].

Recent data suggest that the balance of activation of autophagy-related molecules is crucial to preserve the normal physiology of the liver, while an imbalance in either direction can result in harmful effects. For instance, in autophagy-deficient mice both hyper- and hypo-activation of mTOR molecules was found to be linked to the development of hepatic tumors, including HCC [145••]. This underlines the potential detrimental effects that can occur by the chronic use of mTOR inhibitors due to disruption of the mTOR balance [145••].

ER Stress and Autophagy Interaction

Summarizing the current knowledge, it is evident that ER stress and autophagy play an important role in NAFLD/NASH initiation and progression.

Hyperglycemia, insulin resistance, and lipid accumulation (from dietary lipid influx and de novo lipogenesis) can induce proteostasis and trigger ER stress in hepatic cells [11] so that the UPR adaptive signaling pathway is activated to restore it, by promoting autophagy. Autophagy stimulates also the degradation of intracellular lipid droplets (lipophagy).

Of interest, there is an interplay between ER stress and autophagy, as ER stress is a well-established positive regulator of autophagy via mainly ATF4 and Xbp1 [12], while autophagy can reduce ER stress, so that it can act protectively by inhibiting apoptosis through caspase inactivation [146].

Nevertheless, under chronic stress, the UPR turns from adaptive to terminal, reduces autophagic process, and activates pro-apoptotic pathways, leading thus to hepatocyte death [12].

AMPK is a known positive regulator of autophagy. Zhou et al. demonstrated that the inhibition of a key enzyme in lipid metabolism (Scd-1) increased AMPK activity and autophagy, attenuating thus hepatic steatosis, in HFD-fed mice [147]. Moreover, in line with the aforementioned, we showed that a sodium-glucose cotransporter-2 (SGLT-2) inhibitor (SGLT-2i) can increase autophagy through AMPK phosphorylation, and reduce the HFD-induced ER stress and hepatic cell apoptosis in ApoE(-/-) mice, alleviating the progression of NAFLD [85••].

However, it should be noted that in HCC, the ER stress and autophagy are differentially regulated and interact, probably under the influence of other critical oncogenic signaling pathways.

Treatment

Both genetic and lifestyle factors appear to contribute to the pathogenesis of NAFLD, and, at least at the early stages of the disease, lifestyle changes—namely improved diet, weight management, and increased physical activity—can be an effective strategy to manage NAFLD [148]. Although further research is clearly required, this beneficial effect appears to involve autophagy-related mechanisms. Indeed, a study on rats showed that exercise can reduce NASH effects on mitochondrial permeability transition pore and promote autophagy and mitochondrial fusion towards a protective direction [149]. Furthermore, exercise can be beneficial in NAFLD by promoting autophagy in the liver which could protect from hepatic fat accumulation, as shown in HFD-fed mice [150, 151]. Recent data also confirmed that physical activity, even without dietary changes, can diminish the progression of NAFLD to NASH and tumorigenesis by activating liver autophagy [152•]. However, apart from the changes in lifestyle, in many cases pharmacological intervention is further needed.

Current guidelines on the management of NAFLD do not recommend any drug treatment; however, given the strong association between T2DM, obesity and NAFLD, various anti-diabetic and anti-obesity drugs have been assessed regarding their usefulness in the treatment of NAFLD (Table 1).

Among the main categories of the anti-diabetic drugs that have been tested in clinical trials are insulin sensitizers (metformin and glitazones), glucagon-like peptide-1 (GLP-1) analogues, dipeptidyl-peptidase-4 (DPP-4) inhibitors, and SGLT-2i.

Jalali et al. conducted a meta-analysis of clinical trials evaluating the role of metformin in NAFLD. They included six randomized controlled studies with 307 individuals and their results emphasized the importance of metformin administration for improving liver function in non-diabetic NAFLD patients [157].

Table 1 Effects of anti-diabetic drugs and anti-obesity treatments on NAFLD in humans

Anti-diabetic drugs and anti-obesity treatments			
Family/ Drug name	Effects	NAFLD entity with therapeutic potential	Reference(s)
Antidiabetic drugs			
Metformin	Reduction in concentrations of liver enzymes (transaminases), improvement in insulin sensitivity and decrease in liver volume as detected by ultrasound, improvement in liver function, insulin resistance and body mass index (BMI) to some extent, but no impact on histological response in NAFLD patients, reduced risk of progression to HCC	NAFLD, NASH	[153–157]
Pioglitazone [a thiazolidinedione (TZD)]	Increase in BMI, reduction in the levels of the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (γ GT), reduction in alkaline phosphatase levels, reduction in liver fibrosis, steatosis and inflammation, reduction in hepatocellular injury, ballooning and lobular inflammation, reduction in the activity score for NAFLD, significant improvement in insulin resistance	NAFLD, NASH	[158–161]
Rosiglitazone [a thiazolidinedione (TZD)]	Decrease in the levels of the liver enzymes ALT, AST, γ GT and alkaline phosphatase, reduction in liver steatosis	NASH	[161–164]
SGLT-2 inhibitors (SGLT-2i)	Decrease in BMI, reduction in the levels of the liver enzymes ALT and γ GT, no significant change in the levels of the liver enzyme AST, no significant change in liver fibrosis and steatosis, inadequate data for the estimation of the change in inflammation	NAFLD	[158]
Dipeptidyl-peptidase-4 (DPP4) inhibitors	No significant change in BMI, significant reduction in the levels of the liver enzyme ALT, no significant changes in the levels of the liver enzymes AST and γ GT, no significant change in liver fibrosis and steatosis, inadequate data for the estimation of the change in inflammation	NAFLD	[158]
Glucagon-like peptide-1 (GLP-1) agonists	Decrease in BMI, reduction in the levels of the liver enzymes ALT and γ GT, no significant change in the levels of the liver enzyme AST, reduction in liver steatosis, inadequate data for the estimation of the change in liver fibrosis and inflammation	NAFLD	[158]
Anti-obesity treatments			
Orlistat	Decrease in the levels of the liver enzyme ALT, reduction in serum insulin level, reduction in fatty liver as assessed by ultrasound, amelioration of steatosis. However was not confirmed by all studies	NAFLD, NASH	[161, 165, 166]
Bariatric surgery	Improvement in the levels of the liver enzymes aminotransferases and γ GT, improvement in histology, steatosis, steatohepatitis and fibrosis, decrease in lobular inflammation, reduction in BMI	NAFLD, NASH	[161]
Liraglutide (3mg daily)	Reduction in weight, hepatic steatosis, hepatocyte damage and apoptosis, as well as insulin resistance, although the reductions in weight and liver fat were not sustained after liraglutide was discontinued	NAFLD	[167]

A recent meta-analysis assessed the efficacy of pioglitazone, DPP-4 inhibitors, GLP-1 analogues and SGLT-2i agents. Of note, according to their findings, pioglitazone demonstrates significant improvements in liver enzymes and in liver steatosis, fibrosis and parenchymal inflammation in NAFLD/NASH patients with diabetes as well as without diabetes [158]. Regarding the effects of the other three drug classes, there are findings suggesting possible improvement in liver enzymes in NAFLD subjects without diabetes; however, current data in this area are still very limited, especially for

DPP-4i and SGLT2-i in NAFLD patients without diabetes and in NASH patients.

Anti-obesity treatment with orlistat has also been evaluated for its effectiveness to improve NAFLD. Two small prospective studies have yielded contradictory results, showing either improvement or no effect of orlistat in decreasing liver enzymes and liver fat as estimated by ultrasound [161, 165, 168]. Of note, a recent open-label, 24-week, randomized clinical trial identified orlistat treatment as an independent predictor of steatosis improvement [166].

Table 2 Selected key modulators of the ER stress pathway and their corresponding targets/mechanism(s) of action for the treatment of NAFLD

Name	Targets/Mechanism of action	Effects	NAFLD entity with therapeutic potential	NAFLD model tested	Reference(s)
Natural compounds					
Geniposide (GEN) (iridoid glycoside extracted from <i>Gardenia jasminoides</i> fruit)	Activation of Nrf2 and PPAR α , upregulation of AMPK signaling pathway, inhibition of mTOR phosphorylation	Protection against lipid accumulation	NAFLD	HepG2 cell line	[169]
Resveratrol (trans-3,4',5-trihydroxystilbene) (polyphenolic compound derived from the roots of white hellebore)	Elevation of XBP-1 splicing and CHOP expression	ER stress induction and amplification of palmitate-induced cell death	HCC	HepG2 HCC cell line	[170]
Bufalin and Bufalin extracts (used in traditional Chinese medicine Chansu) Chemical inhibitors and substances	Induction of ER stress via the IRE1-JNK pathway	Blockage of cell proliferation and apoptosis induction	HCC	HepG2 HCC cell line	[171, 172]
STF083010	IRE1 α RNase	Glucose tolerance improvement	NAFLD/NASH	HFD-induced mouse model of NAFLD	[56, 173]
4 μ 8C	IRE1 α RNase	Glucose tolerance improvement	NAFLD/NASH	HFD-induced mouse model of NAFLD	[56, 173]
Selonsertib	Inhibition of apoptosis signal-regulating kinase 1 (ASK-1) which is an intermediate between IRE1 α -JNK	Liver fibrosis reduction	NASH	NASH patients	[174]
Ezetimibe (cholesterol absorption inhibitor)	Nrf2 activation	NAFLD alleviation and protection from development of hepatic steatosis, inflammation, and fibrosis	NASH	Methione- and choline- deficient diet-induced NASH mouse model	[175]
TBE-31 [acetylenic tricyclic bis-(cyano enone)]	Nrf2 activation, ER stress inhibition as shown by the decreased expression of <i>Sreb1</i> (the gene for SREBP-1c) and <i>Xbp1s</i>	Alleviation of NASH manifestations, namely reversion of insulin resistance, decrease of hepatosteatosis and expression of lipid synthesis genes, increase of hepatic expression of fatty acid oxidation and lipoprotein assembly genes, decrease of ER stress, expression of inflammation genes, and markers of apoptosis, fibrosis, and oxidative stress	NASH	HFD plus fructose-fed wild-type mice	[176*]
Salubrinal	eIF2 α dephosphorylation prevention, prolongation of PERK activation and reduction of protein load in the ER	Increase in cell viability in the presence of tunicamycin-induced ER stress	HCC	HepG2 cell line	[61]
Guanabenz (α 2-adrenergic receptor agonist used in the treatment of hypertension)	Dephosphorylation disruption of eIF2 α in ER stress conditions by binding to GADD34	Protein load reduction in the ER and favoring of protein folding	Potentially HCC ?	Pancreatic beta cells and HeLa cells exposed to cytotoxic tunicamycin-induced ER stress	[177]

Table 2 (continued)

ER stress pathway modulators							
Name	Targets/Mechanism of action	Effects	NAFLD entity with therapeutic potential	NAFLD model tested	Reference(s)		
4-phenyl butyric acid (PBA) (chemical chaperone)	Activation reduction of two UPR pathways (IRE1 and PERK) and their targets	ER stress reduction by increasing protein folding capacity	NAFLD	NAFLD rat model	[178]		
Hormones and endogenous substances							
Melatonin (the main hormone of the pineal gland)	Increase in ATF6 and CHOP expression	Increase in ER stress induced apoptotic cell death and improvement of survival in rats with HCC	HCC	HCC cells, rats with DEN-induced HCC	[179]		
Tauroursodeoxycholic acid (TUDCA)	Activation reduction of two UPR pathways (IRE1 and PERK) and their targets	ER stress modulation	NAFLD	NAFLD rat model	[178]		
Reaction products							
Carbon monoxide (CO) [reaction product of heme oxygenase (HO) activity]	Autophagy activation via the AMPK/mTORC1 axis in a manner that depends on PERK/eIF2 α /ATF4 signaling pathway	Autophagy induction, Protection from hepatic steatosis	NAFLD	Methionine/choline-deficient diet-induced mouse model of NAFLD	[180]		
Drugs							
Empagliflozin (SGLT-2i)	Mitigation of ER stress induction by decreasing eIF2 α phosphorylation and expression of the ER stress related genes <i>Chop</i> , <i>Atf4</i> , and <i>Gadd45</i> and via suppression of the IRE1 α pathway, the PERK-eIF2 α -ATF4 and the ATF6 arms	Increase in energy expenditure, reduction of body mass and improvement of glucose tolerance, and insulin sensitivity. Attenuation of NAFLD progression, improvement of fasting blood glucose, lipid profiles and hepatic lipid accumulation and reduction of the expression of lipogenic enzymes and inflammatory markers	NAFLD	HFD- induced mouse models of NAFLD	[85, 181]		
Exendin-4 (GLP-1 analogue)	Increase in GRP78 expression and decrease in CHOP	Inhibition of an uncontrolled ER stress response and protection of hepatocytes from cell death	NAFLD	Fatty acid loaded hepatocytes	[182]		
Viruses							
M1 oncolytic virus with valosin-containing protein (VCP) inhibitors	Suppression of the IRE1 α -XBP1 pathway	Irresolvable ER stress	HCC	Several mouse models of HCC and primary HCC tissues	[183]		

Table 3 Selected key modulators of autophagy and their corresponding targets/mechanism(s) of action for the treatment of NAFLD

Name	Targets /Mechanism of action	Effects	NAFLD entity with therapeutic potential	NAFLD model tested	Reference(s)
Natural compounds					
Ginsenoside Rb2 (one of the major ginsenosides in Panax ginseng, a traditional Chinese medicine)	Increase in autophagy through induction of AMP-activated protein kinase (AMPK) and activation of silent information regulator 1 (sirt1) pathways	NAFLD improvement and reduction of hepatic lipid accumulation	NAFLD	Genetically diabetic <i>db/db</i> mice, HepG2 hepatoma cell line and primary mouse hepatocytes	[184]
Catalpol (CAT) [a bioactive compound from the herb <i>Rehmannia</i> (Di Huang) <i>glutinosa</i>]	Autophagy induction as displayed by upgraded LC3II levels and decreased p62 levels in HepG2 hepatocytes, mechanism dependent on AMPK/TFEB	Attenuation of insulin resistance, histological effects of NAFLD and hepatic steatosis	NAFLD	Genetically <i>ob/ob</i> and HFD-induced obese mice	[185]
Akebia saponin D (ASD) (a typical bioactive triterpenoid saponin isolated from the rhizome of <i>Dipsacus asper Walli</i>)	Autophagy activation as shown by increased levels of autophagosomes and decreased p62, LC3II and Beclin protein levels	Reduction of hepatic steatosis and hepatocyte apoptosis	NAFLD	Genetically obese <i>ob/ob</i> mice, Buffalo rat liver cells	[186]
Nicotinamide [the amide form of nicotinic acid (vitamin B3, Niacin)]	Upregulation of Sirt1 via activation of the cAMP/PKA/CREB pathway and autophagy	Protection against palmitate-induced hepatotoxicity	NAFLD	HepG2 cell line (a human hepatoma cell line) and Alpha mouse liver (AML)-12 cells (a non-transformed mouse hepatocyte cell line)	[187]
Pectic bee pollen polysaccharide (RBP-P) (isolated from the rose species <i>Rosa rugosa</i>)	Increase autophagy via the AMPK/mTOR-mediated signaling pathway	Reduction of insulin resistance, glucose intolerance and hepatic steatosis	NAFLD	High glucose and fatty acid-treated HepG2 cells and HFD-fed mice	[188]
Caffeine	Autophagy induction through downregulation of mTOR signaling, downregulation of ER stress-associated genes (PERK, IRE1, ATF6, BIP and CHOP) and autophagy associated genes (Atg12 and Beclin-1)	Intrahepatic lipid content reduction and stimulation of β -oxidation in hepatocytes, and decrease of hepatic steatosis and lipid accumulation in zebrafish	NAFLD	HepG2 hepatocytes and HFD-fed mice, zebrafish model of NAFLD	[189, 190]
Epigallocatechin gallate (EGCG) (a major polyphenol in green tea)	Autophagy induction through increased phosphorylation of AMPK	Hepatosteatosis reduction and increased autophagy	NAFLD	HFD-fed mice	[191]
Oleuropein (one of the most plentiful phenolic compounds with antioxidant, anti-inflammatory, anti-atherogenic, hypoglycemic and hypolipidemic effects)	Autophagy induction through AMPK-dependent phosphorylation of ULK1	Hepatic steatosis counteraction	NAFLD	HFD-fed mice	[192]
Betaine (an endogenous and dietary methyl donor essential for the methionine-homocysteine cycle)	Autophagy induction through increased expression of autophagy activators Beclin-1, Atg4 and Atg5 and decreasing the autophagic target p62	Hepatic steatosis reduction	NAFLD	Livers of mice with methionine-choline deficient diet (MCD)-induced NAFLD	[193]
Conophylline (a vinca alkaloid extracted from the leaves of the	Autophagy stimulation in the liver through increasing LC3II and decreasing p62 levels	Inhibition of hepatic steatosis	NAFLD	HFD-diet induced NAFLD mouse models	[194]

Table 3 (continued)

Autophagy modulators							
Name	Targets /Mechanism of action	Effects	NAFLD entity with therapeutic potential	NAFLD model tested	Reference(s)		
tropical plant <i>Tabernaemontana divaricate</i>)							
Micheliolide (a sesquiterpene lactone isolated from the tree <i>Michelia champaca</i>)	Induction of AMPK/mTOR-dependent autophagy, as shown by increased LC3B, Atg7 and Beclin-1 expression and LC3B-II/I ratio	Hepatic steatosis amelioration in mice and decrease of lipid accumulation in hepatocytes	NAFLD	<i>db/db</i> mice (liver steatosis model), lipid mixture-treated normal mouse and human hepatocytes	[195]		
Cortilagin (a polyphenols tannic acid compound of many ethnopharmacological plants)	Restoration of autophagy that was blocked by HFD and autophagic flux among other mechanisms, as shown by upgraded LC3II and reduced p62 protein levels	Alleviation of hepatic steatosis	NAFLD	HFD-fed NAFLD mice and mouse hepatocyte cell line with palmitic and oleic acid-induced lipid accumulation	[196]		
Polydatin (natural precursor of resveratrol)	Autophagy induction via inhibition of mTOR signaling	Protection against hepatic lipid accumulation and reduction of inflammation and hepatocyte damage in mice. Reduction of palmitic acid-induced lipid accumulation in cultured hepatocytes	NAFLD/NASH	Mice fed a methionine-choline deficient diet, cell line model of steatosis	[197]		
EF25-(GSH)2 (an analogue of curcumin, which is a spice component also used as a traditional Asian medicine)	Autophagy induction	Inhibition of HCC cell proliferation	HCC	Hepatoma cell line	[198]		
Bufalin and Bufalin extracts (a part of the traditional Chinese medicine Chansu)	Autophagy activation dependent on AMPK-mTOR pathway, as shown by LC3I to LC3II conversion, increased Beclin-1 protein levels, decreased p62 expression and mTOR signaling activation and stimulation of autophagic flux	Blockage of cell proliferation and apoptosis induction	HCC	HepG2 HCC cell line	[171, 172]		
Luteolin (flavonoid from traditional Chinese medicine)	Promotion of apoptosis through increasing autophagosome formation, LC3B-I to LC3B-II conversion and Beclin-1 expression	Reduction in the viability of human HCC cells	HCC	HCC cell line	[199]		
Cryptotanshinone [an active compound isolated from <i>Radix Salviae Miltiorrhizae</i> ; a plant used in traditional Chinese medicine]	Autophagy induction by inhibition of the PI3K/Akt/mTOR signaling pathway as shown by increased Beclin1 and Atg5 expression and LC3II conversion and downregulated p62 expression	Antitumor effects against HCC	HCC	HCC cell lines	[200]		
Crocin (a glucosidic derivative from saffron, the dried dark-red stigma of <i>Crocus sativus L.</i>)	Autophagy induction by inhibition of Akt/mTOR signaling pathway as shown by increased Beclin-1, decreased p62 and accumulated LC3II protein levels	Growth inhibition of HCC cells in a dose- and time-dependent pattern by autophagic apoptosis	HCC	HCC cell lines	[201•]		
Cantharidin and its acid form cantharidinate (first extracted	Induction of autophagy via LC3, since its expression levels were much higher in	Powerful antitumor activity (sodium cantharidinate), growth inhibition of HCC	HCC	HepG2 HCC cell line	[202]		

Table 3 (continued)

Autophagy modulators		Name	Targets /Mechanism of action	Effects	NAFLD entity with therapeutic potential	NAFLD model tested	Reference(s)
	from the Chinese blister beetle; used in traditional Chinese medicine)		treated cells and the LC3II/LC3I ratio was increased				
	Tangeretin (biological compound with antitumor properties extracted from traditional Chinese herbs).		Autophagy induction partially dependent on Beclin-1, as shown by the upgraded LC3II/LC3I ratio and reduced p62 expression and the fact that knockdown of BECLIN-1 expression partly reversed the beneficial effect of tangeretin	Decreased cell proliferation and migration, and induction of cell cycle arrest in HCC cells	HCC	HepG2 HCC cell line	[203]
	Armillaridin (the bioactive compound from the honey mushroom <i>Armillaria mellea</i>)		Autophagy activation as shown by LC3 aggregation and increased levels of Atg5 and Beclin-1 proteins	Cytotoxic effects and growth inhibition in HCC cells	HCC	HepG2 HCC cell line	[204]
	Resveratrol (trans-3,4', 5-trihydroxystilbene) (polyphenolic compound derived from grapes and originally isolated from the roots of white hellebore)		Autophagy activation as shown by upregulated autophagosome formation, LC3II/I ratio and Beclin-1 protein levels and reduced p62 expression	Antitumor effects on hepatocellular carcinoma	HCC	HCC cell line	[205••]
	Dioscin (natural product from medicinal plant)		Autophagy induction as shown by decreased p-mTOR expression levels and increased LC3 and Beclin-1 levels	Inhibition of proliferation and migration of HCC cells and inhibition of diethylnitrosamine-induced primary liver cancer in rats and cell xenografts in nude mice	HCC	HCC cell lines, livers of rats and tumour tissues of nude mice	[206]
	Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (the primary active constituent extracted from the plant <i>Curcuma longa</i>)		Autophagy activation as shown by increased protein levels of both LC3 and Beclin-1	Proliferation prevention and induction of apoptosis in HCC cells	HCC	HepG2 HCC cell line	[207]
	Quercetin (a member of the flavonoids family and a dietary antioxidant)		Autophagy induction in part via AKT/mTOR pathway inhibition since autophagosomes and autolysosomes were increased, LC3A/B-II and Beclin-1 protein levels were upregulated and p62 protein levels were downregulated in HCC cells while in the tumor tissue from a xenograft model in mice LC3A/B protein levels were increased and p62 protein levels were decreased	HCC growth inhibition	HCC	HCC cell lines, tumor tissue from a xenograft model in mice	[208]
	Hormones, metabolism products, and endogenous proteins						

Table 3 (continued)

Autophagy modulators		Name	Targets /Mechanism of action	Effects	NAFLD entity with therapeutic potential	NAFLD model tested	Reference(s)
		Thyroid hormones diiodothyronine (T2) and triiodothyronine (T3)	Autophagy induction	Decrease in hepatic fat content	NAFLD	HFD-fed rats	[209]
		Fibroblast growth factor 21 (FGF21) (Hormone)	Autophagy induction through increased expression of LC3, Beclin-1 and Atg5	Reversion of hepatic steatosis	NAFLD	Monosodium L-glutamate (MSG)-induced obese mice (NAFLD model) and fat-loaded HepG2 cells	[210]
		Ursodeoxycholic acid (UDCA) (Metabolism product)	Autophagy improvement via activating AMP-activated protein kinase (AMPK), increasing Beclin-1, LC3II/I expression and decreasing p62 expression	Hepatic steatosis alleviation	NAFLD	HFD-induced NAFLD rats	[211]
		Caveolin-1 (CAV1) (a human endogenous protein component of the caveolae, which are flask-shaped cholesterol-, and glycosphingolipid-rich membrane microdomains)	Autophagy promotion through inhibition of the Akt/mTOR pathway	Alleviation of lipid accumulation	NAFLD	HFD-fed mice with NAFLD, and alcohol and oleic acid mixture treated human normal liver (L02) cells, which serve as an in vitro model of hepatic steatosis	[212]
		Chemical inhibitors and compounds					
		Pertetinoin (oral acyclic retinoid with a vitamin A-like structure that targets the retinoid nuclear receptor)	Autophagy induction characterized by upregulated co-localized expression of LC3B-II and lysosome-associated membrane protein 2 (LAMP2), increased formation of autophagosomes and upgraded autophagic flux, increased expression of Atg5-12 complex and decreased p62 in mice livers, as well as by increased protein expression of the Atg5-12 complex and LC3B-II in mouse primary hepatocytes. Upregulation of Atg 16L1 expression, which is an important regulator of autophagy, and anti-inflammatory proteins	Improvement in liver histology by suppression of NASH progression and tumorigenesis reduction	NASH/HCC	NASH-HCC mouse models, mouse primary hepatocytes	[213]
		OSU-HDAC42 and SAHA [histone deacetylase (HDAC) inhibitors]	Autophagy activation by inhibition of Akt/mTOR signaling. Autophagy induction was shown by LC3II accumulation	Reactivation of tumor suppressor genes	HCC	HepG2 HCC cell line	[214]
		Propyl gallate (synthetic antioxidant used in preserving food and medicinal preparations)	Autophagy induction through reinforcing the rate of LC3I to LC3II conversion and increasing autophagosome formation	Inhibition of HCC cell proliferation in vitro and in vivo	HCC	Zebrafish models, HCC cell lines	[215]
		E5; N-(3-((4-(benzofuran-2-yl)pyrimidin-2-yl)oxy)-4-methylphenyl)-	Autophagy activation through downregulation of the	Proliferation prevention and induction of apoptosis in HCC cells	HCC	HCC cell lines	[216]

Table 3 (continued)

Name	Targets /Mechanism of action	Effects	NAFLD entity with therapeutic potential	NAFLD model tested	Reference(s)
Autophagy modulators					
4-(4-methylpiperazin-1-yl)methyl) benzamide [a novel 2-phenyloxy-pyrimidine derivative which is a platelet-derived growth factor receptor α (PDGFR α) inhibitor]	PDGFR α /PI3K/AKT/mTOR pathway. Autophagy induction shown by induced LC3I to LC3II conversion, increased Atg5 expression, and autophagy flux restoration				
Reversine (a small molecular agent that induces myogenic lineage-committed cells into multipotent progenitor cells)	Autophagy blockage as shown by the decreased LC3II/LC3I ratio and the increased p62 expression	Proliferation inhibition and induction of apoptosis	HCC	HepG2 HCC cell line	[217]
Bafilomycin A1 (BafA1: lysosome inhibitor) (Chemical inhibitor, antibiotic)	Autophagy blockage as shown by increased LC3I to LC3II conversion and increased p62 levels	Inhibition of human HCC cell growth and induction of cell death	HCC	HepG2 HCC cell line	[218]
Drugs					
Celecoxib [selective cyclooxygenase-2 (COX-2) inhibitor]	Restoration of autophagic flux as shown by increased LC3II/I ratio and reduced p62 levels	NAFLD amelioration	NAFLD	Human hepatocytes treated with palmitate that displayed lipid accumulation, and HFD-fed rats	[219]
Pioglitazone [a peroxisome proliferator-activated receptor gamma (PPAR γ) agonist]	Autophagy activation as shown by increased expression of the mTOR, Atg7, LC3I and LC3II proteins and enhanced expression of LC3 and Atg7 mRNAs, an activation mainly dependent upon PPAR γ	Attenuation of hepatic steatosis	NAFLD	HFD-induced NAFLD mice model, in vitro hepatic steatosis model of mouse liver cells treated with palmitic acid	[220]
Liraglutide (GLP-1 analogue)	Autophagy promotion by Sirtuin1/Sirtuin3 restoration and activated p-FOXO3a protein levels, as well as by the AMPK/mTOR signaling pathway, via AMPK activation and mTOR inhibition. Autophagy promotion was displayed by reduced p62 mRNA and protein levels, upregulated LC3II protein levels and increased Beclin-1 and LC3II/I ratio	Reduction of hepatic lipid accumulation	NAFLD	HFD-fed mice, free fatty acids-induced hepatic cells	[221, 222]
Exendin-4 (GLP-1 analogue)	Autophagy activation as shown by increased Beclin mRNA and protein levels and the conversion of LC3B-I to LC3B-II	Steatosis reduction	NAFLD	Fatty acid loaded hepatocytes	[182]
Aspirin	Autophagy induction via Beclin-1 activation. Autophagy activation was shown by an increased LC3II/LC3I ratio, autophagic flux and decreased p62 expression	Inhibition of HCC development	HCC	HCC cell lines	[223*]
Lapatinib (orally administered small-molecule tyrosine kinase inhibitor)	Autophagy induction as displayed by increased autolysosome formation, increased LC3II protein levels and	Cytotoxicity in human hepatoma cell lines	HCC	HCC cell lines	[224]

Table 3 (continued)

Autophagy modulators		Reference(s)
Name	Targets /Mechanism of action	Effects
Metformin (1,1-dimethylbiguanide hydrochloride) (a biguanide derivative and one of the most used first-line anti-diabetic drugs) (alone or in combination with caloric restriction)	conversion, upregulated Atg5, Atg7, Beclin-1 and decreased p62 protein levels Autophagy activation as shown by the downregulation of p62 and LC3II, with the decrease in LC3II being attributed to activation of autophagic flux and not formation of autophagosomes. Metformin activated AMPK, inhibited mTOR phosphorylation and reduced the hepatic mTOR content, showing that the metformin-induced effect on autophagy is exerted via the AMPK/mTOR signaling pathway.	Improvement of several NAFLD clinical manifestations, inhibition of HCC cell viability
Mitoxantrone (antineoplastic agent that inhibits the type II topoisomerase)	Induction of mTOR-dependent autophagy activation, as shown by the increased protein expression of LC3II and Beclin-1, and the downregulation of the p62 protein	Inhibition of the growth and proliferation of HCC cells in vitro
Empagliflozin (SGLT-2i)	Activation of hepatic autophagic flux as shown by the reduced mTOR mRNA levels and p62 protein levels and the increased LC3B mRNA levels and Beclin-1 protein levels as well as by AMPK activation	Attenuation of NAFLD progression, improvement of fasting blood glucose, lipid profiles and hepatic lipid accumulation and reduction of the expression of lipogenic enzymes and inflammatory markers
Anthistamine deptropine	Autophagy inhibition via blocking the fusion between autophagosomes and lysosomes	Hepatoma cell death
Sorafenib (a multi-kinase inhibitor) & Cisplatinum (a chemotherapy drug) (combination treatment)	Autophagy induction as shown by downregulated mTOR mRNA and protein levels and increased LC3II expression	Inhibition of HCC cell proliferation
Dietary compounds	Autophagy induction shown by LC3II increase	NLRP3 inflammasome inactivation
Dietary polyunsaturated fatty acids (PUFA) (fatty acids that contain more than one double bond in their backbone)		

NAFLD entity with therapeutic potential

NAFLD, HCC

NAFLD rat model, HepG2 HCC cell line

HCC

HCC

HCC

HCC

NAFLD

HepG2 cell line

HFD-fed ApoE(-/-) mice

HepG2 cell line

HepG2 HCC cell line

Liver tissue from dietary PUFA-fed mice

[225, 226]

[227]

[85••]

[228]

[229••]

[230]

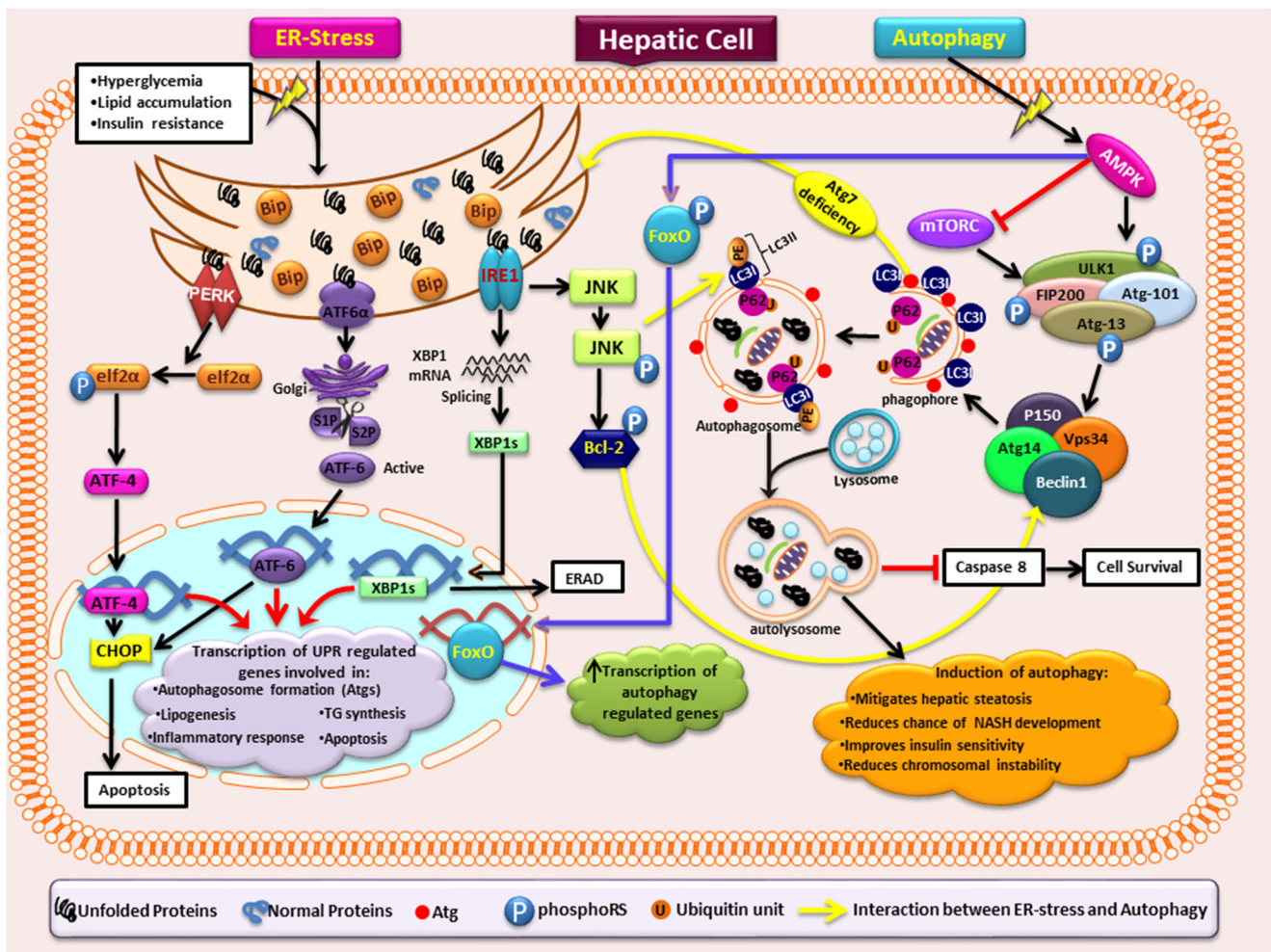


Fig. 1 Cross-talk between ER stress and autophagy. The ER stress pathway is induced by hyperglycemia, the ER stress response is initiated upon accumulation of misfolded and unfolded proteins in the ER lumen. Dissociation of Bip from the ER stress sensors (PERK, IRE1, and ATF6) leads to subsequent activation of 3-arms of UPR signaling. Phosphorylation of PERK results in activation of the downstream translation factor eIF2 α , leading to the activation of ATF4 transcription factor and over-expression of CHOP. Both ATF-4 and CHOP can regulate the expression of a number of Bcl-2 family proteins and autophagy regulatory proteins such as Atg5 and p62. The ATF6 α , upon UPR activation, is transported to the Golgi apparatus where it is processed by Site-1 protease (S1P) and S2P and becomes activated. Activation of IRE1 induces XBP1 splicing to generate active XBP1s. XBP1s enters the nucleus to transcribe proteins involved in ER associated degradation (ERAD). Activation of IRE1 also activates the JNK signaling cascade which in turn increases both phosphorylation of Bcl-2 as well as the formation of LC3II. Phosphorylation of Bcl-2 disrupts the Bcl-2/Beclin1 interaction and therefore induces activation of autophagy. All three transcription factors (XBP1s, ATF4, and ATF6) are translocated to the nucleus where they induce the expression of target

genes including genes related with autophagosome formation. The autophagy pathway is mainly controlled by the AMPK/mTOR-signaling axis. AMPK inhibits mTORC1 leading to initiation of autophagy. AMPK activation induces the phosphorylation of FoxO transcription factor and its translocation into the nucleus where it can bind to the promoter of autophagy genes and elevate their expression. AMPK activation is also the essential step towards activation of ULK1 complex which in turn leads to the activation of the class III PI3K complex (Atg14/Beclin 1/Vps34/p150) by phosphorylating Beclin 1. These steps are required for the formation of autophagosome which is then transformed into a double-membranous vesicle by recruitment of the Atg (12, 5, 16) complex and the lipidation of LC3I into phosphatidylethanolamine-conjugated(PE) LC3II. The LC3II participates in the formation and expansion of phagophore along with Atg8 and Atg7 and ubiquitinated proteins such as p62. The mature autophagosome undergoes fusion with a lysosome to form autolysosome. Activation of autophagy can induce cell survival by clearing damaged organelles and inhibit apoptosis through inhibition of caspase-8 activation

Of great interest, several compounds have been identified to modulate ER stress or autophagy levels as a therapeutic strategy to attenuate the progression of NAFLD. To this aim, several natural compounds, hormones, and drugs have been evaluated in NAFLD animal models and human studies

regarding attenuating NAFLD progression through modulating ER stress and autophagy. Tables 2 and 3 summarize selected key modulators of the ER stress pathway and autophagy, respectively, and their corresponding targets/mechanism(s) of action for the treatment of NAFLD.

Table 2 shows that focusing on the implication of ER stress as a mediating mechanism, most of the chemical compounds that exert favourable effects on NAFLD/NASH have been tested in animal models. Only selonsertib, an ASK1 inhibitor, was assessed in patients with nonalcoholic steatohepatitis and demonstrated reduction of liver fibrosis [174].

Moreover, the effect of natural compounds on ER stress pathway has been evaluated mainly in hepatocarcinoma cell lines.

It is of great interest that although almost all the categories of anti-diabetic drugs have been assessed for their usefulness in NAFLD in clinical trials, the ER-stress as target mechanism have been studied only for SGLT2-i, and only in animal models [85, 181].

In contrast, autophagy has been found to be involved in the favorable effects of four anti-diabetic drug classes (GLP-1, glitazones, metformin and SGLT2-i) on NAFLD/NASH progression albeit again only in animal studies [85, 182, 220–222, 225, 226].

As in the case of ER stress, Table 3 shows that an important number of natural and chemical compounds have been tested for their efficacy to modulate the autophagy process only in hepatocarcinoma cell lines. Interestingly, not even one clinical study has investigated the implication of autophagy as target mechanism that mediates the favorable effect of any compound or drug on NAFLD.

Conclusion and Perspectives

The incidence of NAFLD spectrum is rising and is estimated to affect more than 130 million patients worldwide by 2030 [231•].

The involvement of ER stress and autophagy in the pathogenesis and progression of NAFLD is gaining increasing interest.

Herein, we reviewed *in vitro*, animal and human studies on the deterioration of the aforementioned mechanisms in hepatic cells leading to NAFLD, NASH and HCC.

It is evident from the existing evidence that the complex etiology and pathophysiology of NAFLD with the implication of genetic and environmental factors make it difficult to extrapolate from *in vitro* and animal research on humans, and this is reflected in the divergent results that emerged from various studies.

Undoubtedly, the plethora of genetically engineered mouse models, diets or synthetic compounds that are now available can further facilitate the research of NAFLD.

However, it still remains a challenge to create ideal animal models reproducing the whole spectrum of the NAFLD, in order to fully clarify the disease pathophysiology and ascertain effective treatments. Realizing that the “humanizing” of NAFLD animal models can become possible to a limited extent, humanizing of computational models derived from animal experiments could further aid the attempts of translating relevant research findings from animal to humans [232].

Most studies—especially focusing on diet-induced NAFLD progression—indicate that there is a counterbalance between ER stress and autophagy, where ER stress induces autophagy which sequentially attenuates ER stress and apoptosis of hepatic cells (Fig. 1). It appears that a shift of this balance to the side of ER stress, due to either sustained terminal ER stress or diminished autophagy, can result to hepatic cell apoptosis and liver injury.

Taking into account that other than hepatic cells can also contribute to the pathogenesis of steatohepatitis and tumorigenesis such as Kupffer, endothelial, epithelial and immune cells, studies on the role of ER stress and autophagy should be expanded to these cells which also shape the milieu in the spectrum of NAFLD. To this direction, a recent study showed that a myeloid-specific IRE1 α deletion results in an altered transcriptional profile of hepatic macrophages and diminishes the diabetes-induced NASH, as well as HCC development [233]. Moreover, recent data suggest that cell-type-specific reprogramming of the liver cell transcriptomes is linked to NASH pathogenesis [234•]. Multi-omics approaches could offer a new strategy to investigate the interactive processes of ER stress and autophagy from the perspective of NAFLD and its progression.

Better understanding of the interrelations between ER stress and autophagy, and how both these pathways can interact with inflammation and apoptotic mechanisms is expected to further aid the development of new therapeutic options which can better target and reprogram these underlying pathways. Indeed, both these processes are now intensively explored as targets of various compounds and drugs aiming to attenuate NAFLD progression.

Data Availability Not applicable

Code Availability Not applicable

Compliance with Ethical Standards

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of Interest The authors declared no conflict of interest.

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- [org/10.1016/j.ejphar.2018.01.031](https://doi.org/10.1016/j.ejphar.2018.01.031) **A widely used medication, aspirin, inhibited HCC development by inducing autophagy as shown by the increased LC3II/LC3I ratio, autophagic flux and decreased p62 expression in HCC cell lines. Interestingly these effects were dependent on Beclin-1 activation.**
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