Review

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# Autophagy in liver diseases: Time for translation?

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## Summary

Autophagy is a self-eating catabolic pathway that contributes to liver homeostasis through its role in energy balance and in the quality control of the cytoplasm, by removing misfolded proteins, damaged organelles and lipid droplets. Autophagy not only regulates hepatocyte functions but also impacts on non-parenchymal cells, such as endothelial cells, macrophages and hepatic stellate cells. Deregulation of autophagy has been linked to many liver diseases and its modulation is now recognized as a potential new therapeutic strategy. Indeed, enhancing autophagy may prevent the progression of a number of liver diseases, including storage disorders (alpha-1 antitrypsin deficiency, Wilson's disease), acute liver injury, non-alcoholic steatohepatitis and chronic alcohol-related liver disease. Nevertheless, in some situations such as fibrosis, targeting specific liver cells must be considered, as autophagy displays opposing functions depending on the cell type. In addition, an optimal therapeutic time-window should be identified, since autophagy might be beneficial in the initial stages of disease, but detrimental at more advanced stages, as in the case of hepatocellular carcinoma. Finally, identifying biomarkers of autophagy and methods to monitor autophagic flux *in vivo* are important steps for the future development of personalized autophagy-targeting strategies. In this review, we provide an update on the regulatory role of autophagy in various aspects of liver pathophysiology, describing the different strategies to manipulate autophagy and discussing the potential to modulate autophagy as a therapeutic strategy in the context of liver diseases.



Keywords: Autophagy; Fibrosis; Inflammation; Hepatocyte apoptosis; NASH; Hepatocellular carcinoma; Alcoholic liver disease.

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## Introduction

Autophagy is a self-eating catabolic pathway, conserved in eukaryotic cells, with the lysosome as the final destination.[1–3] Autophagy plays a major role in organ homeostasis and the immune response,[4] as well as being an anti-aging mechanism.[5,6] Dysregulation of autophagy is observed in a wide range of pathological conditions, including obesity and type 2 diabetes, inflammatory and infectious diseases, neurodegenerative diseases, and cancer.[7,8] The term autophagy encompasses 3 processes in mammalian cells: macroautophagy, microautophagy (and endosomal microautophagy), and chaperone-mediated autophagy.

***Macroautophagy*** is initiated by the formation of a double membrane bound vacuole named the autophagosome.[1,3,6,9] During autophagosome formation, part of the cytoplasm, which may include organelles, proteins aggregates, and lipid droplets, is sequestered in a bulk in a selective manner. Many forms of selective autophagy exist (in particular mitophagy for the selective sequestering of mitochondria, ER-phagy for the selective sequestering of fractions of the endoplasmic reticulum, xenophagy for the selective sequestering of microorganisms invading the cytoplasm).[10–12] During selective macroautophagy, autophagy adaptors (*e.g.* SQSTM1/p62, NDR1, NDP52/CALCOCO2, optineurin) specifically target cargo to the autophagosomal membrane for sequestering in a ubiquitin-dependent or ubiquitin-independent pathway (reviewed in[10]). The autophagosome moves along microtubules to deliver cargo to the lysosome for degradation.[13,14] In multicellular organisms, autophagosomes acquire acidic and degradative properties by fusing with endosomal compartments before finally fusing with the lysosomal compartment.[15,16]

Macroautophagy is dependent on the activity of proteins encoded by autophagy-related genes (ATGs) and class III phosphatidylinositol 3-kinase (VPS34) in a complex with Beclin 1 (the orthologue of the yeast Atg6) and ATG14L to produce phosphatidynlinositol 3-phosphate (PI3P) necessary to form the autophagosome.[17–19] The maturation of the autophagosome into an autolysosome also relies on certain ATG proteins such as ATG14L, on the activity of Rab GTPases, on tethering factors (HOPS complex) and adaptors (PLEKHM1), and on SNAREs to execute the membrane fusion process.[20]

***Microautophagy and endosomal microautophagy*** are catabolic pathways that directly send cytoplasmic proteins or organelles such as peroxisomes to the endosomal/lysosomal lumen.[21] The molecular machinery engaged in microautophagy is not well defined in mammalian cells. Endosomal microautophagy, which delivers cytosolic proteins to multivesicular bodies, relies on ESCRT I and III systems and depends on the protein chaperone Hsc70.[22]

***Chaperone-mediated autophagy*** is exclusively for proteins that contain a KFERQ motif,[23] which are recognized in the cytosol by the chaperone protein Hsc70. The KFERQ motif is present in about 30% of cytosolic proteins, including enzymes of the intermediate metabolism.[24] The

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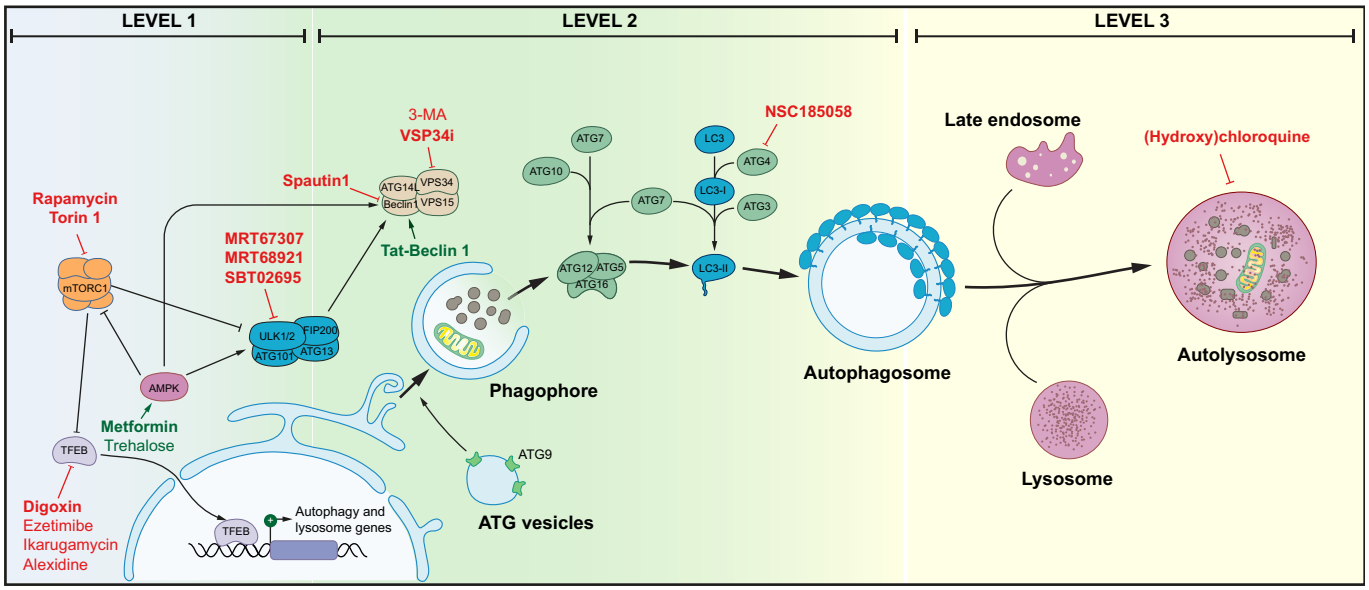


**Fig. 1. Pharmacological targeting of the autophagic pathway.** Autophagy can be regulated at 3 levels: *Level 1*, upstream of autophagosome formation, by modulating the signalling pathways that control the formation of autophagosomes. Only mTOR and AMPK, 2 major kinases that regulate autophagy in opposite ways, are indicated in the figure. These signalling pathways impact on the autophagy machinery to trigger autophagosome formation and/or the transcription of ATGs and of lysosomal genes by transcription factors such as TFEB. *Level 2* during the formation of autophagosome. Autophagosome formation starts with the recruitment of ATG proteins at the endoplasmic reticulum to initiate phagophore elongation. This step is generally initiated by the ULK complex (composed by ULK1 or 2, ATG13, ATG101 and FIP200), that activates the PI3KC3 complex I (composed by VPS34 or class III phosphatidylinositol 3-kinase, VPS15, ATG14L and Beclin 1). Activation of the ULK and PI3KC3 complex I occurs upstream of the 2 ubiquitin-like conjugation systems (where ATG12 is covalently linked to ATG5). The conjugate is stabilized by its interaction with ATG16 and where the C-terminus of LC3-I is covalently linked to the polar head of phosphatidylethanolamine to form LC3-II. ATG7 acts as an E1 enzyme and ATG10 and ATG3 as E2 enzymes systems that elongates the phagophore membrane to form the double-membrane bound autophagosome. ATG9, the only transmembrane ATG, contributes to phagophore elongation. *Level 3* during maturation of autophagosome into autolysosome and the degradation of autophagic cargo into the lysosomal compartment. In most cases, the autophagosome maturation engages fusion with late endosomes before delivery of cargoes into the lysosomal compartment. At each level, some of the activators (in green) and of the inhibitors (in red) of autophagy are indicated. ATG, autophagy-related gene.

translocation through the lysosomal membrane is dependent on the lysosomal membrane protein Lamp2a. Chaperone-mediated autophagy has recently gained attention because of its importance in the regulation of metabolism and its roles in antigen presentation and aging.[24,25]

Macroautophagy (hereafter referred to as autophagy) is the form of autophagy that we will mainly discuss in the present review. Autophagy was first described in the kidney and liver,[3,17,21] as a response to starvation. Autophagy results in the recycling of amino acids, carbohydrates, and fatty acids to the cytosol through the lysosomal degradation of autophagy cargo in hepatocytes. Pioneering works have delineated its regulation by pancreatic hormones (insulin and glucagon) and amino acids.[26–29] Autophagy also contributes to liver homeostasis through its role in the quality control of the cytoplasm, as autophagy removes misfolded proteins and damaged organelles.[30] Moreover, through lipophagy (a selective form of autophagy that targets lipid droplets) autophagy contributes to lipid metabolism.[31]

Recently, ***non-canonical forms of autophagy*** were described, that result in the formation of autophagosomes or autophagosome-like vacuoles that fuse with the lysosomal compartment or the plasma membrane.[32,33] These pathways only use a subset of the core ATG machinery. Among these, LC3-associated phagocytosis (LAP) has gained attention because of its role in immune regulation. LAP involves the recruitment of LC3-II to the phagosomal membrane[21,34–36] and is initiated in macrophages by the engagement of innate immune receptors, such as Toll-like receptors or Fc immunoglobulin receptors, and by pathogens during phagocytosis. A major difference with autophagy is that the LAPosome that emanates from the plasma membrane is a single membrane-bound vacuole. At the molecular level and in contrast to autophagy, ULK1 is not required for LAP. Moreover, Rubicon, which inhibits the maturation of the autophagosome during autophagy, is required at the early stage of LAPosome formation to produce PI3P in a complex that contains Beclin 1, UVRAG and VPS34. These steps act upstream of the ATG5–ATG12 and LC3 conjugations systems.[21,34]

The aim of the present review is to discuss the potential of manipulating autophagy as a therapeutic approach for liver diseases.

**Key points**

Autophagy contributes to liver homeostasis and hepatic lipid metabolism, making it a potential therapeutic target in a number of liver diseases.

## Strategies to modulate autophagy

Autophagy can be modulated at 3 levels of the pathway (Fig. 1): i) upstream of autophagosome formation, by modulating the activity of transcription factors (*e.g.*, TFEB and TFE3) which regulate

the expression of autophagy genes and lysosomal proteins; ii) during autophagosome formation at the stages of initiation, nucleation of the phagophore (or isolation membrane), elongation and maturation, and sealing of the autophagosome; and iii) during maturation of the autophagosome when it fuses with the endolysosomal compartment or by targeting lysosomal hydrolase activity or nutrient recycling from the lysosome lumen to the cytosol. Manipulating autophagic proteins by specific gene deletion or overexpression has demonstrated that targeting components of the autophagic machinery may confer a therapeutic benefit in clinical settings.

### Lessons from genetic interventions

Preclinical studies have shown that activation of autophagy following selective overexpression of certain ATG proteins counteracts disease progression.[37] For example, ATG7 overexpression has a beneficial effect on obesity because it reduces insulin resistance,[38] and overexpression of Atg5 extends life in mice and reduces the onset of aged-related disease (diabetes, cardiovascular disease and neurodegeneration).[39] Targeted gene expression has also been used to modulate autophagy in the brain, muscle, and liver. In particular, overexpression of the transcription factor TFEB, which coordinately regulates autophagy, lysosomal biogenesis and fatty acid oxidation, protects mouse liver from damage caused by a high-fat diet.[40]

### Pharmacological approaches

Historically the first reported autophagy inhibitor was 3-methyladenine (3-MA), which blocks autophagosome formation by interfering with the activity of VPS34.[41] Since then, more specific VPS34 inhibitors have been developed[42–44] and used in preclinical studies. Other inhibitors have been developed that target the activity of specific ATGs, such as ULK1[45,46] or ATG4B (an enzyme responsible for the activation of LC3 and release of LC3-II from the autophagosome membrane)[21,47] (Fig. 1). Pharmacological activators of TFEB prevent steatosis,[48] and eliminate tau aggregates in models of Alzheimer's disease.[49] However rapamycin or rapalogs, which stimulate autophagy by inhibiting the kinase mTOR,[50,51] and the lysosomotropic agent chloroquine and its derivative hydroxychloroquine, which block autophagy by accumulating in acidic compartments,[52] are still the most widely used in clinical trials aimed at modulating autophagy (https://clinicaltrials.gov).

A major drawback of drugs targeting kinases and the lysosomal compartment is that autophagy-independent effects may occur.[53] Similarly, drugs that target some ATG proteins, such as ULK1 and ATG4B, can bear autophagy-independent effects (reviewed in[54,55]). Peptides derived from autophagy proteins may also show promising therapeutic effectiveness. A typical example is a peptide from the evolutionarily conserved domain of Beclin-1 coupled to HIV-1 Tat transducing peptide, which stimulates autophagy in different organs *in vivo* (muscles, bones, liver and brain) and reduces mortality in mice infected by chikungunya and West Nile virus.[56,57]

### Nutritional interventions

Caloric restriction or increases in natural nutrients, such as spermidine, stimulate autophagy[58] and contribute to life extension and protection against age-related diseases.[59,60] Spermidine has beneficial effects on cardiac hypertrophy in old mice via an autophagy-dependent pathway, since autophagy-deficient mice exposed to spermidine are no longer protected.[61] Interestingly, a high level of dietary spermidine is correlated with a lower incidence of cardiovascular diseases in humans.[61,62] Late parenteral nutrition (supplementation with macronutrients) in critically ill patients is correlated with an increase of autophagic structures in striated muscles, leading to beneficial effects on muscular weakness and recovery time.[63]

### Targeting multiple autophagy pathways for therapeutic purposes

An archetypal example of targeting multiple autophagy pathways has been illustrated in the context of hyperammonia.[64] Hepatic ureagenesis eliminates ammonia accumulation that can cause neuronal damage and death. Hepatic gene transfer of *TFEB* by a helper-dependent adenoviral vector reduced the levels of blood ammonia and increased the level of urea production in a mouse model of acute hyperammonemia. The same study showed that animals injected with Tat-Beclin 1 peptide are protected against the toxicity of ammonia accumulation. Importantly, stimulation of autophagy helps to eliminate ammonia by increasing the levels of an intermediate of the urea cycle in the liver. In addition, rapamycin treatment protects against acute and chronic hyperammonemia via the induction of hepatic autophagy. The possibility of manipulating autophagy in other pathological settings in the liver will be discussed in the following sections.

## Modulation of autophagy for therapeutic purposes: A promising approach for the management of liver diseases

### Inducing autophagy: a potential therapeutic strategy in the context of acute liver injury, non-alcoholic fatty liver disease, fibrosis

*Autophagy and acute liver failure*

In animal models, loss of autophagy promotes spontaneous hepatomegaly and liver injury, that can be reversed by a variety of autophagy activators or by using an inducible shRNA targeting *Atg5*.[65] Studies have revealed the crucial role of autophagy in limiting hepatocyte death in

**Key points**

Autophagy has a crucial role in limiting hepatocyte death in response to stress-induced liver injury. Thus, pharmacological activators of autophagy are an exciting therapeutic option for treatment of acute liver failure.

response to stress-induced liver injury, promoted by hepatotoxic drugs, death receptors, and ischemia-reperfusion.

Liver injury promoted by acetaminophen (APAP) overdose is a major cause of acute liver failure in humans. The toxicity of APAP is due to the formation of the reactive metabolite N-acetyl-p-benzoquinone imine, which causes the depletion of cellular glutathione (GSH) and the formation of APAP protein adducts. This results in mitochondrial damage, oxidative stress, activation of mitogen-activated protein kinases and hepatocyte necrosis.[66] Autophagy is enhanced after APAP overdose, acting as a defense mechanism to remove damaged mitochondria.[67] Pharmacological activation of autophagy by rapamycin protects against APAP-induced liver injury, whereas inhibition of autophagy by chloroquine or inducible deletion of ATG7 in hepatocytes further enhances liver damage.[67,68] The hepatoprotective effects of autophagy on APAP-induced liver injury also rely on the formation of APAP protein adducts, as shown by enhanced levels of soluble APAP protein adducts in p62-deficient hepatocytes exposed to APAP.[69] However, the picture is more complex than anticipated, since mice bearing a hepatocyte-specific deletion of Atg5 are protected against the liver damage elicited by APAP overdose, most likely via compensatory mechanisms, leading to GSH repletion and hepatocyte proliferation.[70] Moreover, unexpectedly, recent data also demonstrate that by promoting activation of c-Jun N-terminal kinase signalling the kinase activities of ULK1/2 are essential in APAP-induced liver injury, independently of their activity in autophagy.[71] Based on the data from these studies, it cannot be excluded that autophagy-dependent and independent ULK1 pathways result in opposing regulation of APAP-induced liver injury.

Sterile inflammation has also been proposed to contribute to APAP-induced toxicity, via inflammasome-dependent release of IL-1β.[72] The identity of the cell(s) involved is still controversial, but neutrophils and Kupffer cells are thought to contribute.[73] Interestingly, it is now well documented that mitochondrial damage may be a cause of inflammasome activation. Elimination of damaged mitochondria by autophagy may thus be critical to prevent this excessive inflammasome activation.[74,75] Whether mitophagy in neutrophils and/or Kupffer cells may contribute to protection against APAP-induced liver injury remains to be investigated.

Autophagy also protects hepatocytes from death receptor-mediated acute liver injury, a feature of viral hepatitis, acute alcoholic hepatitis and non-alcoholic steatohepatitis. The underlying mechanism involves both direct and indirect effects on hepatocytes and macrophages. Direct effects occur through autophagy-dependent inhibition of caspase 8 in hepatocytes.[76] Indirect effects on macrophages may rely on p62-dependent mitophagy, which has been shown to limit NF-κB-mediated inflammation and inflammasome-dependent IL-1β production.[77,78]

During ischemia-reperfusion (I/R) injury, the liver is transiently subjected to a reduction of blood supply, and thus lacks nutrients and oxygen, resulting in liver injury due to ATP depletion, hypoxia and alterations in the microcirculation. Liver damage is further amplified by reperfusion, due to Kupffer cell activation, resulting in infiltration by activated polymorphonuclear cells and neutrophil-dependent liver dysfunction. The role of autophagy in I/R injury is controversial, with opposite effects depending on the type or model of ischemia (warm or cold) and/or the preservation solution used. Understanding the role of autophagy in I/R injury is further complicated by the lack of accurate evaluation of autophagy *in vivo* (reviewed in[79]).

The hepatoprotective and anti-inflammatory effects of autophagy during acute liver failure suggest that newly developed pharmacological activators of autophagy may represent an interesting therapeutic option, in particular in the context of APAP overdose. Further studies are needed to better delineate the benefit of pharmacological modulation of autophagy in the context of liver I/R injury.

*Autophagy and inherited liver diseases*

Wilson's disease is caused by copper overload in hepatocytes, as a consequence of mutations in the Cu pump ATP7B. The hepatotoxicity of copper results from its capacity to damage cellular organelles and affect the redox balance. Recent data highlight the protective role of autophagy against copper toxicity during Wilson's disease.[80] Interestingly, autophagy is increased both in the liver of patients with Wilson's disease, in mice bearing a deletion in *Atp7b* and in hepatoma cells silenced for ATP7B and exposed to $CuCl_2$. *In vitro* or *in vivo* inhibition of autophagy with spautin-1 accelerates hepatocyte death, mitochondrial damage and oxidative stress, whereas activation of autophagy by starvation or TFEB overexpression promotes hepatocyte survival.[80] The potential for activators of autophagy to counteract copper toxicity in the context of Wilson's disease needs to be further explored and extended to other metal-related liver disorders, such as haemochromatosis.

Alpha-1 antitrypsin deficiency (AATD) is caused by homozygosity for alpha-1 antitrypsin mutant Z protein (ATZ), and is associated with an increased risk of liver disease in adults and children, as well as lung disease in adults.[81] The mutant ATZ folds improperly during biogenesis and is retained within the endoplasmic reticulum of hepatocytes. The hepatocytes of patients with AATD display an increased accumulation of autophagosomes containing ATZ.[82] Likewise, *ATG5*-KO cells exhibit retarded degradation of ATZ with characteristic cellular inclusions of ATZ,

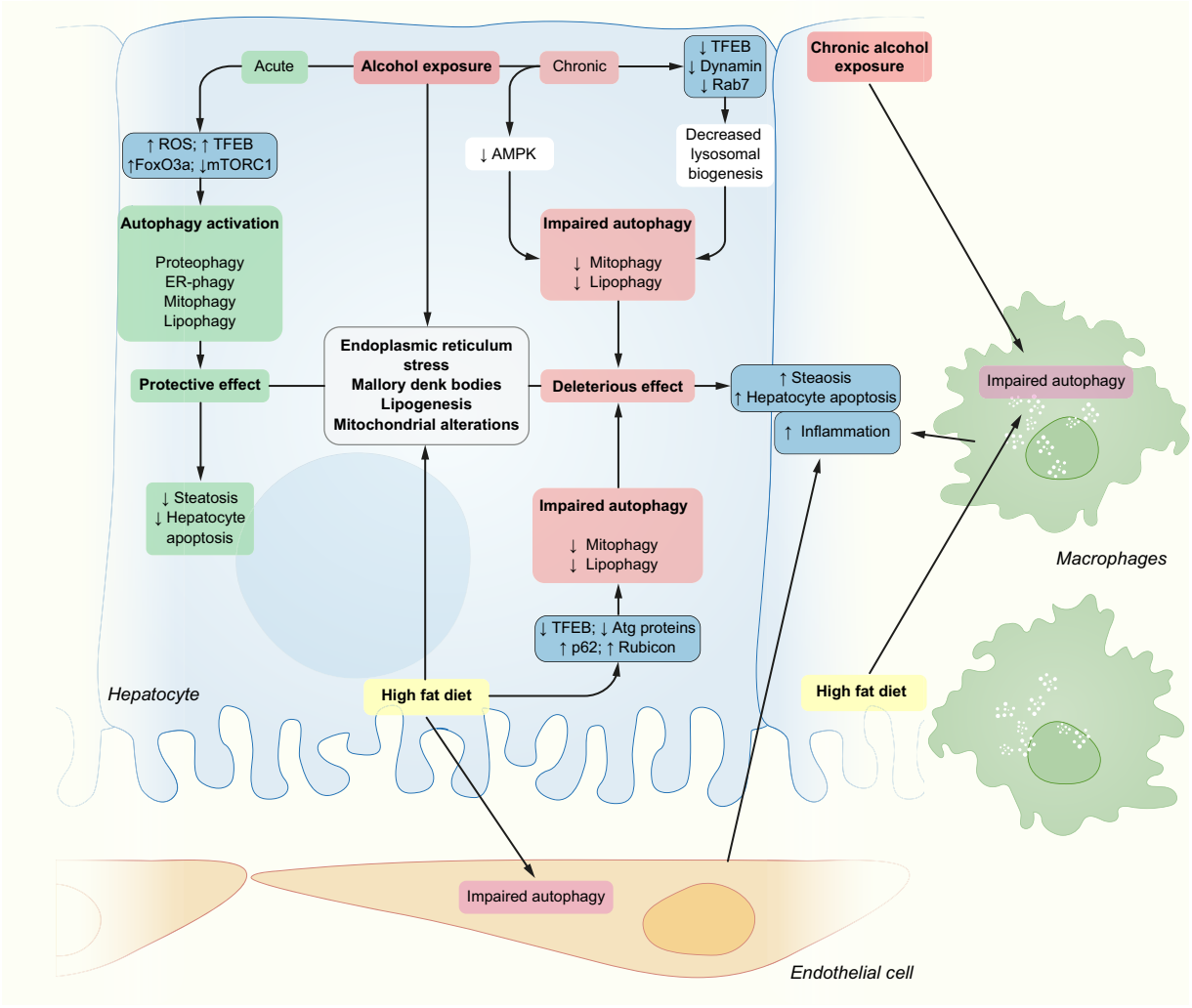


**Fig. 2. Autophagy and fatty liver diseases: Common and specific pathways.** Chronic alcohol consumption and overfeeding cause similar damage to hepatocytes by promoting endoplasmic reticulum stress, lipogenesis, mitochondrial alterations and emergence of Mallory-Denk bodies. In both conditions, autophagy is impaired, promoting disease progression. In addition, in macrophages and endothelial cells, impaired autophagy contributes to increased inflammation. In contrast, acute alcoholic consumption will activate autophagy and decrease hepatocyte apoptosis. ATG, autophagy-related gene.

suggesting that autophagy plays a major role in the degradation of ATZ.[83] However, degradation is insufficient and a proportion of ATZ persists in intractable inclusions, with toxic effects on hepatocytes, leading to liver injury and fibrosis.

Several preclinical studies have shown that induction of autophagy improves the liver injury and fibrosis caused by AATD. Hepatocyte *TFEB* gene transfer in mice with AT deficiency results in a dramatic reduction of hepatic ATZ, liver apoptosis and fibrosis.[34,84] Moreover, activation of autophagy by carbamazepin decreased the hepatic load of ATZ and reduced liver fibrosis in a mouse model of the disease. These results provided the basis for an ongoing phase II trial testing the effect of 52 weeks of carbamazepin in patients aged 14 to 80 with AATD-related cirrhosis (https://clinicaltrials.gov/ct2/show/NCT01379469). The beneficial effect of activating autophagy in AATD has also been observed with other drugs that induce autophagy, including rapamycin,[85] and norursodeoxycholic acid.[86]

Thus, activating autophagy appears to be an attractive strategy for human disorders caused by intracellular accumulation of toxic proteins or metals. It should also be emphasized that autophagy deficiency has been reported in other inherited liver metabolic diseases, such as glycogen storage disease type Ia (GSD-Ia). GSD-Ia is characterized by a deficiency in glucose-6-phosphatase-α (G6Pase-α), resulting in metabolic defects including impaired glucose homeostasis and hepatomegaly. Preclinical studies have recently shown that G6Pase-α deficiency leads to autophagy impairment, associated with downregulation of mTOR and SIRT1 signalling.[87,88] Restoration of hepatic G6Pase-α expression corrects metabolic abnormalities, and normalizes defective autophagy, suggesting that patients with GSD1a could also benefit from the use of autophagy activators.

**Key points**

Activating autophagy is a promising strategy for the treatment of human disorders characterised by the intracellular accumulation of toxic proteins/metals.

*Autophagy in non-alcoholic fatty liver disease*

With the rising incidence of obesity and diabetes, non-alcoholic fatty liver disease (NAFLD) has become a leading cause of chronic liver diseases and hepatocellular carcinoma (HCC) worldwide, with non-alcoholic steatohepatitis (NASH) projected to become the most common indication for liver transplantation in the next decade.[89] Accumulating evidence suggests that autophagy may represent a valuable target in NAFLD, because of its anti-steatogenic properties in hepatocytes (lipophagy), and its beneficial effects on the transition to NASH, through the hepatoprotective effects of mitophagy in hepatocytes and anti-inflammatory properties in macrophages.

Autophagy is decreased by a wide variety of signals that contribute to the pathogenesis of NAFLD, including insulin resistance, excess triglycerides and free fatty acids, endoplasmic reticulum stress and oxidative stress.[90] Accordingly, reduced autophagy during NAFLD has been well documented in animal models and in humans, although accurate methods for measuring autophagic fluxes in humans are still lacking. ATG proteins and TFEB expression are decreased in patients with NASH and in mice fed a high-fat or a methionine-choline-deficient diet, whereas p62 and the autophagy inhibitor Rubicon are increased (Fig. 2).[90–92] Compared to patients with simple steatosis or normal livers, decreased levels of autophagy are observed in the liver endothelial cells of patients with NASH.[93] In keeping with these data, mice deficient in components of the autophagic pathway in specific liver cells, including deletion of Atg7,[31] Atg14[94] or Tfeb proteins[40] in hepatocytes, deletion of Atg5 in endothelial cells[93] and myeloid cells,[95] or animals exposed to 3-MA, show exacerbated features of NAFLD when undergoing high-fat diet feeding. They show enhanced accumulation of lipid droplets[31,40,94] and/or Mallory-Denk bodies containing ubiquitinated p62 in hepatocytes, as well as increased hepatocyte injury, endoplasmic reticulum stress and/or production of inflammatory cytokines.[93,95] Conversely, mice bearing a deletion of the autophagy inhibitor Rubicon or adenoviral delivery of ATG7 show improvement in both steatosis and liver injury, associated with restoration of autophagy and decreased endoplasmic reticulum stress.[38,92] Recent data also indicate that impaired mitophagy may also contribute to liver injury during NAFLD and result in megamitochondria formation.[96]

However, controversy still exists about the role of some components of the autophagic pathway in steatosis, since hepatocyte-specific deletion of Atg7 exacerbates[31] or blunts hepatic steatosis,[97] and hepatocyte deletion of focal adhesion family kinase-interacting protein of 200 kDa (FIP200), a core component of the complex initiating autophagosome formation, also reduces hepatic triglyceride content in mice fed a high-fat diet.[98] Although these findings need to be reconciled, the use of pharmacological activators of autophagy has provided encouraging results in animal models and patients with NAFLD.

Currently, the only therapeutic options for NAFLD remain lifestyle interventions, typically involving dietary modifications and changes in physical activity. Interestingly, caloric restriction and exercise are 2 main autophagic stimuli,[99] and may at least in part underlie some of their beneficial consequences on liver dysfunction and steatosis. Targeting AMP-activated protein kinase (AMPK) with metformin or the disaccharide tetrahelose,[100] or administering caffeine to enhance lipophagy and beta-oxidation[101] have also shown promising anti-steatogenic effects. In addition, the use of TFEB agonists has recently been the focus of a number of studies, based on the demonstration that TFEB overexpression in hepatocytes protects against steatosis via autophagy in mice fed a high-fat diet. In a recent preclinical study, digoxin, ikarugamycin and alexidine dihydrochloride were selected from a 15,000-chemical library for their ability to enhance autophagic flux and capacity to promote TFEB nuclear translocation. These TFEB agonists improved lipid metabolism, insulin resistance and steatosis in mice fed a high-fat diet, and were associated with reversion of p62 accumulation in hepatocytes.[48] Similar findings were obtained when using an autophagy inducer, identified by high-throughput screening of a chemical library against metabolic syndrome, which conferred beneficial effects by inducing nuclear TFEB translocation.[102] In line with these reports, activating TFEB by ezetimibe, an inhibitor of NPC1L1-dependent cholesterol transport,[103] also protects against steatosis and hepatocyte injury. Because recent data also indicate that TFEB overexpression in macrophages induces autophagy and limits IL-1β production,[104] further studies should evaluate whether agonists of TFEB could also limit the inflammation associated with progression to NASH. Encouraging data obtained with ezetimibe[103] and tetrahelose[104] show that these compounds inhibit NRLP3-dependent IL-1β production in macrophages, and that ezetimibe reduces NAFLD lesions, whereas tetrahelose displays atheroprotective properties in experimental models. Interestingly, all the aforementioned drugs are already FDA-approved, and ezetimibe has been evaluated in clinical trials for patients with NASH, although conclusive results need larger studies.[105]

In conclusion, dysregulation of autophagy during NAFLD and the demonstration of beneficial effects of molecules targeting autophagy both in animal models and clinical settings is promising. Whether the effects of available drugs are fully linked to their effects on autophagy remains to be elucidated, and further development of more selective autophagy inducers may be valuable for therapeutic purposes.

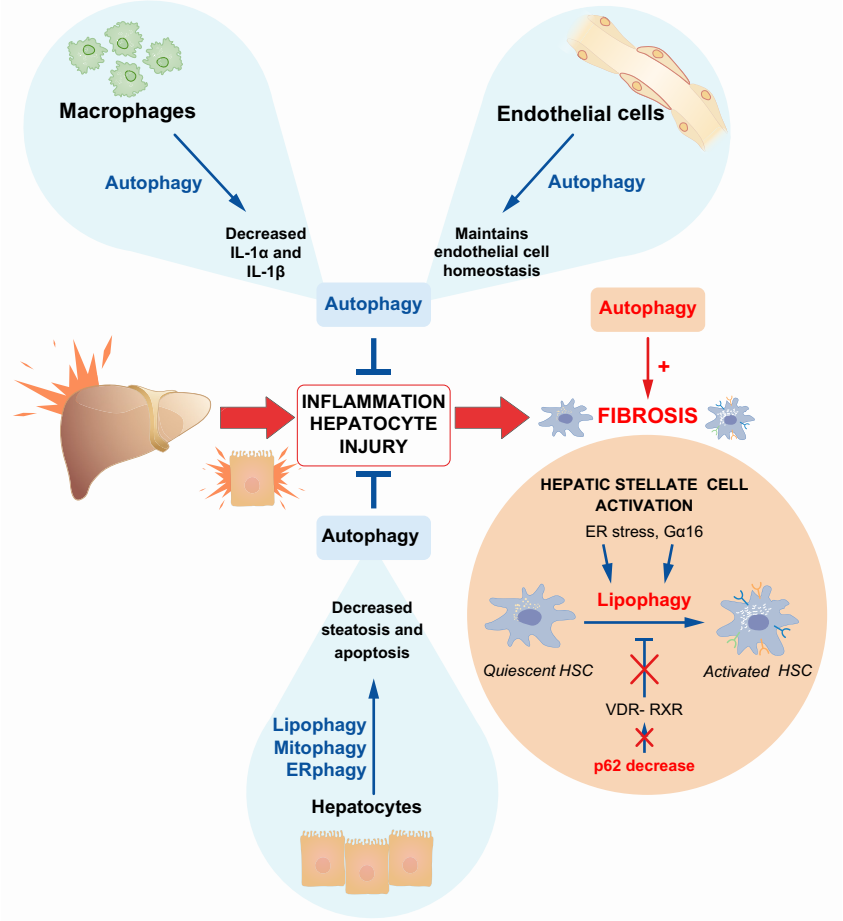


**Fig. 3. Autophagy and liver fibrosis: the good and the dark side**. Hepatocyte injury initiates the fibrogenic process by triggering inflammation and HSC activation, during which quiescent HSCs lose their lipid vacuoles and acquire myofibroblastic features associated with the capacity to secrete matrix components and inhibitors of matrix degradation. Autophagy plays a complex role in the fibrogenic process: in hepatocytes, macrophages and endothelial cells, autophagy indirectly protects against liver fibrosis i) by limiting hepatocyte injury, ii) maintaining endothelial cell homeostasis iii), and reducing the production of inflammatory cytokines by macrophages and endothelial cells. However, in HSC, autophagy displays fibrogenic properties via 2 distinct processes, i) lipophagy which allow lipid droplet digestion and release of ATP required to promote the activation process, and ii) via p62 loss, which impairs VDR-RXR interaction, a complex critical for maintaining HSC in a quiescent state. ER, endoplasmic reticulum; HSC, hepatic stellate cell.

## Fibrosis

A key step in the fibrogenic process is the activation of hepatic stellate cells (HSCs), the main fibrogenic cell population, following a phenotypic switch from a lipid-rich to a fibrogenic myofibroblastic phenotype. Phenotypic changes are initiated by repeated hepatocyte or biliary cell death and via complex interactions between parenchymal, endothelial and immune cells. In parallel, quiescent HSCs lose their lipid vacuoles, which contain retinoid stored as retinyl esters, cholesteryl esters and triglycerides.[106] Recent data demonstrate that the role of autophagy on fibrosis is complex and depends on the cell type (Fig. 3).

### *Activation of hepatic stellate cells by autophagy: The dark side*

Autophagic flux is increased in HSCs upon activation, with a number of reports now showing that autophagy in HSCs contributes to the phenotypic switch to a myofibroblastic phenotype. In landmark studies, Hernandez-Gea *et al.*[107] and Thoen *et al.*[108] showed that autophagy promotes digestion of lipid droplets in quiescent HSCs, thereby facilitating HSC activation. The link between autophagy and the loss of lipid droplets was confirmed *in vitro* with pharmacological inhibitors of autophagy and small interfering RNAs against ATG5 or ATG7. These studies demonstrated that autophagy promotes catabolism of retinyl esters by lipases, thereby generating free fatty acids that increase adenosine triphosphate levels following mitochondrial ß-oxidation. *In vivo* studies further showed that mice bearing a specific *Atg5* or *Atg7* deletion in HSCs are resistant to fibrosis and display decreased HSC activation upon chronic carbon tetrachloride or thioacetamide challenge.[107] Mechanistic studies demonstrated that endoplasmic reticulum stress (via the XBP1 arm of the unfolded protein response[74,109]), oxidative stress or G proteins (G alpha 12)[110] promote HSC activation by enhancing autophagy, resulting in enhanced fibrosis.

**Key points**

The role of autophagy in fibrosis is complicated, with opposing outcomes depending on the cell type in which it is activated.

In addition to lipophagy, downregulation of the autophagy substrate p62 may serve as an additional mechanism of HSC activation. This adaptor protein is constantly degraded via autophagy through its LIR domain, which binds to LC3 on autophagosome membranes, although autophagy-independent effects may also occur.[111] During experimental fibrosis, p62 expression is downregulated in HSCs in humans and mice. HSCs with p62 knocked-down show enhanced expression of α-SMA protein and *COL1A1* mRNA.[112] In addition, mice with p62 deficiency in HSCs, either via the GFAP or L-RAT Cre-LoxP system, are more prone to fibrosis than wild-type counterparts.[112] Interestingly, the loss of p62 in HSCs impairs vitamin D receptor-retinoid X receptor interaction,[112] a complex critical for maintaining HSCs in a quiescent state.[113] These data demonstrate that by promoting vitamin D receptor-retinoid X receptor heterodimerization, p62 is a cell autonomous repressor of HSC activation and the fibrogenic phenotype. Whether this effect is autophagy-dependent remains to be evaluated.

*Autophagy in macrophages, endothelial cells and hepatocytes: The good side*

Because hepatocyte apoptosis is a central event in the initiation and maintenance of the fibrogenic process, autophagy may be considered an anti-fibrogenic pathway because it generates a survival signal for hepatocytes. Old mice with hepatocyte-specific deletion of Atg5 spontaneously develop liver fibrosis, because of proteotoxicity and disruption of pro-and anti-apoptotic protein homeostasis.[114] In endothelial cells, selective disruption of Atg5 or Atg7 impairs endothelial phenotype and favours liver injury, inflammation and fibrosis when mice are exposed to carbon tetrachloride or to prolonged high-fat diet feeding.[93,115]

Autophagy is an anti-inflammatory pathway in macrophages of various origins, including Kupffer cells, because it limits IL-1β production, a survival and profibrogenic pathway for HSC. Co-culture of ATG5-deficient Kupffer cells with HSCs increase the fibrogenic properties of HSCs, an effect blunted in the presence of an IL-1R antibody.[116] Moreover, mice with an Atg5 deficiency in the myeloid lineage produce enhanced hepatic Il-1β and are more prone to liver injury and fibrosis than wild-type counterparts. Therefore, macrophage autophagy directly prevents fibrosis via paracrine interactions with hepatic myofibroblasts. Macrophage autophagy may also indirectly limit fibrosis via hepatoprotective properties[116] and/or possible crosstalk with other immune cells that require IL-1α and IL-1β for their activation, such as Th17 lymphocytes.[117]

*Targeting autophagy as an anti-fibrogenic strategy?*

Autophagy is undoubtedly a major player in liver fibrosis. However, because autophagy promotes HSC activation but generates anti-fibrogenic signals via its hepatoprotective and anti-inflammatory effects[76] in hepatocytes, macrophages and endothelial cells, targeting autophagy for anti-fibrogenic therapies will require cell type specific approaches. Interestingly, activation of autophagy in macrophages using molecules targeting the endocannabinoid system, such as agonists of the cannabinoid receptor type 2 or monoacylglycerol lipase inhibitors, have shown anti-inflammatory and anti-fibrogenic effects.[118,119] However, conflicting studies have reported reductions in liver fibrosis in response to autophagy activators such as rapamycin[120,121] or carbamazepine.[122] Because these pharmacological modulators may also have off target effects, the use of more specific autophagy modulators (see earlier) may be more promising.

## Targeting autophagy: A more complex approach as far as hepatocellular carcinoma and alcohol-related liver disease are concerned

### Hepatocellular carcinoma

HCC is the second leading cause of cancer-related death worldwide, mainly occurring in the context of cirrhosis.[123,124] HCC results from a unique combination of somatic genetic alterations in various signalling pathways (*i.e.* cell cycle, telomere maintenance, oxidative stress pathway, PI3K/Akt/mTOR, Ras/Raf/MAP kinase and Wnt-β-catenin pathways) that cooperate to promote oncogenesis.[123–125] Autophagy can interact with some of these pathways and it plays a dual role in the carcinogenic process, inhibiting the initiation process, while promoting tumour growth, metastasis and therapeutic resistance during tumour progression.[126,127]

*Protective effects of autophagy in hepatocellular carcinoma initiation*

Several lines of evidence suggest that autophagy protects against tumour initiation by maintaining intracellular homeostasis. Through deletion of Beclin-1, Atg5 or Atg7, impairment of autophagy promotes spontaneous liver tumorigenesis in aged mice.[30,114,128–133] It also leads to accumulation of p62 which, on the one hand, blocks the antioxidant functions of nuclear factor erythroid-2-related factor 2 (NRF2) by binding to Kelch-like ECH-associated protein 1 (KEAP1).[134–139] On the other hand, accumulation of p62 also contributes to carcinogenesis through crosstalk with NF-kB, PI3K/Akt/mTOR and Wnt-β-catenin signalling pathways.[140]

The protective effects of autophagy also involve enhanced degradation of Yap, the major nuclear effector of the Hippo signalling pathway. The Hippo pathway controls liver growth and Yap overexpression is an early event in hepatocarcinogenesis.[141] Mice with a liver-specific Atg7 deficiency display increased Yap protein levels and overexpression of Yap target genes. Concordantly, Yap deletion in these mice with liver-specific Atg7 deficiency decreases HCC incidence, without

**Table 1. Clinical trials targeting autophagy in liver diseases.**

| ClinicalTrials.gov Identifier: | Title | Patients | Objective |
|---|---|---|---|
| NCT03208868 | Leucine enriched essential amino acid mixture to reverse muscle loss in cirrhosis | Cirrhosis, Child Pugh score 5–9 | Test whether administration of leucine, a direct stimulant of mTOR, for 90 days reduces autophagy in skeletal muscles and thus improves sarcopenia associated with cirrhosis |
| NCT03037437 | Modulation of sorafenib induced autophagy using hydroxychloroquine in hepatocellular carcinoma | Adults with advanced or metastatic hepatocellular carcinoma | Determine whether adding the autophagy blocker hydroxychloroquine on top of sorafenib improves control of hepatocellular carcinoma as compared with sorafenib alone in patients progressing on sorafenib alone |
| NCT01379469 | Carbamazepine in severe liver disease due to alpha-1 antitrypsin deficiency | Patients aged 14 to 80, with severe liver disease (HVPG ≥10 mmHg) due to alpha-1-antitrypsin deficiency | To determine if 52 weeks carbamazepine therapy leads to a significant reduction in the hepatic accumulation of ATZ |

ATZ, alpha-1 antitrypsin mutant Z protein; HVPG, hepatic venous pressure gradient.

affecting the p62-NRF2 axis. Therefore, by controlling the induction of Yap, autophagy acts as a gate keeper of carcinogenesis, independently of the p62-NRF2 axis.[129]

*Promotion of hepatocellular carcinoma progression by autophagy*

In established HCC, autophagy is increased and tumour cells recruit energy through autophagy, which improves their survival ability in hypoxic and low-nutrient environments, promoting cancer progression.[142–144] In addition, autophagy could promote HCC cell invasion through activation of the epithelial–mesenchymal transition.[145] Finally, a prognostic role of LC3 and Beclin-1 has been supported by several studies.[146]

In conclusion, autophagy plays opposing roles in HCC by protecting from carcinogenesis at early stages and by promoting tumour progression at more advanced stages. This dual role illustrates the complexity of targeting autophagy for the treatment of HCC.

*Targeting autophagy in hepatocellular carcinoma: A complex approach*

In established tumours (*e.g.* glioblastoma, brain metastasis, melanoma, pancreatic cancer), several phases I/II trials support the idea that inhibiting autophagy (*e.g.* (hydroxy)chloroquine) in combination with classical anticancer treatments (*e.g.* chemotherapy, targeted therapy, radiation therapy) could improve clinical outcomes without serious adverse events.[126] These preliminary results were confirmed in a prospective controlled randomized trial, where chronic administration of chloroquine in combination with conventional surgery, chemotherapy and radiotherapy improved survival in patients with glioblastoma multiform.[147] Modern management of HCC is complex and separates early stages accessible to curative treatment from advanced stages treated using a palliative approach.[124] Currently, no data about autophagy modulation combined with conventional HCC therapy are available in humans. However, preclinical data suggest autophagy as a potential therapeutic target in HCC. Activation of autophagy has been observed following percutaneous treatment and transarterial chemoembolization in residual tumour cells in rodents and rabbits. A combination of these treatments with an autophagy inhibitor (*e.g.* (hydroxy)chloroquine, 3-MA and Lys05) was associated with enhanced tumour cell necrosis and apoptosis,[148–151] suggesting that inhibiting autophagy to target residual tumour cells could be beneficial. Moreover, autophagy is implicated in tumour cells' resistance to systemic treatments.[126] Indeed, sorafenib induces autophagy,[152] and preclinical data show that combined therapy with autophagy inhibitors (*i.e.* chloroquine, miR-375) improves tumour response.[153,154] However, attention should be paid to the deleterious consequences of blocking autophagy in dying tumour cells, as autophagy is required for effective antitumor T cell response. Considering that immune-mediated clearance of tumour cells is crucial to suppress HCC development, a combination of immune checkpoint inhibitors together with autophagy enhancers could boost antitumor immune responses.[155–157]

## Alcohol-related liver disease

Binge and chronic alcohol consumption are major healthcare problems, and it is known that repeated binge-drinking sensitizes the liver to progression toward steatohepatitis and/or cirrhosis. Chronic ethanol exposure impairs lysosome function, promoting hepatomegaly and hepatic protein accumulation. The involvement of autophagy in alcohol-related liver disease is complex, since acute alcohol consumption activates autophagy, whereas the impact of ethanol upon chronic exposure is more controversial.

**Key point**

Targeting autophagy for the treatment of hepatocellular carcinoma is complicated by the fact that it has different effects at different disease stages.

Acute alcohol consumption activates autophagy through different mechanisms. Ethanol causes the generation of reactive oxygen species, endoplasmic reticulum stress, lipogenesis and mitochondrial alterations.[158] Ethanol also inhibits the mTORC1 complex, leading to activation of ULK1, and increases the nuclear translocation of FoxO3a which elevates the transcription of many autophagy-related genes.[158,159] Finally, acute ethanol exposure increases the nuclear level of TFEB.[160,161] As a consequence, autophagy is hepatoprotective in response to acute alcohol

exposure, by decreasing hepatocyte apoptosis and steatosis through degradation of damaged mitochondria and lipid droplets.[162]

A recent study has reconciled the controversial existing data on autophagy upon chronic alcohol exposure. Previous studies showed that chronic ethanol feeding increases autophagosome numbers in rodent liver, suggesting the induction of autophagy.[163,164] Nevertheless, even if an increase in the number of autophagosomes was reported in these studies,[161,163,164] the number of lysosomes is decreased by ethanol and associated with reduced autophagic flux.[161] Defects in lysosome number are associated with a decrease in nuclear TFEB accumulation and impairment of lysosomal functions.[160,161] Adenoviral overexpression of TFEB reverses the deleterious effects of alcohol on lysosome biogenesis and mice become resistant to steatosis and hepatocyte injury.[161] An additional mechanism that could underlie reduced autophagy following chronic alcohol exposure is the inactivation of the small guanosine triphosphate Rab7 and reduced dynamin 2 activity, which causes depletion of lysosomes and inhibits hepatocyte lipophagy.[165,166] Finally, inhibition of AMPK by alcohol results in decreased mitochondrial beta-oxidation and increased lipogenesis.[167] Additional anti-inflammatory effects of autophagy in macrophages are likely to reduce cytokine production by macrophages and therefore to block neutrophil recruitment to the liver (Fig. 2).[118]

*Targeting autophagy in alcohol-related liver disease*

Currently, no specific therapy against alcohol-related liver disease is available, and the main option remains alcohol withdrawal. Preclinical data indicate that enhancing autophagy using carbamazepine or rapamycin decreases steatosis and liver injury in mice fed chronic ethanol.[163] Due to common features of NASH and alcohol-related liver diseases, it is likely that autophagy-targeted approaches validated in NASH could be evaluated in the context of alcohol-related liver disease. In particular, the use of TFEB agonists that combine both hepatoprotective effects on hepatocytes and anti-inflammatory effects on macrophages deserves particular attention.

## Challenge for translation into human clinical trials

Autophagy is now recognized as a process that can be usefully modulated in liver diseases to eliminate protein aggregates, damaged organelles, and lipid droplets, but also to regulate inflammatory signalling. Some clinical trials targeting autophagy are already ongoing in specific liver diseases (Table 1). Yet, as a result of the broad range of cellular functions regulated by autophagy in physiological and pathological conditions, several challenges will have to be addressed before widely testing autophagy-modulating approaches in clinical trials.[53] First, a better characterization of pathways regulating autophagy in liver diseases might uncover targetable autophagy-specific regulatory steps. In particular, it should be considered that ATG proteins are not exclusively involved in canonical autophagy[53] and that manipulating autophagy may impact other interconnected pathways.[9] Second, identification of autophagy biomarkers and methods to assess the autophagic flux (*i.e.* the flux of material from the autophagosome into the lysosomal lumen) *in vivo* are important steps for the development of autophagy-targeting strategies, that should be disease-specific. Third, identification of the optimal therapeutic time-window for chronic liver disease will also be needed, since the type and level of autophagy likely varies during liver disease progression from initial steps to cirrhosis. Autophagy might indeed be beneficial at initial steps and detrimental at more advanced stages, and vice-versa. Fourth, strategies targeting a specific liver cell type using dedicated vectors would be highly desirable since they would likely limit side effects related to the physiological role of autophagy in all organs. Finally, defining careful dose adjustments and frequency of administration will be needed, since excessive activation of autophagy might be deleterious,[168] and therapeutic effects of autophagy-modulating agents might also be achieved with only intermittent therapy.[37] In addition, the circadian rhythm of autophagy in the liver is a parameter to be considered for optimization of treatment efficiency.[169] Extensive research within the last 10 years has unravelled the central role of autophagy in liver pathophysiology and highlighted its potential as a novel therapeutic target. The time has now come for translation.

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## Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

## Supplementary data

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