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AUTOPHAGY IN LIVER DISEASES: TIME FOR TRANSLATION?

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Abbreviations

3-MA: 3-methyladenine
AMPK: AMP-activated protein kinase
APAP: acetaminophen
APAP-AD: APAP protein adducts
AT : alpha-1 antitrypsin
ATG : autophagy-related genes
ATZ : alpha-1 antitrypsin mutant Z protein
FIP200: family kinase-interacting protein of 200 kDa
G6Pase- α : glucose- 6-phosphatase- α
GSD-Ia : Glycogen storage disease type Ia
GSH: cellular glutathione
HCC : hepatocellular carcinoma
HSC: hepatic stellate cells
I/R: ischemia-reperfusion
KEAP1: Kelch-like ECH-associated protein 1
LAP: LC3-associated phagocytosis
MAPK: mitogen-activated protein kinases
miR: microRNAs
NAFLD: Non-Alcoholic Fatty Liver Disease
NASH: non-alcoholic steatohepatitis
NRF2: nuclear factor erythroid-2-related factor 2
PI3P: phosphatidylinositol 3-phosphate

Abstract

Autophagy is a self-eating catabolic pathway that contributes to liver homeostasis through its role on 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 impact 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 in the context of storage disorders (alpha 1 antitrypsin deficiency, Wilson's disease), acute liver injury, non-alcoholic steato-hepatitis and chronic alcoholic liver disease may prevent the progression of these diseases. Nevertheless, in some situations such as fibrosis, targeting specific liver cells must be considered, as autophagy displays opposite functions depending on the cell type. In addition, optimal therapeutic time-window should be identified, since autophagy might be beneficial at initial steps and detrimental at more advanced stages as in the case of hepatocellular carcinoma. Finally, identification of autophagy biomarkers and methods to monitor autophagic flux *in vivo* is an important step for future development of personalized autophagy-targeting strategies. This review provides an update on the regulatory role of autophagy in various aspects of liver pathophysiology, describe the different strategies to manipulate autophagy and discusses the potential for modulating autophagy as a therapeutic strategy in the context of liver diseases.

A. 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, is an anti-aging mechanism [4, 5], and an actor of the immune response [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 three 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, 5, 9]. During autophagosome formation, part of the cytoplasm, which may include organelles, proteins aggregates, and lipid droplets, is sequestered in a bulk or in a selective way. Many forms of selective autophagy exist (in particular mitophagy for the selective sequestration of mitochondria, ER-phagy for the selective sequestration of fractions of the endoplasmic reticulum, xenophagy for the selective sequestration 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 sequestration 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 the fusion with endosomal compartments before the final fusion with the lysosomal compartment [15, 16].

Macroautophagy is dependent on the activity of autophagy-related genes (ATG) proteins and class III phosphatidylinositol 3-kinase (VPS34) in a complex with Beclin 1 (the ortholog of

the yeast Atg6) and ATG14L to produce phosphatidylinositol 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 address 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 exclusive 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 translocation through the lysosomal membrane is dependent on the lysosomal membrane protein Lamp2a. Chaperone-mediated autophagy has recently gained attention because 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 will be mainly discussed in the present review. Autophagy was first described in kidney and liver [3, 17, 21], as a response to starvation and results in recycling of amino acids, carbohydrates, and fatty acids to the cytosol through the lysosomal degradation of autophagy cargo in

hepatocytes. Pioneering works 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 to 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 consists in 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 levels and in contrast to autophagy, ULK1 is not required for LAP. Moreover, Rubicon, which inhibits the maturation of autophagosome during autophagy, is required at the early stage of LAPosome formation to produce PI3P by 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.

B. Strategies to modulate autophagy (Figure 1)

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

regulate the expression of autophagy genes and that of lysosomal proteins. 2. during autophagosome formation at steps of initiation, nucleation of the phagophore (or isolation membrane), its elongation and maturation, and sealing of the autophagosome; and 3. 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. Manipulation of autophagic proteins by specific gene deletion or overexpression have demonstrated that components of the autophagic machinery may show therapeutic benefit in clinical settings.

1. 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 by reducing 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 brain, muscle, and liver. In particular, overexpression of the transcription factor TFEB, which coordinately regulates autophagy, lysosomal biogenesis and fatty acid oxidation and protects mouse liver from damage caused by a high-fat diet [40].

2. Pharmacological approaches.

Historically the first reported autophagy inhibitor was 3-methyladenine (3-MA) that 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 ATG, 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] (**Figure 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 efficiency. 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].

3. 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 structure in striated muscles and show beneficial effects on muscular weakness and recovery time [63].

4. Targeting multiple autophagy pathways for therapeutic purpose

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 increases the levels of an intermediate of the urea cycle in the liver to eliminate ammonia. In addition, rapamycin treatment protects against acute and chronic hyperammonemia via the induction of hepatic autophagy. The possibility of manipulating autophagy in other liver pathological settings will be discussed in the following sections.

C. Modulation of autophagy for therapeutic purposes: a promising approach for the management of liver diseases

1. Inducing autophagy: a potential therapeutic strategy in the context of acute liver injury, NAFLD, fibrosis

1.1. Autophagy and acute liver failure (ALF)

In animal models, loss of autophagy promotes spontaneous hepatomegaly and liver injury, that can be restored by a variety of autophagy activators or by using an inducible shRNA targeting ATG5 [65]. Concordant studies have revealed the crucial role of autophagy in

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

Autophagy and paracetamol overdose. Liver injury promoted by acetaminophen (APAP) overdose is a major cause of acute liver failure in humans. Toxicity of APAP is due to the formation of a reactive metabolite, presumably N-acetyl-p-benzoquinone imine, which causes the depletion of cellular glutathione (GSH) and the formation of APAP protein adducts (APAP-AD). This results in mitochondrial damage, oxidative stress, activation of mitogen-activated protein kinases (MAPK) and hepatocyte necrosis [66]. As a defense mechanism, APAP overdose leads to enhanced autophagy in hepatocytes to remove damaged mitochondria [67]. Pharmacological activation of autophagy by rapamycin protects against APAP-induced liver injury, whereas autophagy blockade 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 relies on the formation of APAP-AD protein adducts, as shown by enhanced levels of soluble APAP-AD in p62-deficient hepatocytes exposed to APAP [69]. However, the picture is more complex than anticipated, since mice bearing a specific deletion of ATG5 in hepatocytes are protected against 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 the kinase activities of ULK1/2 are essential in APAP-induced liver injury by promoting activation of c-Jun N-terminal kinase signaling, independently of their autophagy activity [71]. From these data and others [70], it cannot be excluded that autophagy-dependent and independent ULK1 pathways result in opposite regulation of APAP-induced liver injury.

Sterile inflammation has also been proposed to contribute to APAP-induced toxicity, *via* inflammasome-dependent release of IL-1 β [72]. The identity of the cell(s) involved is still controversial, but contribution of neutrophils and Kupffer cells has been put forward [73]. Interestingly, it is now well described 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 and liver injury mediated by death receptors. Autophagy also protects hepatocytes from death receptor-mediated acute liver injury, a feature of viral hepatitis, acute alcoholic hepatitis or 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 described to limit NF- κ B-mediated inflammation and inflammasome-dependent IL-1 β production [77, 78].

Ischemia reperfusion injury. 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 type or model of ischemia (warm or cold) and/or the preservation solution used, and further complicated by the lack of accurate evaluation of autophagy *in vivo* (reviewed in [79]).

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

1.2. Autophagy and inherited liver diseases

Wilson's disease. Wilson's disease is caused by copper overload in hepatocytes, as a consequence of mutations in the Cu pump ATP7B. The toxicity of copper for hepatocytes 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 CuCl₂. *In vitro* or *in vivo* inhibition of autophagy with spautin-1 accelerates hepatocyte death, mitochondrial damages and oxidative stress, whereas activation of autophagy by starvation or TFEB overexpression promotes hepatocyte survival [80]. The potential of autophagy activators 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 hemochromatosis.

Alpha-1 Antitrypsin (α1-AT) Deficiency. α1-AT deficiency is caused by homozygosity for alpha-1 antitrypsin mutant Z protein (ATZ), and associated with an increased risk of liver disease in adults and children, and with lung disease in adults [81]. The mutant ATZ folds

improperly during biogenesis and is retained within endoplasmic reticulum in hepatocytes. Hepatocytes of patients with α 1-AT deficiency display an increased accumulation of autophagosomes containing ATZ [82]. Likewise, ATG5-KO cells show a retarded degradation of ATZ with characteristic cellular inclusions of ATZ, 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 functions on hepatocytes, leading to liver injury and fibrosis.

Targeting autophagy as a therapeutic strategy for α 1-AT deficiency. Several preclinical evidences showed that induction of autophagy improves liver injury and fibrosis due to α 1-AT deficiency. Hepatocyte TFEB gene transfer in mice with AT deficiency results in dramatic reduction of hepatic ATZ, liver apoptosis and fibrosis [34, 84]. Moreover, activation of autophagy by carbamazepin decreases the hepatic load of ATZ, but also liver fibrosis in a mouse model of the disease. These results provided the basis for an ongoing phase 2 trial testing the effect of 52 weeks of carbamazepin in patients aged 14 to 80 with α 1-AT deficiency-related cirrhosis (<https://clinicaltrials.gov/ct2/show/NCT01379469>). The beneficial effect of activating autophagy in α 1-AT deficiency has also been observed with other drugs inducing autophagy, including rapamycin [85], and Nor-Ursodeoxycholic Acid [86].

Activating autophagy thus appears as an attractive strategy for human disorders due to 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). GSD1a 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 down-regulation of mTOR and SIRT 1 signaling [87, 88]. Restoration of hepatic G6Pase- α expression corrects metabolic abnormalities, and normalizes defective autophagy, suggesting that GSD1a patients could also benefit from the use of autophagy activators.

1.3. Autophagy in Non-Alcoholic Fatty Liver Disease (NAFLD).

With the rising incidence of obesity and diabetes, NAFLD has become a leading cause of chronic liver diseases and hepatocellular carcinoma (HCC) worldwide, and non-alcoholic steatohepatitis (NASH) is 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 via lipophagy, and its beneficial effects on the transition to NASH, via hepatoprotective effects of mitophagy in hepatocytes and anti-inflammatory properties in macrophages.

Autophagy is decreased by a wide variety of signal that contribute to NAFLD pathogenesis, including insulin resistance, excess triglycerides and free fatty acids, endoplasmic reticulum and oxidative stress [90]. Accordingly, reduction of 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 (**Figure 2**) [90-92]. Autophagy is also decreased in liver endothelial cells of patients with NASH as compared with patients with simple steatosis or normal liver [93]. In keeping with these data, mice deficient in components of the autophagic pathway in specific liver cells, including

hepatocytes deleted in ATG7 [31], ATG14 [94] or TFEB proteins [40], endothelial cells deleted in ATG5 [93], myeloid cells deleted in ATG5 [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, 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 decrease in 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 have shown encouraging effects in NAFLD patients and animal models.

Targeting autophagy, a promising approach in NASH. Currently, the only therapeutic options for NAFLD remain lifestyle interventions, that typically involve dietary modifications and physical activity change. Interestingly, caloric restriction and exercise are two 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) by 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 high fat diet fed mice. 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 high fat diet-fed mice, 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, that showed 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-1 β production [104], further studies should evaluate whether agonists of TFEB could also additionally limit inflammation associated with progression to NASH. Encouraging data obtained with ezetimibe [103] and tetraheleose [104] show that these compounds inhibit NLRP3-dependent IL-1 β production in macrophages, and that ezetimibe reduces NAFLD lesions, whereas tetraheleose displays atheroprotective properties in experimental models. Interestingly, all the above-mentioned drugs are already FDA-approved, and ezetimibe has been evaluated in clinical trials with NASH patients, 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.

1.4. Fibrosis

A key step in the fibrogenic process is the activation of hepatic stellate cells (HSC) activation, the main fibrogenic cell population, following a phenotypic switch from a lipid-rich to a fibrogenic myofibroblastic phenotype. Phenotypic changes are initiated by reiterative hepatocyte or biliary cell death and via complex interactions between parenchymal, endothelial and immune cells. In parallel, quiescent HSC lose their lipid vacuoles containing 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 **(Figure 3)**.

Activation of HSC by autophagy: the dark side. Autophagic flux is increased in HSC upon activation and a large number of reports have now shown that autophagy in HSC contributes to the phenotypic switch to a myofibroblastic phenotype. In landmark studies, Hernandez-Gea et al [107] and Thoen et al [108] described that autophagy promotes digestion of lipid droplets in quiescent HSC, thereby facilitating HSC activation. The link between autophagy and the loss of lipid droplets was evidenced *in vitro* with pharmacological autophagy inhibitors or the use of small interfering RNAs against ATG5 or ATG7. These studies allowed to demonstrate that autophagy promotes catabolism of retinyl esters by lipases, thereby generating free fatty acids that increase adenosine triphosphate levels following mitochondrial β -oxidation. *In vivo* studies further showed that mice bearing a specific ATG5 or ATG7 deletion in HSC 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.

In addition to lipophagy, down-regulation 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 that binds to LC3 on autophagosomes membranes, although autophagy-independent effects may also occur [111]. During experimental fibrosis, p62 expression is down-regulated in HSC in humans or mice. HSC knocked-down for p62 show enhanced expression of a α -SMA protein and COL1A1 mRNA [112]. In addition, mice with p62 deficiency in HSC, 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 HSC impairs Vitamin D Receptor-Retinoid X receptor interaction [112], a complex critical for maintaining HSC in a quiescent state [113]. These data demonstrate that p62 is a cell autonomous repressor of HSC activation, by promoting Vitamin D Receptor-Retinoid X receptor heterodimerization and repression of 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 the maintenance of the fibrogenic process, autophagy may be considered as an anti-fibrogenic pathway, by generating survival signal for hepatocytes. Old mice with specific deletion of ATG5 in hepatocytes 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 favors 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 from various origins, including Kupffer cells, by limiting IL1- β production, a survival and profibrogenic pathway for HSC. Co-culture of ATG5-deficient Kupffer cells with HSC increase the fibrogenic properties of HSC, an effect blunted in the presence of an IL1-R antibody [116]. Moreover, ATG5-deficient mice in the myeloid lineage produce enhanced hepatic IL-1 β 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 cross-talk with other immune cells that require IL-1 α and IL-1 β 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 by 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 reduction of liver fibrosis in response to autophagy activators such as rapamycin [120, 121] or carbamazepine [122]. Because these pharmacological modulators may also have

off targets, the use of more specific autophagy modulators (see above) may also be more promising.

D. Targeting autophagy: a more complex approach as far as HCC and alcoholic liver disease are concerned

1. Hepatocellular carcinoma.

HCC is the second leading cause of cancer-related death worldwide, which mainly occurs in the context of cirrhosis [123, 124]. HCC results from a unique combination of somatic genetic alterations in various signaling pathways (*i.e.* cell cycle gene, 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 plays a dual role in carcinogenic process, inhibiting the initiation process, while promoting tumor growth, metastasis and therapeutic resistance during tumor progression [126, 127].

Protective effects of autophagy in HCC initiation. Several lines of evidence suggest that autophagy protects against tumor initiation by maintaining intracellular homeostasis. Impaired autophagy through deletion of Beclin-1, ATG5 or ATG7 in mice promote spontaneous liver tumorigenesis in aged mice [30, 114, 128-133]. It also leads to accumulation of the p62 which on the one hand prevents 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- κ B, PI3K/Akt/mTOR or and Wnt- β -catenin [140].

The protective effects of autophagy also involve enhanced degradation of Yap, the major nuclear effector of the Hippo signaling pathway. Hippo pathway controls liver growth and Yap overexpression is an early event in hepatocarcinogenesis [141]. ATG7 liver-specific deficient mice display increased Yap protein levels and overexpression of Yap target genes. Concordantly, Yap deletion in these ATG7 liver specific-deficient mice decrease HCC incidence, without affecting the p62-NRF2 axis. Therefore, autophagy is a gate keeper in carcinogenesis through the induction of Yap and independently of the p62-NRF2 axis [129].

Promotion of HCC progression by autophagy. In established HCC, autophagy is increased and tumor cells recruit energy through autophagy, which improves their survival ability under hypoxia and low-nutrient environment and promotes 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 opposite roles in HCC by protecting from carcinogenesis at early stages and by promoting tumor progression at more advanced stages. This dual role illustrates the complexity of targeting autophagy for HCC treatments.

Targeting autophagy in HCC, a complex approach. In established tumors (*e.g.* glioblastoma, brain metastasis, melanoma, pancreatic cancer), several phases 1/2 trials support the idea that inhibiting autophagy (*e.g.* (hydroxychloroquine) in combination with classical anticancer treatments (*e.g.* chemotherapy, targeted therapy, radiation therapy) could improve clinical out-comes 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 tumors cells in rodents and rabbits. Combination of these treatments with an autophagy inhibitor (*e.g.* (hydroxyl)chloroquine, 3-MA and Lys05) was associated with enhanced tumor cell necrosis and apoptosis. [148-151], suggesting that inhibiting autophagy to target residual tumor cells could be beneficial. Moreover, autophagy is implicated in tumor 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 tumor response [153, 154]. However, attention should be paid to the deleterious consequences of blocking autophagy in dying tumor cells, as autophagy is required for effective antitumor T cell response. Considering that immune-mediated clearance of tumor cells is crucial to suppress HCC development, combination of immune checkpoint inhibitors together with an autophagy enhancers, enhancers could boost antitumor immune responses [155-157].

2. Alcoholic liver disease

Binge and chronic alcohol consumptions are major healthcare problems, and it is admitted that repeated binge-drinking sensitizes the liver to progression toward steatohepatitis and/or cirrhosis. Chronic ethanol exposure impairs lysosome function, and promotes hepatomegaly and hepatic protein accumulation. The involvement of autophagy in alcoholic liver disease is

complex, since acute alcohol consumption activates autophagy, whereas the impact of ethanol upon chronic exposure is more controversial.

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 autophagosomes 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 is associated with 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 will result in decrease mitochondrial beta-oxidation and increased

lipogenesis [167]. Additional effects on autophagy in macrophages is likely to reduce cytokine production by macrophages and blocks neutrophil recruitment to the liver (**Figure 2**) [118].

Targeting autophagy in alcoholic liver disease. Currently, no specific therapy against alcohol liver disease is available, and the main option remains alcoholic 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 alcoholic liver diseases, it is likely that autophagy-targeted approaches validated in NASH could be evaluated in the context of alcoholic liver disease. In particular, the use of TFEB agonists that combine both hepatoprotective effects on hepatocytes and anti-inflammatory effects in macrophages would deserve particular attention.

E. 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 signaling. 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 disease 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 ATGs 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 appreciate the autophagic flux (i.e. the flux of material from the autophagosome into the lysosomal lumen) *in vivo* is an important step for the development of autophagy-targeting strategies, that should be disease-specific. Third, identification of optimal therapeutic time-window for chronic liver disease will also be needed since 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 unraveled the central role for autophagy in liver pathophysiology and highlighted its potential as a novel therapeutic target. Time has now come for translation.

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 | To test whether administration of leucine, a direct stimulant of mTOR, for 90 days reduces autophagy in skeletal muscles and thus improves sarcopenia associated with in cirrhosis |
| NCT03037437 | Modulation of sorafenib induced autophagy using hydroxychloroquine in hepatocellular carcinoma | Adults with advanced or metastatic hepatocellular carcinoma | To 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 \geq 10 mm Hg) due to alpha-1-antitrypsin deficiency | To determine if 52 weeks Carbamazepine therapy leads to a significant reduction in the hepatic accumulation of ATZ |

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

LEGEND TO FIGURES

Figure 1 : Pharmacological targeting of the autophagic pathway. Autophagy can be regulated at 3 levels: *Level 1*, upstream of autophagosome formation, by modulating the signaling pathways that control the formation of autophagosomes. Only mTOR and AMPK, two major kinases that regulate autophagy in opposite ways, are indicated in the Figure. These signaling pathways impact on the autophagy machinery to trigger autophagosome formation and/or the transcription of autophagy-related genes (ATG) 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 two 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 is the only transmembrane ATG, and 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.

Figure 2 : Autophagy and fatty liver diseases : common and specific pathways

Chronic Alcohol consumption and overfeeding cause similar damages in hepatocytes by promoting endoplasmic reticulum stress, lipogenesis, mitochondrial alterations and emergence of Mallory Denk bodies. In both conditions, autophagy is impaired and promote progression of the disease. 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.

Figure 3 : Autophagy and liver fibrosis: the good and the dark side. Hepatocyte injury initiates the fibrogenic process by triggering inflammation and hepatic stellate cell activation, during which quiescent hepatic stellate cells 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) reducing the production of inflammatory cytokines by macrophages and endothelial cells. However, in HSC, autophagy displays fibrogenic properties via two distinct processes, i) lipophagy which allow lipid droplet digestion and release of ATP required to promote the activation process ii) via p62 loss, which impairs VDR-RXR interaction, a complex critical for maintaining HSC in a quiescent state.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests

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