Chapter 21

# Autophagy in Non-Alcoholic Fatty Liver Disease (NAFLD)

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Additional information is available at the end of the chapter

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**Abstract**

Autophagy is a mechanism involved in cellular homeostasis under basal and stressed conditions delivering cytoplasmic content to the lysosomes for degradation to macronutrients. The potential role of autophagy in disease is increasingly recognised and investigated. To date, a key role of autophagy in hepatic lipid metabolism is recognised and dysfunctional autophagy might be an underlying cause of non-alcoholic fatty liver disease (NAFLD). Nevertheless, the exact role of autophagy in lipid metabolism remains controversial, with both a lipolytic function of autophagy and lipogenic function reported. This chapter aims to review the current knowledge on autophagy in NAFLD, with a special focus on its role in hepatic lipid metabolism, hepatic glucose metabolism and insulin resistance, steatohepatitis, hepatocellular injury and hepatic fibrogenesis. Finally, interaction with another cellular homeostatic process, the unfolded protein response (UPR), will be briefly discussed.

**Keywords:** autophagy, non-alcoholic fatty liver disease (NAFLD), non-alcohlic steatohepatitis (NASH), lipid metabolism, glucose metabolism, insulin resistance, fibrogenesis, hepatocellular carcinoma

## 1. Introduction

The term autophagy has been introduced by de Duve *et al*. over 40 years ago [1] to define a process of vacuolisation for the transport of intracellular material to lysosomes for degradation. Because the importance of autophagy in (patho)physiology became more and more recognised, the knowledge and number of autophagy-related publications increased exponentially in the last decade. Indeed, autophagy is progressively acknowledged as an important regulator of

intracellular homeostasis. Dysfunction of this process has been linked with cardiovascular, respiratory, neurodegenerative and metabolic diseases and with cancer [2, 3].

A growing body of evidence indicates that autophagy and lipid metabolism are correlated. Dysfunctional autophagy may therefore contribute to the pathogenesis of non-alcoholic fatty liver disease (NAFLD). However, controversies still exist and the exact role of autophagy in hepatic lipid metabolism is not entirely elucidated yet. This chapter aims to give a brief introduction about NAFLD and autophagy and subsequently reviews the current knowledge on autophagy in NAFLD.

## 2. Non-alcoholic fatty liver disease (NAFLD)

Even though histological features of NAFLD were recognised for decades, the first formal definition was introduced in 1980 to describe a small cohort with striking fatty changes in the liver with lobular hepatitis and focal necrosis, termed non-alcoholic steatohepatitis (NASH) [4]. Since then the concept has evolved to the definition of NAFLD, which covers a spectrum of fatty liver without evidence for any secondary cause of hepatic fatty liver accumulation, such as alcohol consumption or inherited disorders [5]. NAFLD is epidemiologically associated with the metabolic syndrome that encompasses obesity, diabetes mellitus, arterial hypertension and dyslipidaemia [6].

The hallmark of NAFLD is macrovesicular fat accumulation in more than 5% of the hepatocytes, ranging from scarce to panacinar steatosis and usually starting in the acinar zone 3 [7]. It is important to discriminate non-alcoholic fatty liver (NAFL, also known as simple steatosis) from non-alcoholic steatohepatitis (NASH). In the latter, not only macrovesicular steatosis is present, but also hepatocellular ballooning and lobular inflammation [5, 7] (**Figure 1**).

Simple steatosis is currently still considered as relatively innocent, as it has a slow evolution to advanced disease (though a subgroup of fast progressors was identified [8]) and in the absence of fibrosis mortality does not seem to be increased [9]. Potential consequences might therefore mainly be confined to the operative setting [10].

Once NASH has been established, patients are subjected to an increased risk of hepatic and non-hepatic comorbidities and mortality (**Figure 1**) [6, 11, 12]. NAFLD is an independent risk factor for cardiovascular disease, and cardiovascular events are the most important cause of death [12, 13]. Of all the features of advanced disease, fibrosis appears to be the most predictive for NAFLD-related outcomes [9, 14].

Since liver biopsy is the current gold standard for diagnosis but cannot be used routinely for population-based studies, the current prevalence of NAFLD can only be estimated based on a range of non-invasive diagnostic methods and/or in highly selected patient groups. The overall prevalence in the Western societies is estimated 20–30% for NAFLD and 3–5% for NASH. The incidence has substantially increased in the last decades and is expected to rise further [6, 16]. By 2025, NAFLD-related liver disease is anticipated to become the most important indication for liver transplantation in the United States of America [17].

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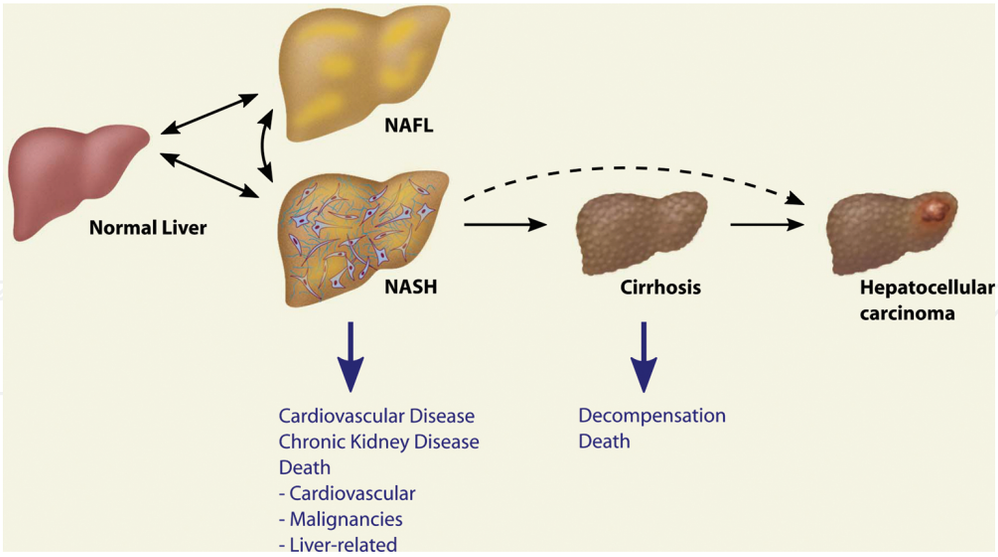


**Figure 1.** Non-alcoholic liver disease (NAFLD) spectrum. NAFLD encompasses a spectrum of fatty liver disease ranging from non-alcoholic fatty liver (NAFL, also simple steatosis) to non-alcoholic steatohepatitis (NASH). The latter might further evolve to advanced fibrosis and eventually cirrhosis that can be complicated by hepatocellular carcinoma (HCC). HCC can also develop outside the setting of cirrhosis. NASH is also associated with increased hepatic and non-hepatic comorbidities and mortality (adapted from Cohen *et al*. [15] and Torres *et al*. [11]).

With the growing importance of NAFLD, research on this topic further increased, which led to the formation of major research consortia in the United States and Europe [18]. Nevertheless, there are still major knowledge gaps concerning the exact pathophysiology behind the development and progression of NAFLD. It is generally believed that NAFLD is a very dynamic and multifactorial disease, in which different 'hits' contribute simultaneously and/or sequentially to the pathogenesis of NAFLD [19, 20]. Autophagy gained interest recently as one of those potential hits.

## 3. Autophagy

The term autophagy is derived from the Greek language and literally means "self-eating". Damaged or dysfunctional cellular contents are continuously removed via basally active autophagy to conserve cellular homeostasis and to supply the cell with substrates for energy production, though autophagy can be further stimulated via oxidative or metabolic stress [21, 22].

In mammalian cells, three types of autophagy are described: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy [2, 23, 24]. Microautophagy describes the direct engulfment of a small portion of cytoplasm by the lysosome. When proteins containing a special targeting motif are recognised by heat-shock cognate protein 70 (HSC70) and its cochaperones, they are selectively delivered to the lysosome via CMA. In macroautophagy, cytoplasmic material is either non-selectively or selectively (e.g. 'mitophagy', selective autophagy of mitochondria) [2, 3, 24, 25] sequestrated in a double membrane structure, the

autophagosome. This process starts with the formation of an isolation membrane (also known as phagophore), which will lengthen to create an autophagosome. Autophagosomes fuse with a lysosome, after which the sequestrated content will be degraded.

Macroautophagy (henceforth autophagy) is generally considered to play the most important role in the (patho)physiology and is extensively studied in the last decades. The process of autophagy is dynamic and strictly regulated, with control mechanisms at the transcriptional and post-transcriptional level [26]. It is regulated at the molecular level by autophagy-related (*Atg*) genes and their products, which form the core machinery of autophagy [27]. Paramount in the regulation of autophagy is the mammalian target of rapamycin (mTOR) [28]. The initiation of autophagosome formation by phosphorylating UNC51-like kinase 1 (ULK1) is inhibited by mTOR. In response to growth factors (e.g. insulin), mTOR gets stimulated by the class I phosphatidylinositol 3-kinase (PI3K)/AKT pathway. However, in case of starvation, the AMP/ATP ratio increases and leads to adenosine 5′-monophosphate-activated protein kinase (AMPK) activation and consecutive mTOR inhibition and thus activation of autophagy [27, 28]. The nucleation of the phagophore is mediated by a beclin-1/VSP34 (a class III PI3K)-interacting complex [27, 28]. The elongation of the phagophore to form an autophagosome is performed by two ubiquitin-like conjugated complexes: the ATG5-ATG12-ATG16L1 complex and light chain 3 (LC3). The ATG7 protein (an E1-like protein) is needed to mediate the conjugation of both complexes and is an interesting target for the study of autophagy [29]. The active conjugated form of LC3, LC3-II, is frequently used as a marker for autophagy [30]. For further information on autophagy regulation, we refer to previously published reviews [24, 26, 27].

## 4. Autophagy in lipid metabolism

Autophagy was convincingly correlated to lipid metabolism for the first time by Singh and his colleagues [31] and considered as a novel selective pathway in lipid breakdown known as ‘lipophagy’. Others claimed in the same year that autophagy was indispensible for the genesis of lipid droplets (LDs) rather than for the breakdown of LDs [32]. Ever since, supporting evidence for both lipid breakdown and lipogenesis has been published. After describing some common findings, both the opposing theories and contextual variations of autophagy in lipid metabolism will be discussed.

### 4.1. Common findings in autophagy and lipid metabolism

In spite of the opposing views present in current literature, some common findings supporting the relationship between autophagy and lipid metabolism in the liver deserve to be mentioned.

First, a close association between LDs and LC3, as well as between LDs and lysosomes, has been demonstrated. As demonstrated by immunofluorescence microscopy, LC3-positive structures and markers of LDs colocalise in liver tissue [32] and in cell lines [33–35]. Increased colocalisation of LDs with lysosomal markers such as lysosomal-associated membrane protein 1 (LAMP1) [31] or lysotracker [35] in fat-loaded cells was also demonstrated by immunofluor-

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escence microscopy. Similarly, immunohistochemical LC3B-positive dots were localised on the surface of LDs [36]. Immunogold staining of LC3 on transmission electron microscopy (TEM) slides confirmed the colocalisation of LC3 with LDs and implies a LD-regulating function of autophagy [31–33].

The colocalisation of LC3 with LDs was not influenced by inhibition of autophagosome–lysosome fusion or knockout of autophagy. However, colocalisation of LDs with lysosomes decreased after inhibition of autophagosome formation or by knockdown of autophagy [31]. These findings suggest that processing of LC3 into LC3-II (the active form) not only occurs on autophagosomes but also on the surface of LDs [31].

Secondly, in parallel with the histological pattern of NAFLD [7], immunohistochemical staining of LC3 is more localised in acinar zone 3 (i.e. around the central veins) [36, 37]. Findings in glutamine metabolism postulate a theoretic zonal distribution of autophagy [38]. In this view, low rates of autophagy in the periportal areas and constitutively high levels in the pericentrally areas are assumed in case of well-nourished conditions. Accordingly, it might be a potential explanation for the pattern found in NAFLD.

### 4.2. Autophagy as a lipolytic mechanism

The liver is capable of mobilising free fatty acids (FFAs) rapidly when needed. Autophagy as contributing factor to lipolysis is hence an attractive theory, as it helps explaining this capability while hepatocytes have relatively low concentrations of cytosolic lipases [39].

When hepatocytes were cultured in the presence of lipid stimuli, hepatocyte triglyceride (TG) levels increase and LDs accumulate. The pharmacological inhibition or knockdown of autophagy (targeting ATG5) enhanced these findings [31]. It was shown that impaired lipolysis (fuelling β-oxidation) and not increased TG synthesis were responsible for these findings. When autophagy was pharmacologically induced, the opposite happened with decreased lipid stores in hepatocytes. Hepatocellular-specific, autophagy-deficient mice (targeting *Atg7*) confirmed these *in vitro* results. Indeed, compared with wild-type littermates, liver TG and cholesterol content increased [31]. Fasting-induced steatosis, which can be observed after 24 h starvation, was less pronounced in wild-type mice compared with autophagy-deficient littermates. Moreover, lysosomes and lipid-containing autophagosomes increased after fasting in the autophagy-competent mice, supporting lipolysis [31].

The oxidation of FFA and the production of very low-density lipoprotein (VLDL) appeared to be dependent on autophagy. Inhibition of autophagy decreases both FFA oxidation and VLDL production, while stimulation induces the opposite [40, 41]. The distribution of lysosomal lipases (LAL) changed towards the autophagosome fraction after starvation in rat liver and supports an increase in autophagy-mediated lysosomal lipolysis [40].

It has been shown that dietary-induced obesity induces decreased autophagy flux [42, 43]. In mice with dietary-induced obesity as well as in genetically induced obesity, ATG7 protein levels were reduced (although the mRNA expression was comparable) [42]. Autophagy induction via liver-specific overexpression of ATG7 in ob/ob mice [42] or via calcium channel

blockers [43] restored autophagy flux, improved the metabolic state and reduced steatosis significantly. These findings further support a lipolytic function of autophagy.

Trafficking of autophagosomes and lysosomes, as well as their interaction, is just modestly understood. The Rab guanosine triphosphatases (GTPases) serve as master regulators of intracellular membrane traffic [44] and might be involved in regulation of lipophagy as well. Indeed, Rab7 is a fundamental component of both LDs and endolysosomal membranes and a central regulator for LD breakdown by autophagy [45]. Dynamin 2, another GTPase, is also involved in maintenance of lysosomal homeostasis by recycling of autophagosomes. Ablation of Dynamin 2 compromised the autolysosomal compartment, with subsequent depletion of lysosomes, and inhibited lipophagy [46].

Steatosis and dyslipidaemia are linked to defects in forkhead box class O (FOXO) [47], and liver-specific triple knockout of FOXO1/3/4 (LTKO) causes steatosis and hypertriglyceridemia [48]. Regulation of the key autophagy genes mediated by FOXO1 was demonstrated [49]. The autophagy gene *Atg14* is regulated by FOXO1 and 3. Hepatic and serum TG increase after knockdown of hepatic ATG14, whereas overexpression decreases steatosis in HFD fed animals. Overexpression of *Atg14* in LTKO mice could counteract the observed lipid disturbances including steatosis [48]. Surprisingly, an increase instead of a decrease in FOXO1 levels was described in a small cohort of NASH patients [50].

The longer-term transcriptional regulation of autophagy becomes further unravelled [51] and seems to be in favour of lipophagy. The transcription factor EB (TFEB) appears to be a master regulator of autophagy [52] and is involved in lipid metabolism as well. Steatosis is induced when TFEB is suppressed, while steatosis is inhibited when TFEB is overexpressed [53]. Both autophagy and the stimulation of the peroxisome proliferator-activated receptor $\gamma$ coactivator 1 $\alpha$–peroxisome proliferator-activated receptor $\alpha$ (pgc-1$\alpha$-PPAR$\alpha$) pathway mediated the observed effects. The dependency of TFEB function on autophagy mechanisms was demonstrated by the inability to counteract steatosis caused by disruption of autophagy [53]. The cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) is able to promote lipophagy in the fasted state via activation of TFEB and transcription of autophagy genes, while the farnesoid X receptor (FXR) suppresses many autophagy genes in fed conditions [54]. Likewise, starvation-induced activation of PPAR$\alpha$ impedes the inhibitory effects of FXR on autophagy [55].

It is well known that steatosis can be the consequence of some HIV antiretroviral drugs. Thymidine analogues were able to inhibit the autophagic flux of hepatocytes *in vitro* in a dose-dependent manner, with subsequent induction of lipid accumulation and mitochondrial dysfunction [56]. Even though currently only used as clinical treatment in trial [57], glucagon like peptide-1 (GLP-1) analogues were able to reduce fat accumulation *in vitro* and *in vivo* by the activation of autophagy [58–60], and by the reduction in endoplasmic reticulum (ER) stress [58]. Moreover, Roux-en-Y gastric bypass in obese diabetic rats was able to improve metabolic parameters and to restore hepatic autophagy and was correlated with increased plasma GLP-1 levels [61]. Carbamazepine and rapamycin induce autophagy and were effective in reducing steatosis in models of alcoholic and non-alcoholic fatty liver disease [62]. Also caffeine, of which epidemiological data suggest a protective effect on NAFLD, was shown to

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induce autophagy dose dependently with increased lipid clearance [35, 63]. Finally, the antioxidants resveratrol [64, 65] and tert-butylhydroquinone [66], but also metformin [67], were able to attenuate hepatic steatosis by inducing autophagy via activation of AMPK/Sirtuin-1. Other possible mechanisms parallel to changes in autophagy that might explain the observed effects are formally not excluded, as illustrated by the reduced expression of genes related to ER stress and inflammation and increased expression of genes involved in lipid oxidation in zebrafish exposed to caffeine [63]. However, given the alike effects of different compounds, involvement of autophagy in lipolysis is at least partially feasible.

Very recently, the role of autophagy appeared to be even more complex, since CMA emerged to control lipid homeostasis. Blockade of CMA induces severe hepatosteatosis, partially explained by defective breakdown of key enzymes involved in lipid binding, transport and synthesis [68]. Moreover, CMA is required for breakdown of LDs. LDs are covered with perilipins (PLINs), which regulate the accessibility of the LD for lipases. PLIN2 and PLIN3 are substrates of CMA, and upon fasting, they are removed of the LD surface in a CMA-mediated fashion. Afterwards, the lipid droplets are accessible for both cytosolic neutral lipases and for autophagy-mediated lipolysis. When CMA is blocked, lipid oxidation decreases and LDs accumulate [69].

Even though the exact relevance of microautophagy in normal cell physiology remains largely unknown at present, microautophagy might have a role in lipid breakdown. In a recent study, yeast cells were capable of translocating LDs in lysosomes by a process morphologically resembling microautophagy and independent of core autophagy proteins [70]. Whether this also holds true for mammalian cells remains to be proven.

Experimental restrictions, for example the impossibility to use specific drugs or to perform consecutive biopsies, limit the availability and interpretation of human data. Data of liver biopsy represent a snapshot of a very dynamic process and cannot accurately discern between increased autophagy and decreased degradation of autophagosomes [30, 36]. Furthermore, some markers need to be overexpressed for accurate identification of autophagic structures [36]. Nonetheless, a small post-mortem study demonstrated decreased LC3 and increased p62 staining in relation with the degree of steatosis, suggesting decreased autophagy in more severe steatosis [37]. Likewise, in proven NAFLD p62 accumulation, increased numbers of autophagic vesicles were demonstrated [71]. mRNA and protein analysis of liver biopsies were also indicative of an impaired autophagic flux in both NAFL and NASH patients [72]. A more in-depth analysis with gene set enrichment analysis of liver biopsies demonstrated that NASH has distinct patterns, compared with normal livers or NAFL. In these livers, the gene categories for apoptosis and autophagy were enriched for upregulated genes, while the gene categories for ER stress and lipogenesis were enriched for downregulated genes. In NAFL, no genes were significantly enriched except for an enrichment for upregulated genes related to autophagy [73]. This correlation can either indicate upregulation in an attempt to attenuate lipotoxicity and to increase lipolysis or indicate the direct involvement of autophagy in fat accumulation in the liver.

Finally, two clinical observations need to be mentioned. Hypothyroidism is more prevalent in patients with NAFLD [74–76] as well is hypovitaminosis D [77]. The thyroid hormone ($T_3$) is

a known regulator of the basal metabolism and recently shown to be a powerful inducer of autophagy *in vitro* and *in vivo*. Autophagy plays a crucial role in $T_3$-stimulated β-oxidation [34]. Vitamin D acts also as a potent inducer of autophagy [28]. As a result, these associations might be explained via autophagy and are in line with lipophagy. A direct effect via autophagy, however, has hitherto not been investigated.

### 4.3. Autophagy as a lipogenic mechanism

*Caenorhabditis elegans* is a nematode increasingly used in metabolic research. Its intestine fulfils the role of a multifunctional organ reflecting the roles of the liver and adipose tissue [78]. Inhibition of autophagy via knockdown of several different genes involved in the autophagy process results in a strong reduction in lipid content. Importantly, there were no arguments for altered food uptake or defecation, nor for influenced differentiation of the tissue in case of autophagy deficiency [79].

The body possesses an adaptive mechanism to maintain homeostasis in case of fasting. The declined insulin levels no longer inhibit lipolysis in the adipose tissue (AT) and release FFA to the serum. The liver captures FFA, either for the formation of ketone bodies, or for temporary storage as TG in LDs [32, 80]. The latter can be seen with $^1$H-magnetic resonance spectroscopy after 36-h fasting [81]. A substantial accumulation of TG in the liver, consequent to this mechanism, can be seen in rodents and is known as fasting-induced steatosis. The C57Bl/6 mouse strain showed to be very prone to develop fasting-induced steatosis [80]. However, compared with wild-type littermates, hepatocyte-specific autophagy-deficient mice lack fasting-induced steatosis. The total TG content in their livers is lower, and the remaining LDs are decreased in size and numbers [32]. This observation was not only the case in very young mice (22 days old) but was also demonstrated in 8- to 12-week-old mice [32, 36, 82]. As a consequence, autophagy seems to be implicated in the formation and growth of LDs. The colocalisation of LC3 (necessary for autophagosome formation) with LDs in starved wild-type mice further supports these findings [32]. Consistent with the overall nutrient shortage by fasting, mice exposed to a one-week dietary protein deficiency develop hepatic steatosis, accompanied by autophagy and ER stress. Leucine supplement, a known autophagy inhibitor, lowered autophagy, ER stress and liver TG content [83]

In different cell lines, amongst which hepatocytes, the indispensability of autophagy for LD formation was confirmed [33]. The knockdown of LC3 in these cells leads to reduced formation of LDs and reduced TG content compared with their controls. Since FFA uptake, TG synthesis or TG breakdown are unaltered after knockdown of LC3, an impaired ability to preserve synthesised TG within these cells is suggested [33].

Hepatocyte- or skeletal muscle-specific autophagy-deficient mice exhibit an improved metabolic profile [84]. When hepatocellular-specific autophagy-deficient mice are fed a control diet [82, 84], the aforementioned fasting-induced steatosis did not occur. Moreover, when these mice were fed a HFD, lipid accumulation was absent [84] or did not increase [82]. The expression of genes involved in fatty acid and TG synthesis, but also of those involved in β-oxidation and TG secretion were reduced in comparison with autophagy-competent littermates [82, 84]. Therefore, it is not clear whether these findings are epiphenomena or directly

involved in the prevention of steatosis. Kim and colleagues held the 'mitokine' fibroblast growth factor 21 (FGF21), which is induced by mitochondrial stress, responsible as a central mediator of the metabolic alterations [84].

Studies with dietary and genetic models of obesity report decreased levels of autophagy, while the overexpression of ATG7 had beneficial metabolic effects [42] as discussed before. Nevertheless, in the same paper the suppression of ATG7 in lean mice failed to alter lipid accumulation in the liver (as well as TG or FFA in serum), while hepatic glycogen content did show an increase [42]. This study may therefore be considered as non-conclusive about a lipolytic or lipogenic function of autophagy.

### 4.4. Contextual variability of autophagy in lipid metabolism

Besides the opposing views regarding autophagy as a lipolytic or lipogenic process, autophagy is also subjected to context-dependent alterations. In most of these cases, these differences were described by those who support autophagy as a lipolytic mechanism.

Lipid metabolism seems to be more dependent on basal autophagy than on induced autophagy, since (sudden) lipid stimuli did not reveal signs of induced autophagy or autophagic flux in cultured hepatocytes [31]. *In vivo* there is also impaired adjustment of autophagy, as external lipid load by prolonged HFD decreases autophagy efficiency [31, 41, 42, 58]. Intriguingly, a detailed follow-up of autophagy reveals fluctuating levels of autophagy over time. Increased autophagic flux was observed after 2 weeks [41] or 4 weeks [85] of HFD, and a decrease was observed after, respectively, 10 or 16 weeks of HFD. Other data (only published in abstract form) suggest that autophagy decreases after short-term HFD (3 days) and normalises after long-term HFD (10 weeks) [86]. Instead of a decrease, 8 weeks of a diet high in fat load generates an increase in autophagy [87]. Recently, autophagy was shown to behave dynamically with an oscillating damping pattern under HFD, probably the consequence of a feedback loop mechanism between mTORC1 and TFEB (X.M. Yin, personal communication at the AASLD 2015). Overall, autophagic flux seems to be very dynamic in case of overnutrition.

The detrimental effects of fatty acids on the cellular integrity are called lipotoxicity [88, 89]. Thus, it is not surprising that lipids by itself may have impact on autophagy. Autophagy can be induced by short chain fatty acids [90] and ω3-fatty acids (mTOR independently) *in vitro* [91]. Unsaturated fatty acids (e.g. oleic acid) stimulate autophagy and protect against apoptosis, while saturated fatty acids (e.g. palmitic acid) inhibit autophagy and promote apoptosis [58, 92, 93]. Tu *et al.* observed the opposite effects, with inhibition of autophagy by oleic acid and induction by palmitic acid [94]. These conflicting results might reflect differences in cell type, concentration and duration of FFA application, but more importantly emphasise the contextual variability of autophagy.

The impairment of autophagy in case of saturated fatty acids is considered to be due to a diminished fusion capacity of autophagosomes with lysosomes [43, 95]. Long exposure to high lipid concentrations alters the lipid composition of membranes or vesicular compartments and in this way impairs their fusion [95]. Another explanation includes an inhibitory effect of increased cytoplasmic calcium concentrations via inhibitory effects of SFA on the sarco-ER

calcium pump (SERCA) [43]. This may explain the altered autophagy after prolonged fatty diets. Attenuation of CMA was also observed after lipid challenge [23]. However, some authors did not observe an attenuated fusion capacity. Instead, they report a decrease in clearance of autophagosomes due to a disturbed acidification of lysosomal compartments [96, 97] and/or downregulated cathepsin expression [71, 96].

Variation is not only the case within the liver, but is also dependent on tissue type. Adipogenesis and transdifferentiation towards white AT, for example, depend on autophagy [98, 99], thus arguing against a potential lipolytic function of autophagy in AT. This is opposite to the observations in liver tissue, which mainly claim a lipolytic function (as discussed above). Additionally, in the AT of patients with metabolic syndrome or type 2 diabetes mellitus, autophagy increased [100–102].

### 4.5. Discrepancies and hypotheses

The role of autophagy in the liver seems to be more complicated than expected. At present, a clear-cut explanation for the discrepancies of autophagy with respect to lipid metabolism as stated above is missing [103]. However, several hypotheses have been put forward.

Firstly, some concerns were related to the age of the laboratory animals [104], since autophagy declines with age [105]. Older mice might be less dependent on autophagy than juvenile mice. However, experiments with both younger and older mice provide comparable results [32, 36, 82, 84], implying that only age cannot explain the observed differences.

Secondly, *in vivo* experiments can be subject to small variances in the mouse strains used [84]. However, this issue is not likely to offer an explanation. Most of the experiments were performed on a C57Bl/6 background, which is an inbred strain. Moreover, manual backtracking the cited resources of the hepatocyte-specific *Atg7* knockout mice leads to the same origin of the mice. *Atg7* flox mice were created by Komatsu *and colleagues* [29], while the albumin-Cre mice were created by the group of Magnuson [106]. In addition, conflicting results were also seen in hepatocyte cell lines as described above.

Thirdly, lipid accumulation and autophagy can be examined by many different methods. Liver steatosis can be induced by fasting, and by genetic and/or dietary interventions. However, these genetic modifications or pharmacological approaches can also alter autophagy by themselves. Genetic modification can target different autophagy-related genes as well, which might be reflected in the results. Whereas papers supporting autophagy as lipophagy use a wide range of methods (see above), articles claiming the contrary chiefly applied *in vivo* knockout and knockdown models and fasting-induced steatosis (see above). One also has to be aware of the potential influential consequences of knockout models on the developmental stages, for example as seen by the transdifferentiation of white adipocytes [98, 99]. As a result, the observed differences in autophagic lipid handling might be consequent to altered hepatocellular maturation as well as to the experimental method applied.

Fourthly, basal autophagy and stimulated autophagy have to be distinguished. The former is supposed to be the most important in the pathogenesis of NAFLD [31, 107]. Discernment between basal and induced autophagy is hampered by the fact that mostly total blockage of

autophagy is used. Selective blockage of stimulus-induced autophagy (i.e. by exercise or starvation) can be achieved in a recently described *Bcl-2* knock-in model [108]. These mice have an exercise-related impairment of glucose metabolism and exhibit increased serum lipid levels when fed a HFD. Importantly, liver and pancreas morphology did not alter after HFD, supporting the importance of basal autophagy in lipid metabolism. These differences consequent to basal versus stimulated autophagy deserve further study to elucidate each particular role and potential influence on the divergent findings in lipid metabolism.

Whether the observed effects are a secondary/adaptive process or primary caused by autophagy is sometimes difficult to differentiate [99, 109], as well as the potential modified effects due to (compensatory upregulation of) other forms of autophagy (e.g. CMA) [107]. Furthermore, other organelles and cellular processes are impacted by dysfunctional autophagy and could in part explain observed differences in liver metabolism. Since autophagy can degrade apoB, a necessary protein for the VLDL formation, impaired VLDL production might be involved as well [109]. The involved ATG proteins might even exert non-autophagic (and autophagy independent) functions [39, 42, 110] in lipid metabolism.

While focussing on the role of autophagy in lipid metabolism, the role of cytosolic lipases may not become overlooked, while they still account for a substantial part of the lipolysis [88]. Total blockage of lipolysis by diethylumbelliferyl phosphate (DEUP) causes a greater increase in the cellular TG content than blocking autophagy alone [31]. In addition, if LD formation is autophagy dependent, small LD-like bodies are still observed on TEM in autophagy-deficient cells, suggesting that LDs formed out of the ER are unaffected [32].

Finally, the microscopical techniques currently available do not allow visualisation of the smallest LDs in living cells [111]. Hence, it is possible that the observed effects only reflect autophagy-related modulation once LDs are formed. In that case, autophagy can be considered as a dynamically active process that controls LD size and the amount of lipotoxic FFA in the cytoplasm. The effects of autophagy will be rather context dependent [79, 88]. In this perspective, lipolysis and lipogenesis are no longer mutually exclusive and in fact coexist [112].

## 5. Autophagy in glucose metabolism and insulin resistance

The liver has a key role in glucose metabolism and autophagy substantially contributes to maintain glucose homeostasis. In case of a conditional whole-body knockout of *Atg7* in adult mice, liver glycogen stores were totally depleted and serum amino acids and glycaemia dropped severely when fasted and led to death. The underlying cause was a lack of sufficient substrates because of deficient autophagy, since liver ketogenesis and gluconeogenesis in the liver remained intact [113].

Similar to whole-body autophagy, hepatic autophagy is necessary to deliver sufficient substrates to maintain blood glucose levels in the fasting state [114, 115], which is under strict control of insulin [114]. Additionally, long-term maintenance of blood glucose levels is dependent on growth hormone-stimulated autophagy [115]. Adenoviral overexpression of

TFEB, a master regulator of autophagy, improves the metabolic syndrome in HFD fed and ob/ob mice, amongst which improved glucose metabolism [53].

CMA is also involved in glucose metabolism. Mice with knockout of LAMP2A, necessary for internalisation of CMA-dedicated proteins, demonstrated increased levels of glycolysis. As a consequence, they exhibit lower blood glucose levels after fasting, have decreased glucose tolerance testing and recover less from insulin tolerance testing [68].

Impaired insulin signalling is an important feature of NAFLD [11]. The exact interactions between the action of insulin and autophagy, explaining the observed alterations in glucose metabolism, are not entirely clarified yet. **Figure 2** summarises the current knowledge of autophagy and insulin resistance.

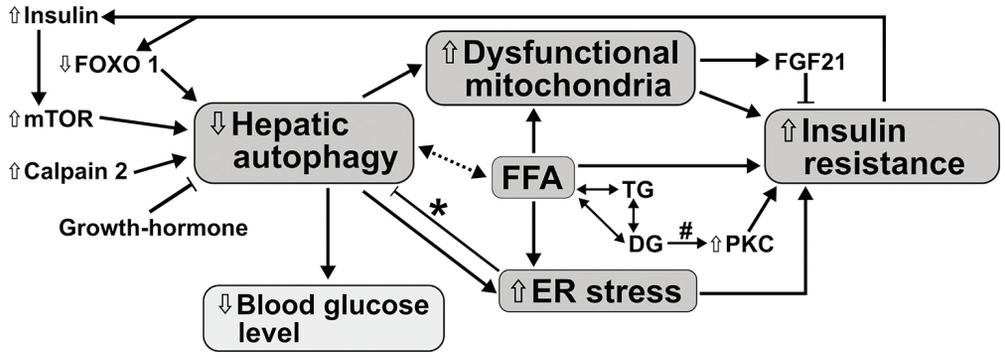


**Figure 2.** Autophagy and insulin resistance. Reciprocal influences are described between autophagy and insulin resistance (IR). Reduced autophagy causes a decrease in blood glucose level and is subject to different influences. Growth hormone regulates blood glucose on long term through the stimulation of hepatic autophagy. Increased levels of calpain 2 can induce IR through the effects of decreased autophagy on mitochondria and the endoplasmic reticulum (ER) stress levels. On the other hand, when normal insulin sensitivity remains present, secondary hyperinsulinism due to IR can decrease autophagy through the effects on mammalian target of rapamycin (mTOR) and through reduced forkhead box class O 1 (FOXO1). IR is also influenced directly through modulation of ER stress and the mitochondrial function by free fatty acids (FFA). Controversy exists on how autophagy might influence the level of lipids and thus FFA. Likewise, the formation of bioactive stereoisomers of diacylglycerol (DG), which might induce protein kinase C (PKC)-dependent IR, is also dependent on the effects of autophagy on FFA. The fibroblast growth factor 21 (FGF21) is produced in response to mitochondrial dysfunction and capable of reducing IR. Arrows indicate a consequence of a certain alteration, bar-headed arrows denote an inhibition. Double-headed arrows present a reciprocal influence. The dashed arrow denotes the uncertain relation between FFA and autophagy. *ER stress actually increases autophagy; #Only certain bioactive stereoisomers induce PKC (adapted from [112]).

Insulin inhibits autophagy via the stimulation of mTOR [2, 27]. When the liver presents a normal insulin sensitivity, reduced hepatocellular autophagy might be explained by insulin-dependent stimulation of mTOR in hyperinsulinaemic states. In case of insulin resistance (IR), an alternative inhibitory pathway of autophagy was described as well [49]. In mice fed HFD, diminished autophagy was the consequence of reduced FOXO1-mediated expression of key autophagy genes. The IR was believed to be due to the reduced clearance of dysfunctional mitochondria, as oxidative stress and altered mitochondrial integrity (and mass) are related to IR [49].

In contrast to the abovementioned study, IR was the result of decreased autophagy rather than the cause of reduced autophagy [42]. Insulin sensitivity and glucose tolerance improved, while

the hepatic glucose production and steatosis decreased when obese mice overexpressed *Atg7*. Knockdown of *Atg7* in lean mice induced severe IR. Decreased protein levels of ATG7, with the following increase in cellular stress, with emphasis on ER stress, might be the cause of the observed IR. For this, increased levels of the calcium-dependent protease calpain 2, capable of cleaving several autophagy-related proteins, were held responsible [42].

In line with the potential underlying mechanism of ER stress-mediated increase in the hepatic IR, developing secondary to decreased autophagy, are the findings that intracellular saturated fatty acids can contribute to IR by an increase in ER stress [116]. However, ER stress-independent mechanism was described as well [116]. Moreover, ER stress can stimulate autophagy [27, 117]. In this viewpoint, autophagy might potentially prevent cell injury and IR particular by serving as an escape mechanism in an attempt to reduce ER stress.

Autophagy not only plays a role on cellular level, but appears to exert endocrine and metabolic functions as well [84]. Defective clearance of mitochondria, due to dysfunctional autophagy, induces cellular stress and subsequent stress responses. One of those is activation of transcription factor 4 (ATF4), which promotes the expression of FGF21. FGF21 in turn has several beneficial metabolic effects including improvement in insulin sensitivity and glucose tolerance [84, 118]. Paradoxically, in patients with NAFLD a positive association was observed between plasma FGF21 levels, IR and steatosis [119]. This phenomenon might be explained by FGF21 resistance, since less IR is actually expected with increasing levels of FGF21, or can either be explained by an adaptive increase in FGF21 once IR has been established. Taking into account these positive metabolic consequences, mitochondrial dysfunction no longer has to be seen as just detrimental, but also as beneficial by improving glucose metabolism and reducing fasting-induced steatosis.

Finally, insulin resistance is linked to protein kinase C (PKC) [120]. PKC in an important cellular effector enzyme involved in several signal transduction cascades and several isoforms exist. Many of them can be activated by diacylglycerol (DG). PKC showed to be able to inhibit and stimulate autophagy [121, 122]. DG is a product of lipolysis, but also a TG intermediate, and might be an additional crosslink between IR and autophagy. Importantly, not all DG are able to activate PKC, only specific stereoisomers can. Of these, no bioactive DG can be produced by lipolysis, and therefore, potential crosstalk is less likely [88, 120]. On the contrary, bioactive stereoisomers of DG (i.e. 1,2-diacyl-glycerol) can still be generated in lipid synthesis and interfere with insulin signalling.

## 6. Autophagy and hepatocellular injury and hepatocellular carcinoma

As mentioned in Section 1, NAFL can evolve to NASH and is prone to subsequent development of advanced fibrosis or even development of hepatocellular carcinoma (HCC) in a subset of patients [6]. Because autophagy plays a central role in cellular homeostasis, dysfunction likely results in cellular injury. Indeed, stimulation of autophagy could reduce liver injury in animal models of ethanol-induced steatohepatitis [62, 123] and NAFLD [62].

Mitochondrial damage is often observed in autophagy deficiency [29, 36, 56, 84, 92, 124]. Dysfunctional mitochondria lead to the production of reactive oxygen species (ROS), which are involved in the pathogenesis of NASH [125]. Increased ROS production was indeed observed in autophagy deficiency [56, 126]. Autophagy-deficient hepatocytes are more susceptible to cell death via menadione-induced oxidative stress [107]. Compensatory upregulation of CMA, capable of protecting against menadione-induced cell death via different mechanisms, is unable to overcome the induced oxidative stress [107]. TNF-induced hepatic injury causes similar alterations as menadione, with increased cell death, JNK/c-JUN overactivation and activation of the mitochondrial death pathway in hepatocyte-specific autophagy-deficient mice. However, this seems to be independent of oxidative stress or impaired cellular energy homeostasis secondary to mitochondrial dysfunction [127].

In autophagy-deficient cells, SQSTM1/p62-positive proteinaceous aggregates accumulate as they are no longer degraded. p62 seems to contribute considerably to the hepatocellular injury seen in autophagy deficiency, as double knockouts (DKO) of autophagy (*Atg7*) and p62 have less hepatocyte injury compared with autophagy knockout (*Atg7*) alone [128]. However, overexpression of p62 is not cytotoxic [129]. The effects of p62 might be executed by NF-E2-related factor 2 (NRF2), of which p62 is an endogenous protein inducer. Indeed, DKO of *Atg7* and *Nrf2* was able to prevent hepatic injury, similar to the DKO of *Atg7* and p62 [129, 130]. This is a paradoxical finding, as NRF2-dependent gene products are known to be cytoprotective, but is believed to be the consequence of increased cellular stress due to imbalance between increased protein synthesis (NRF2-driven) and reduced breakdown (by autophagy) [129]. Of note, despite the alleviation of cellular injury by these DKOs, the observed phenotypes cannot be attributed solely to NRF2 activation. Since turnover of damaged organelles is still not corrected in these DKOs, complete prevention of the cellular injury comparable to control levels cannot be achieved either.

In adipocytes, autophagy is linked to inflammatory cytokines and inflammation. Knockout of p62 in adipocytes induces infiltration of macrophages and the production of pro-inflammatory cytokines in AT [131]. Furthermore, obesity and glucose intolerance are observed, while this is not the case in p62 knockout in hepatocytes. Whether these inflammatory changes also occur in liver is not investigated. Human and mice adipocytes also increase their pro-inflammatory cytokine production when autophagy is inhibited [102, 132].

Autophagy exerts a dual role in tumorigenesis depending on the stage of tumour development. In normal tissue, autophagy acts as a tumour suppressor and thus prevents the development of malignant neoplasia. However, when a tumour already has developed, autophagy aids the survival of tumour cells by supplying nutrients [133].

Hepatocellular autophagy deficiency, either by *Atg5* or *Atg7* knockout, causes the development of multiple spontaneous liver tumours [124, 126, 130, 134]. In line with the observed effects of the contributing role of p62-NRF2 to cellular injury, DKO of *Atg7* and p62 was able to reduce tumour size [124], and DKO of *Atg7* and *Nrf2* totally prevented tumour formation [130]. Intriguingly, all these tumours were not hepatocellular carcinomas (HCCs) but hepatocellular adenoma, which are benign tumours. Even stimulation with diethylnitrosamine, an established chemical inducer of HCC, was not able to induce HCC in autophagy-deficient livers

compared with wild-type livers [126]. The induction of several tumour suppressors (e.g. p53) could explain the prevention of carcinogenesis in case of autophagy deficiency [126]. Once HCC has developed in autophagy-competent rat livers, differences in autophagy pattern correlate with aggressiveness of the tumours, as determined by the marker cytokeratin-19 [135]. For further extensive discussion of the role(s) of autophagy in liver tumour biology, we refer to other published reviews [21, 136].

## 7. Autophagy in liver fibrosis

As stated before, liver fibrosis is the main predictor for long-term outcomes in patients with NAFLD [9, 14]. Interestingly, hepatocellular autophagy affects liver fibrosis too. In mice with hepatocellular autophagy deficiency, the degree of fibrosis was significantly increased [82, 130, 137]. This could be only an indirect effect considering the protective role of hepatocellular autophagy on liver injury and inflammation, which are the driving forces of fibrogenesis [82, 137].

Available evidence suggests also a direct elementary role for autophagy in different fibrogenic cells [138]. Autophagy seems to provide nutrients to fuel the processes involved in the activation of these cells. The hepatic stellate cells (HSCs) are considered major fibrogenic cells in the liver. When these cells transdifferentiate from a quiescent state to active myofibroblasts, their lipid stores (in HSC mainly vitamin A) become depleted [139].

During HSC activation, the autophagic flux increases and pharmacological or genetic inhibition could prevent the activation of HSCs [140, 141]. In these cells, autophagy interferes with LD metabolism, as shown by colocalisation of LC3B with LDs. Interestingly, only larger LDs seem to be affected and colocalisation disappeared once HSCs were activated [140]. More specifically, autophagy plays a role as energy supplier through delivery of FFA out of LDs for β-oxidation, necessary for the transdifferentiation of HSCs [141]. Even though there is no effect on fibrogenesis in autophagy-competent cells, oleic acid could partly restore HSC activation in autophagy-deficient cells [141].

HSC activation through autophagy activation was very recently ratified in an *in vitro* model using rat HSCs [142]. When hypoxic stress was applied, HSCs increased autophagic flux and got activated. Pharmacological intervention and knockdown of autophagy demonstrated that HSC activation was autophagy dependent and mediated by the activation of $Ca^{2+}$-AMPK-mTOR and PKCθ signalling pathways.

## 8. Autophagy and ER stress

Next to autophagy, cells possess another homeostatic mechanism to protect cells by alleviating cellular stress or by inducing cell death under extreme conditions: the unfolded protein response (UPR). The UPR is activated in response to the accumulation of unfolded proteins in

the endoplasmic reticulum (ER) (ER stress) [143, 144]. ER stress results from perturbation of the normal protein folding capacity of the ER and induces inflammation and oxidative stress [145]. The UPR encompasses three major adaptive mechanisms to restore protein homeostasis, named after the respective ER stress sensor: activating transcription factor 6 (ATF6), protein kinase RNA-like ER kinase (PERK) and inositol-requiring enzyme-1$\alpha$ (IRE1$\alpha$) [143].

The UPR and autophagy can function independently, but are dynamically interconnected. The classical view is that ER stress induces autophagy in order to restore cellular integrity, though ER stress can both induce and inhibit autophagy, even in a selective way [144]. Reciprocal feedback also exists, where autophagy influences the turnover of ER and the removal of misfolded proteins and hence regulates ER stress [146].

Interestingly, impaired autophagy is associated with increased levels of ER stress (mRNA and protein levels) in patients with NASH [72]. Gene analysis showed a general enrichment of downregulated genes related to the UPR in patients with NASH [73]. However, microarray data of the different UPR branches showed a more scattered pattern with both up- and downregulated gene expression. Finally, there were increased levels of IRE1$\alpha$-regulated spliced X-box binding protein 1 (XBP1s) at protein level, with congruent increased nuclear staining, and equal levels of other UPR chaperones (though with large variability) [73].

In a methionine-choline-deficient diet (MCDD) and HFD model of NAFLD, an analogous association between impaired autophagy and ER stress was observed [72]. The same authors demonstrated alleviation of ER stress *in vitro* after induction of autophagy in palmitic acid induced fat accumulation. High fructose feeding, a model for diabetes, induces ER stress and reduces autophagy after two weeks. Autophagy and ER stress occurred prior to lipid accumulation, wherein autophagy preceded ER stress. Vice versa, induction of autophagy could alleviate ER stress, restored insulin signalling and reduced liver fat content [147].

In line with these results, knockdown of *Atg7* increased ER stress levels in lean mice, while overexpression of *Atg7* in obese ob/ob mice showed the opposite [42]. Furthermore, HFD induced obesity or *in vitro* addition of SFA was able to impair autophagy by inhibiting the fusion of autophagosomes with lysosomes. This impairment induced increased ER stress. Intriguingly, ER stress *in se* was not able to inhibit autophagy, but the underlying inhibition of SERCA pumps with subsequent rise in cytosolic calcium levels was. Calcium channel blockers were able to restore autophagy, ER stress and the metabolic consequences of HFD or SFA [43].

Finally, C1q/TNF-related protein 9 (CTRP9) is the closest known paralog of adiponectin and also thought to serve as an adipokine. CTRP9 has shown *in vitro* and *in vivo* to induce hepatocellular autophagy, reduce ER stress and subsequently alleviate TG accumulation and apoptosis. The reduction in ER stress was independent of direct effects on UPR chaperones and proven to be dependent on its actions on autophagy [148].

These studies subscribe the reciprocal effects of autophagy on ER stress and their role (albeit possibly indirectly) on lipid metabolism.

## 9. Conclusion

The current literature clearly emphasises the importance of autophagy in the liver. Nevertheless, when focussing on its role in liver lipid metabolism, controversy still exists regarding lipolytic or lipogenic features of autophagy. Moreover, autophagy is a highly dynamic process and appears to act in a context- and tissue-specific way.

Autophagy is not only involved in lipid metabolism but also in glucose metabolism, liver fibrogenesis and cellular injury. At the cellular level, there is a close interaction between ER stress and the corresponding UPR, another cellular homeostatic defence mechanism.

Unravelling the exact function of autophagy in the complex pathophysiology of metabolic disturbances and NAFLD could make autophagy an interesting target for treatment of the metabolic syndrome or for NAFLD.

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