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Intestinal Alkaline Phosphatase Orchestrates Autophagy to Alleviate Hepatocellular Lipid Accumulation in Non-Alcoholic Fatty Liver Disease

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Zusammenfassung

Hintergrund: Eine Dysregulation der Autophagie gilt als zentraler Mechanismus in der Pathogenese der nicht-alkoholischen Fettlebererkrankung (NAFLD). Die Induktion von Autophagie hat sich als vielversprechender therapeutischer Ansatz zur Reduktion der Lipidablagerung in Hepatozyten herausgestellt. Die intestinale alkalische Phosphatase (IAP), ein Bürstensaumenzym, das bereits in Mausmodellen erfolgreich zur Behandlung von Leberfibrose eingesetzt wurde, hat Autophagie-induzierende Effekte in intestinalen Epithelzellen und Makrophagen gezeigt. In der vorliegenden Arbeit wurde das Potenzial von IAP, Autophagie zu induzieren und die Lipidakkumulation in HepG2-Zellen zu verringern untersucht, um eine Grundlage für ihre therapeutische Anwendung bei NAFLD zu schaffen.

Materialien und Methoden: Zunächst wurde ein HepG2-basiertes Zellmodell für die Fettleber etabliert, indem wir die Zellen mit einer Mischung aus freien Fettsäuren (FFA), bestehend aus Ölsäure und Palmitinsäure, behandelt haben. Die Lipidakkumulation wurde mittels Oil-Red-O-Färbung und Durchflusszytometrie nachgewiesen. Die Lipotoxizität wurde mit dem MTT-Assay bewertet. Die Wirkung von IAP auf die autophagische Aktivität wurde anhand von HepG2-Zellen untersucht. Die Induktion der Autophagie wurde durch LC3B-II-Expression mittels Western-Blot analysiert. Die Bildung von Autophagosomen wurde mittels Monodansylcadaverin (MDC)-Färbung nachgewiesen. Die Wirkung von IAP auf die Lipidreduktion wurde sowohl in Anwesenheit als auch in Abwesenheit des Autophagieinhibitors 3-Methyladenin (3-MA) gemessen.

Ergebnisse: Die Oil-Red-O-Färbung zeigte eine deutliche Zunahme der Lipidakkumulation in HepG2-Zellen. Die Durchflusszytometrie quantifizierte diese Akkumulation und zeigte eine signifikante Erhöhung der intrazellulären Lipide nach Behandlung mit einer 400 μM

FFA-Mischung. Der MTT-Assay bestätigte, dass die FFA-Mischung eine lipotoxische Wirkung auf HepG2-Zellen ausübt, wobei die Zellviabilität bei derselben Konzentration (400 µM) unter 50 % sank. Daher wurde eine FFA-Mischung mit 400 µM für nachfolgende Experimente ausgewählt. Die Ergebnisse des Western Blots zeigten eine signifikante Induktion der autophagischen Aktivität in HepG2-Zellen nach IAP-Behandlung, insbesondere nach 24 Stunden Exposition und bei einer IAP-Konzentration von 50 U/ml. Gleichzeitig zeigte die MDC-Färbung, dass im Vergleich zur Vehikel-Gruppe die mit IAP behandelte Gruppe eine deutliche Zunahme der Autophagosomenansammlung aufwies. Die Durchflusszytometrie zeigte eine signifikante Reduktion der Lipidablagerung im Fettleber-Zellmodell nach IAP-Behandlung. Bemerkenswerterweise wurde dieser Effekt durch die Einführung von 3-MA aufgehoben.

Schlussfolgerung: IAP reduziert die Lipidablagerung in Hepatozyten, wobei die Induktion der Autophagie als möglicher zugrunde liegender Mechanismus identifiziert wurde. Unsere Ergebnisse unterstreichen die vielversprechende therapeutische Rolle von IAP bei der Behandlung der NAFLD.

Abstract

Background: Dysregulation of autophagy is recognized as a pivotal mechanism in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). The induction of autophagy has emerged as a prospective therapeutic avenue for mitigating lipid deposition within hepatocytes. Intestinal alkaline phosphatase (IAP), a brush border enzyme implicated in treating liver fibrosis in murine models, has demonstrated autophagy-inducing effects in intestinal epithelial cells and macrophages. In this study, we explore the potential of IAP to induce autophagy and ameliorate lipid accumulation in HepG2 cells, establishing a foundation for its therapeutic utility in NAFLD.

Materials and methods: First, a HepG2-based fatty liver cell model was established by treating the cells with a mixture of free fatty acids (FFAs) consisting of oleic acid and palmitic acid. Lipid buildup was confirmed through Oil Red O staining and flow cytometry. Lipotoxicity was evaluated using the MTT assay. Bovine-derived IAP was applied to HepG2 cells to investigate its impact on autophagic activity. Autophagy induction was assessed by monitoring LC3B-II expression through western blot analysis, and autophagosome formation was detected using monodansylcadaverine (MDC) staining. The effect of IAP on lipid reduction was measured with and without the autophagy inhibitor 3-Methyladenine (3-MA).

Results: Oil Red O staining revealed an increase in lipid accumulation in HepG2 cells. Flow cytometry analysis further quantified this accumulation, demonstrating a significant rise in intracellular lipids following treatment with a 400 μM concentration of the FFA mixture. The MTT assay confirmed that the FFA mixture exerts a lipotoxic effect on HepG2 cells, with cell viability dropping below 50% at the same concentration. Therefore, 400 μM of the fatty acid mixture was selected for subsequent experiments. Western blot results demonstrated a significant induction of autophagic activity in HepG2 cells following IAP

treatment, particularly evident after a 24-hour exposure and at a concentration of 50 U/ml. Simultaneously, MDC staining showed that compared to the vehicle group, the IAP-treated group displayed a notable increase in the accumulation of autophagosomes. Flow cytometry analysis revealed a significant reduction in lipid deposition within the fatty liver cell model upon IAP treatment. Notably, this effect was reversed with the introduction of 3-MA.

Conclusion: IAP has an effect in reducing lipid deposition in hepatocytes, with the induction of autophagy identified as a potential underlying mechanism. Our findings underscore the promising therapeutic role of IAP in treating NAFLD.

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List of Abbreviations

3-MA	3-Methyladenin
APAP	acetaminophen
AASLD	American Association for the Study of Liver Diseases
BSA	bovine serum albumin
CMA	chaperone-mediated autophagy
DNL	de novo lipogenesis
DAMPs	damage-associated molecular patterns
DILI	drug-induced liver injury
DMSO	dimethyl sulfoxide
ER	endoplasmic reticulum
ECM	extracellular matrix
EFV	efavirenz
ERK	extracellular signal-regulated kinase
FA	fatty acid
FAO	fatty acid oxidation
FC	free cholesterol
GPL	glycerophospholipids
GCALP	germ cell alkaline phosphatase
HSL	hormone sensitive lipase
HSCs	hepatic stellate cells
HPCs	hepatic progenitor cells
HBV	hepatitis B
HCV	hepatitis C
HPP	hypophosphatasia
IAP	intestinal alkaline phosphatase

IBD	inflammatory bowel disease
JNK	c-Jun amino-terminal kinase
LD	lipid droplet
LPS	lipopolysaccharide
LC3-1	microtubule-associated protein 1 light chain 3
MAPKs	Mitogen-Activated Kinases
MFI	median fluorescence intensity
MVM	microvillous plasma membrane
MTP	microsomal triacylglycerol transfer protein
mg	milligram
ml	milliliter
NAFLD	nonalcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEFA	non-esterified fatty acid
NAD	nicotinamide adenine dinucleotide
NPCs	non-parenchymal cells
NaOH	sodium hydroxide
OCA	obeticholic acid
OD	optical density
PAMP	pathogen associated molecular patterns
PAS	pre-autophagosome structure
PDK1	phosphoinositide-dependent protein kinase-1
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI(3)P	phosphatidylinositol 3-phosphate
PI3KC3–C1	class III PI3K complex I

PLAP	placental alkaline phosphatase
Pi	inorganic phosphate
PPi	pyrophosphate
ROS	reactive oxygen species
SFAs	saturated fatty acids
SGLT-2	odium-glucose co-transporter-2
TAG	triacylglycerols
TCA	tricarboxylic acid
TGFβ	transforming growth factor-β
TLR4	toll like receptor 4
TNAP	tissue-nonspecific alkaline phosphatase
TSC2	tuberous sclerosis complex 2
UC	ulcerative colitis
VLDL	very low density lipoprotein

1. Introduction

1.1 NAFLD overview

Non-alcoholic fatty liver disease (NAFLD) is characterized by histological or imaging evidence of steatosis affecting more than 5% of liver cells, after ruling out secondary causes of fat accumulation.[1] NAFLD represents a spectrum of liver conditions, starting with simple fat accumulation (nonalcoholic fatty liver, or NAFL) and progressing to chronic inflammation (nonalcoholic steatohepatitis, or NASH), which can result in fibrosis, cirrhosis, and even hepatocellular carcinoma.[2] NAFLD is increasingly acknowledged as the liver-related component of metabolic syndrome, with its global prevalence rising significantly in recent decades, closely mirroring the epidemics of obesity and type 2 diabetes.[3, 4] NAFL used to be considered a benign condition that is reversible and rarely results in cirrhosis or liver cancer.[5] However, according to a meta-analysis, NAFL can also progress into progressive fibrosis. Among patients with NAFL and NASH starting with stage 0 fibrosis, the annual progression rates were 0.07 stages and 0.14 stages, respectively.[6]

NASH, the inflammatory subtype of NAFLD, represents the more aggressive end of the NAFLD spectrum. It is marked by hepatocyte injury (such as ballooning) and inflammation, with or without the presence of fibrosis, and progresses over time, potentially leading to cirrhosis and hepatocellular carcinoma.[7] Given that the diagnosis of NASH requires liver biopsy, accurate data on its prevalence in the general population remains unknown.[8] The global prevalence of NASH is estimated to range between 3% and 5%.[9] While NASH can develop as a potential progression of NAFL, not all cases of NAFL advance to NASH. About 40% of NAFLD patients will progress to NASH.[10] In the development of NASH, steatosis represents the initial stage, while factors that trigger inflammation drive the progression from NAFL to NASH. Lipotoxicity, which causes hepatocytes to release signals that initiate wound-healing responses, is considered a major contributor to NASH.[11]

1.2 Lipid metabolism in the liver

The liver is the central organ in fatty acid metabolism.[12] Lipid processing relies on the balance between the transport of fatty acids (FAs) to the liver and their utilization through esterification or oxidation.[13] Steatosis develops when fat import or synthesis surpasses its export or degradation. FAs serve as the primary form of stored and circulating energy, with triacylglycerols (TGs) being the most prevalent non-toxic form. Neutral lipids, such as TGs and cholesterol esters, are stored in the liver within cytoplasmic organelles known as lipid droplets (LDs).[14] FAs in the liver are derived from three main sources: dietary TGs, direct uptake of FAs from the plasma, and de novo lipogenesis (DNL).

1.2.1 Dietary TG

Dietary TG are broken down into FAs within the intestinal lumen. After emulsification, these lipid molecules are absorbed by intestinal cells and re-esterified into TGs.[15] The TGs are subsequently incorporated into chylomicrons, released into the lymphatic system, and eventually enter the bloodstream.[16] In circulation, most chylomicron triglycerides are absorbed by muscle and adipose tissue. Only a small proportion of dietary FAs from chylomicron remnants or the spillover FAs resulting from peripheral hydrolysis of chylomicron triglycerides is available to the liver. Therefore, dietary FAs are not a major source of triglyceride storage in the liver. Studies found that reducing carbohydrate intake may be more important for decreasing hepatic TG content than reducing calorie intake.[17-19] Moreover, under eucaloric conditions, reducing carbohydrate and increasing fat intake can reduce liver triglyceride content in individuals both with and without steatosis.[20, 21]

1.2.2 Direct fatty acid uptake

The plasma pool of non-esterified fatty acids (NEFAs) significantly contributes to TG formation in the liver during the fasted state, accounting for an estimated 60% of the hepatic TAG content in NAFLD patients.[22, 23] The liver absorbs NEFAs from the

bloodstream in direct proportion to their concentration, with increased fatty acid influx rapidly elevating hepatic lipid content.[24] Plasma NEFA concentrations are elevated in three interrelated pathological conditions: obesity, type 2 diabetes, and hypertriglyceridemia. In both the fasting and fed states, adipose tissue is the primary contributor to the NEFA pool.[23] In insulin-resistant states, impaired suppression of hormone-sensitive lipase (HSL) activity results in enhanced lipolysis within adipose tissue, leading to an elevated release of FAs into the plasma NEFA pool.[25] As a result, insulin-sensitizing agents may offer effective therapeutic strategies for managing NAFLD.[26]

1.2.3 Hepatic *de novo* lipogenesis

Hepatic de novo lipogenesis (DNL) is the biochemical process by which FAs are synthesized from acetyl-CoA subunits, which are generated during glycolysis from dietary carbohydrates and other substrates.[27] Therefore, a high carbohydrate diet has been demonstrated to significantly enhance hepatic DNL.[28] This process occurs in three sequential steps: fatty acid synthesis, fatty acid elongation, and triglyceride assembly. The conversion of acetyl coenzyme A (acetyl-CoA) to malonyl-CoA serves as the rate-limiting step in DNL. Approximately 25% of accumulated TAGs in the liver are derived from DNL. Abnormal increases in hepatic DNL is a key contributor to NAFLD. [23] It is reported that hepatic DNL increases from 2%-5% in healthy individuals to more than 26% in patients with NAFLD, [23] and further increases to approximately 43% in those with NASH and fibrosis.[29]

1.2.4 Fatty acid oxidation (FAO)

FAs are oxidized in the liver through multiple pathways within three key organelles: the mitochondria, peroxisomes, and the endoplasmic reticulum (ER). The primary route for oxidizing short-chain (<C8), medium-chain (C8-C12), and long-chain (C12-C20) fatty acids is mitochondrial β-oxidation. Additionally, very-long-chain fatty acids (>C20), which are

considered more toxic, are oxidized through peroxisomal β-oxidation, while microsomal ω-oxidation of both saturated and unsaturated fatty acids occur in the ER, mediated by CYP4A enzymes.[30, 31] Fatty acids are metabolized within mitochondria through the β-oxidation pathway, leading to the generation of acetyl-CoA. This acetyl-CoA can either undergo condensation to form ketone bodies or be utilized in the tricarboxylic acid (TCA) cycle for further oxidation. When acetyl-CoA is converted into ketone body formation rather than fully oxidized through the TCA cycle, less ATP is produced per mole of fatty acid oxidized. Very-long-chain fatty acids, however, require initial shortening via peroxisomal β-oxidation before their complete oxidation in the mitochondria.[32] An efficient peroxisomal oxidation system is critical for reducing the accumulation of dicarboxylic acids and other toxic fatty acid intermediates, preventing conditions like hepatic steatosis. The P450 CYP4A ω-oxidation pathway also acts as an auxiliary substrate for β-oxidation when mitochondrial oxidation is impaired, such as in obesity and diabetes.[33] In addition to mitochondria, peroxisomes and microsomes are key contributors to reactive oxygen species (ROS) production.[34]

1.2.5 Very low-density lipoprotein (VLDL) excretion

Under normal conditions, the liver contains very little or no fat but plays a key role in exporting significant amounts of VLDL particles. These particles transport fatty acids to muscle tissue for oxidation and to adipose tissue for storage. Most of the triglycerides in VLDL originate from exogenous lipids rather than *de novo* lipogenesis.[35] The assembly of VLDL can be divided into two distinct steps. In the first step, lipid components are added to Apo protein B100 (apoB100) by the microsomal triacylglycerol transfer protein (MTP) , resulting in the formation of a lipid-poor, primordial VLDL particle.[36] In the second step, the primordial VLDL particles fuses with TG-rich particles and carried to the Golgi apparatus to form VLDL particles. Secretory vesicles detach from the Golgi membrane, travel to the

sinusoidal membrane of hepatocyte, fuse with it, and release VLDL particles into the bloodstream.[37] Factors that affect VLDL synthesis and secretion can lead to aggravation of fatty liver. A low or absent level of apoB results in a decreased capacity for triglyceride transport from the liver, leading to increased hepatic fat content.[38, 39] Patients with NASH exhibit impaired synthesis and excretion of lipoproteins, contributing to disrupted lipid metabolism and buildup of fat in the liver.[40]

1.3 The pathogenesis of NAFLD

In the past decades, the most cited theory of NAFLD pathogenesis is the “two-hit hypothesis.” The "first hit" involves lipid accumulation in hepatocytes, driven by factors such as a high-fat diet, obesity, and insulin resistance. This initial lipid buildup sensitizes the liver to further damage. The “second hit” includes oxidative stress, lipid peroxidation, proinflammatory cytokines, adipokines, mitochondrial dysfunction, endotoxins, and gut microbiota, as well as genetic and epigenetic factors, all of which promote liver injury, inflammation, and fibrosis. The gut-liver axis plays a pivotal role in this process. Pathogen-associated molecular patterns (PAMPs) derived from the gut can translocate to the liver and trigger inflammation. For example, bacterial by-products like lipopolysaccharide (LPS) can travel to the liver via the portal vein. As a strong pro-inflammatory agent, LPS activates immune responses in the liver, exacerbating inflammation and promoting the development of fibrosis. (Figure 1).[41]

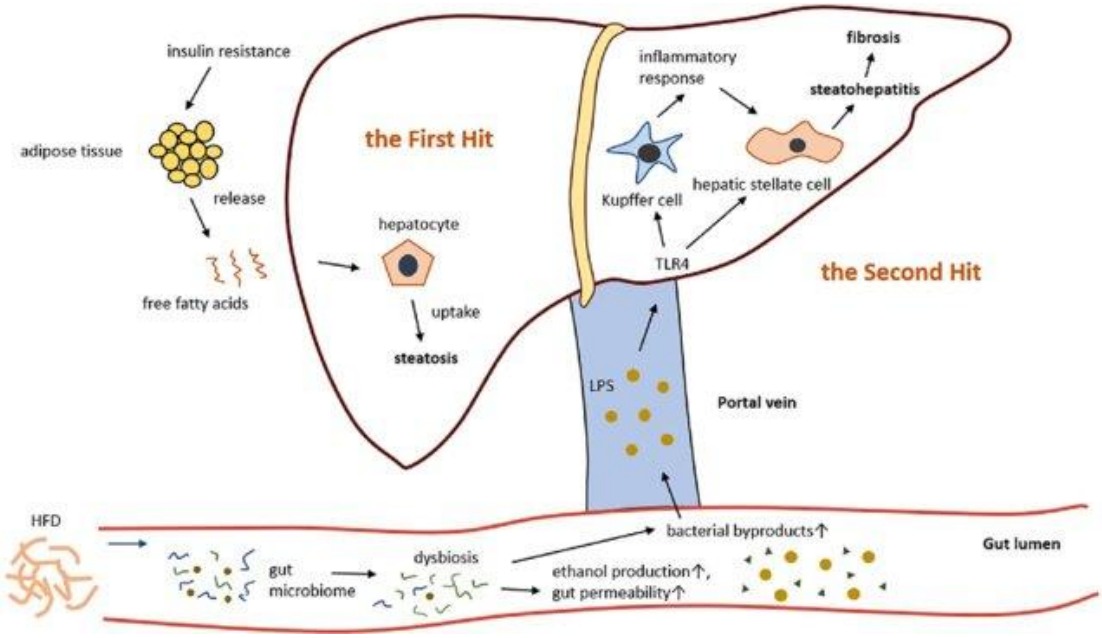


Figure 1. The role of the gut-liver axis in the pathogenesis of NAFLD (adapted from An et al.[41]). A high-fat diet induces gut microbiota dysbiosis and compromises the integrity of the intestinal barrier, allowing increased translocation of bacterial products into the circulation and contributing to systemic and hepatic inflammation. In the liver, the "first hit" involves lipid accumulation in hepatocytes, triggered by obesity or insulin resistance. Gut-derived bacterial products, including pro-inflammatory molecules like LPS, bind to specific receptors on immune cells in the liver, such as Toll-like receptor 4 (TLR4). This interaction activates immune pathways, triggering an inflammatory response and contributing to liver injury and disease progression.

1.3.1 The role of lipotoxicity in NAFLD — from NAFL to NASH

Lipotoxicity describes the detrimental effects of excessive lipid buildup in non-adipose tissues, leading to organelle dysfunction, abnormal intracellular signaling activation, chronic inflammation, and cell death— widely recognized as key hallmarks of NASH. [42, 43] Notably, not all lipids are toxic; for example, TGs, have a less harmful effect and are considered relatively inert lipid species. It is now widely accepted that storing excess lipid molecules as triacylglycerol is a protective mechanism against cellular lipotoxicity.[44]

Polyunsaturated fats and monounsaturated fats are considered beneficial in inhibiting inflammation. In contrast, certain lipids, including saturated fatty acids (SFAs), free cholesterol (FC), glycerophospholipids (GPLs), and sphingolipids, are considered major toxic lipids. They significantly contribute to hepatocyte injury and cell death, thereby accelerating the progression of NASH.[45]

At the cellular level, the mechanisms underlying lipotoxicity involve a variety of cellular processes, such as ER stress, mitochondrial dysfunction, and lysosomal dysfunction. Among these, mitochondrial oxidative stress is the primary driver of NASH.[46] Additionally, the crosstalk between damaged hepatocytes and liver non-parenchymal cells (NPCs) through cytokines, chemokines, and other molecules can trigger tissue inflammation and a wound-healing response, ultimately leading to fibrosis and cirrhosis.[47] The NLRP3 inflammasome recognizes damage-associated molecular patterns (DAMPs) triggered by cellular stress signals, such as lipotoxicity and mitochondrial dysfunction, as well as PAMPs from pathogens in the gut-liver axis. It has been demonstrated to be a critical contributor to the pathogenesis of NASH.[48] When activated, it facilitates the cleavage of pro-caspase-1 into its active form, driving the maturation of pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into their active forms, IL-1β and IL-18, respectively. These cytokines then promote inflammation and hepatocyte injury, playing a significant role in the transition from simple steatosis to NASH.

1.3.2 The role of inflammation in NAFLD — from NASH to fibrosis

The extent of liver fibrosis is the most important predictor of liver-related mortality and the onset of other comorbidities.[49] Liver fibrosis reflects the liver's attempt to heal chronic injuries through overproduction of extracellular matrix (ECM), a process that disrupts normal liver structure. Activated hepatic stellate cells (HSCs) are the main contributors to ECM production. It is now widely accepted that an exaggerated immune response can

trigger a pro-fibrogenic response, with the hepatocyte-macrophage-HSC network serving as the key driver of fibrosis in NASH.[50] According to a systemic review, the presence of inflammation on the initial biopsy independently predicts the progression to advanced fibrosis in patients with NASH.[6] During this process, the inflammation associated with NASH leads to hepatocyte death and apoptosis, resulting in the release of DAMPs and apoptotic bodies.[51] DAMPs activate hepatic progenitor cells (HPCs), while apoptotic bodies are engulfed by HSCs and Kupffer cells. This process stimulates a pro-fibrogenic response driven by the release of transforming growth factor-β (TGFβ), which is the most potent cytokine promoting fibrosis.[52] During prolonged liver injury, the infiltration of immune cells produces pro-fibrogenic cytokines, which activate the transdifferentiation of HSCs into collagen-producing myofibroblasts and stimulate ECM synthesis. Additionally, NLRP3 activation has been shown to enhance fibrosis by activating HSCs, further promoting extracellular matrix deposition.

1.4 Current therapy for NAFLD

The primary treatment strategies for NAFLD emphasize lifestyle changes, pharmacological interventions, and, in some cases, surgical procedures. Despite the increasing prevalence of NAFLD, there are currently no FDA-approved medications specifically designed to treat the condition. Below is an overview of the therapeutic approaches currently being used and studied:

1.4.1 Lifestyle modifications

Lifestyle changes are the foundation of NAFLD management, particularly in its early stages. Among these, weight loss is the most effective approach for decreasing liver fat, improving liver inflammation and fibrosis.[53] A target weight loss of 7-10% is recommended to improve liver health.[54] Caloric restriction, particularly reducing intake of sugars and saturated fats, helps decrease hepatic fat accumulation. Avoiding fructose is also important, as high

fructose consumption is associated with liver fat accumulation. Reducing fructose, commonly found in sugary beverages, can significantly benefit liver health.[55] In this context, the Mediterranean diet is recommended. This diet, rich in fruits, vegetables, whole grains, lean proteins, and healthy fats such as olive oil supports weight loss and lowers the risk of chronic diseases.[56]

In terms of physical activity, both aerobic and resistance exercises help reduce liver fat, even without significant weight loss, although resistance exercise tends to be less effective.[57] To reduce hepatic fat accumulation, it is recommended to engage in at least 150 minutes of moderate-intensity physical activity or 75 minutes of vigorous-intensity exercise per week.[58] In contrast, insufficient physical activity leads to weight gain, insulin resistance, and increased fat accumulation in the liver.[59] A sedentary life style, characterized by prolonged sitting or lying down, is strongly associate with NAFLD.[60]

1.4.2 Pharmacological therapies

Although no drug is approved specifically approved for the treatment of NAFLD, certain medications are used off-label, such as anti-diabetic and anti-dyslipidemia agents. The significance of pharmacological treatment for NAFLD lies in its potential to improve prognosis, particularly in preventing cirrhosis and its associated conditions, including ascites, hepatic encephalopathy, and upper gastrointestinal bleeding. Since only a small proportion of individuals develop these severe outcomes, pharmacotherapy must demonstrate the ability to improve liver histology.[61] Currently available drugs for the treatment of NAFLD primarily target two key mechanisms: insulin resistance and oxidative stress.

Anti-diabetic medications, such as metformin and pioglitazone, have been analyzed in meta-analyses for their efficacy in treating NAFLD. The results indicate that pioglitazone effectively reduces ballooning degeneration, improves lobular inflammation, and

decreases fat accumulation, while also alleviating liver fibrosis in NASH patients.[62] However, the findings for metformin suggest that it may exacerbate lobular inflammation and does not significantly improve intrahepatic fat accumulation.[63] Thus, pioglitazone has shown promise in treating NAFLD, especially in individuals with NASH. Vitamin E has been shown to not only normalize ALT levels in NAFLD patients but also improve liver histology in those with NASH.[63, 64] However, it does not appear to reduce liver fibrosis. The American Association for the Study of Liver Diseases (AASLD) recommends vitamin E at a dose of 800 IU per day as a therapeutic approach for non-diabetic patients with biopsy-confirmed NASH.[65] Despite its potential benefits, concerns remain about its long-term safety, including an increased risk of prostate cancer in men.

Several drugs are under investigation for NAFLD treatment. Statins, primarily used for dyslipidemia, do not reduce liver fat accumulation but help lower cardiovascular risk in NAFLD patients. Omega-3 fatty acids aid in managing high triglycerides. Obeticholic acid (OCA), a bile acid derivative and FXR agonist, has shown promise in reducing liver fibrosis. Similarly, GLP-1 receptor agonists, including liraglutide and semaglutide, have demonstrated potential in lowering liver fat and inflammation. Additionally, sodium-glucose co-transporter-2 (SGLT-2) inhibitors, such as dapagliflozin, commonly used in type 2 diabetes management, have also shown promise in improving liver enzyme levels and reducing liver fat in NAFLD patients.

1.4.3 Surgical therapies

Bariatric surgery such as gastric banding, sleeve gastrectomy and Roux-en-Y gastric bypass (RYGB) have demonstrated improvements in both biochemical markers and liver histology in NAFLD patients with obesity. These surgeries result in significant weight loss, which can reduce liver fat, inflammation, and fibrosis, and may even reverse NASH in some cases.[66] However, bariatric surgery is indicated for individuals with a BMI greater than 40

kg/m² or for those with a BMI of 35 kg/m² or higher accompanied by obesity-related comorbid conditions, as it carries considerable risks and requires long-term lifestyle changes.[67] Liver transplantation is an option for patients with NAFLD who progress to end-stage liver disease, but this is generally reserved as a last option for those with liver failure or advanced cirrhosis.[68]

1.5 Insights into autophagy

In addition to fatty acid uptake, DNL, triglyceride secretion, and fatty acid oxidation, lipophagy is now recognized as the fifth pathway contributing to the development and progression of NAFLD.[69] Autophagy is a highly conserved cellular process in eukaryotes cells, responsible for degrading and recycling unnecessary or dysfunctional cellular components through lysosomes. It has three subtypes: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), with macroautophagy being the predominant form in mammalian cells.[70] Macroautophagy can occur in two forms: non-selective or selective.[71] In the non-selective form, cells respond to nutrient deprivation by randomly engulfing portions of the cytoplasm. In the selective form, macroautophagy enables cells to specifically target and remove particular components, such as protein aggregates and damaged organelles, ensuring cellular quality control and homeostasis. Selective macroautophagic pathways are typically named based on the specific cargo targeted for degradation. For instance, aggrephagy targets protein aggregates, ferritinophagy degrades ferritin, mitophagy focuses on mitochondria, xenophagy eliminates pathogens such as bacteria, and ER-phagy removes components of the endoplasmic reticulum.[72] The selective autophagic process that breaks down intracellular lipid droplets is referred to as lipophagy.

1.5.1 Autophagic process

The process of macroautophagy can be described in several stages: the initiation and

extension of the phagophore, the closure and maturation of the membrane around its cargo to form the autophagosome, the fusion of the autophagosome with a lysosome to form an autolysosome, and, ultimately, the breakdown of the enclosed material.[73]

The autophagy process involves a set of approximately 16–20 core conserved ATG (autophagy-related) genes.[74] The induction of autophagy is mediated by the ULK1 kinase complex (comprising ULK1, FIP200, ATG13 and ATG101) from the pre-autophagosome structure (PAS) under nutrient starvation. Subsequently, the ULK complex recruits the class III PI3K complex I (PI3KC3–C1), which generates phosphatidylinositol 3-phosphate (PI(3)P). This PI(3)P then recruits its effector proteins, such as DFCP1, to the PAS, promoting the formation of the omegasome. Additionally, it attracts WIPI (WD repeat domain phosphoinositide-interacting) proteins and their associated binding partners, ATG2A or ATG2B, to the phagophores, contributing to their elongation.[75-77]

The ATG12 and LC3 conjugation systems (ATG8 in yeast) are essential for the expansion and closure of the autophagosome membrane. The ATG12-ATG5-ATG16 complex plays a pivotal role in facilitating ATG8/LC3 lipidation and the proper localization of LC3 to the PAS. LC3 is initially synthesized as proLC3, which is processed by ATG4, producing the cytosolic isoform LC3-I. This cytosolic form is subsequently conjugated to phosphatidylethanolamine (PE) in a reaction catalyzed by the enzymes ATG7 and ATG3, resulting in LC3-II. LC3-II specifically associates with the elongated autophagosome membrane, marking it for expansion and completion. In mammalian cells, LC3-II serves as an excellent protein marker for investigating autophagy.[78, 79]

1.5.2 Key pathways in autophagy

Autophagy is regulated by several pathways, among which the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathway serves as a central regulator. By coordinating nutrient signals, stress responses, and growth factors,

these pathways ensure that autophagy is properly balanced to maintain cellular health. Disruptions in these pathways are linked to numerous diseases, including cancer, neurodegenerative disorders, and metabolic conditions such as NASH.

1.5.2.1 The PI3K/AKT/mTOR pathway

mTOR, a member of the PI3K-related kinase family, is a conserved serine/threonine protein kinase that acts as both an ATP and amino acid sensor.[80] It acts in two different complexes: mTORC1 and mTORC2, whereby only mTORC1 can be inhibited by rapamycin.[81] mTORC1 consists of mTOR, MLST8, RAPTOR, PRAS40, DEPTOR, RadA-D, and Rheb-GP, while mTORC2 includes mTOR, MLST8, RICTOR, and associated proteins.[82] mTORC1 is a major negative regulator of endosomal biogenesis and autophagy, responding to signals from growth factors, amino acids, glucose, hormones, and stress, with amino acids being the most influential regulator.[83] This regulation is crucial for controlling cell growth and metabolism. Adequate intracellular nutrients enhance mTORC1 activity, which acts as a negative feedback mechanism to suppress autophagy. Conversely, amino acid deprivation can stimulate autophagy. Although mTORC1 is a well-established regulator of autophagy, the role of mTORC2 is less understood. [84]

The pathway begins with the activation of PI3K by various extracellular signals, such as growth factors. PI3K phosphorylates the inositol ring of phosphatidylinositol (PI) lipids at the plasma membrane, producing phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 then recruits AKT to the membrane, where AKT is activated through phosphorylation by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and mTORC2. When AKT is activated, it phosphorylates and inhibits tuberous sclerosis complex 2 (TSC2), a negative regulator of mTORC1. Inhibition of TSC2 results in mTORC1 activation, which suppresses autophagy by phosphorylating and inactivating essential autophagy-initiating proteins such as ULK1. In contrast, during nutrient deprivation or cellular stress, mTORC1 is inhibited,

allowing the activation of ULK1 and the subsequent initiation of the autophagy process.

1.5.2.2 The AMPK pathway

Another important pathway in the regulation of autophagy is the AMP-activated protein kinase (AMPK) signaling pathway. Research indicates that glucose starvation triggers autophagy through AMPK activation.[85] Furthermore, under conditions of amino acid sufficiency, AMPK can sustain autophagy to preserve protein homeostasis.[86] AMPK is a heterotrimeric complex consisting of a catalytic α-subunit and regulatory β- and γ-subunits. AMP binding to the γ-subunit allosterically activates the complex, facilitating the phosphorylation of the α-subunit at threonine 172, which is further phosphorylated by upstream LKB1.[87] AMPK can promote autophagy either by binding to the proline-serine-rich (PS) domain of ULK1 or by activating ULK1 through phosphorylation at specific sites, such as Ser317 and Ser777.[88, 89] The negative regulator of autophagy, mTORC1, inhibits AMPK by disrupting its interaction with ULK1.[90, 91] Conversely, AMPK can stimulate autophagy by inhibiting mTORC1 activity through the direct phosphorylation of TSC2 or RAPTOR. [92, 93] Additional mechanisms include the phosphorylation of the pro-autophagy PIK3C3/VPS34 complex and ATG9A, both of which enhance autophagosome production.[94, 95] Besides this, AMPK also promotes autophagy by activating autophagy regulators such as FOXO3 and nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase Sirtuin 1 (Sirt1), thereby promoting the expression of downstream autophagy-related genes.[96-98]

1.5.2.3 The MAPK pathway

Additionally, the MAPK signaling pathway is a potential regulator of autophagy. Mitogen-Activated Kinases (MAPKs) are well-conserved serine/threonine protein kinases, categorized into three main subfamilies: extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun amino-terminal kinase (JNK).[99] The MAPK signaling cascade involves

sequential phosphorylation of MAPKKK, MAPKK, and MAPK, leading to the rapid activation of downstream targets.[100] ERK can phosphorylate Bcl-2, which in turn affect Beclin-1, promoting autophagy.[101, 102] Activation of ERK can also stimulates autophagy through inhibition of mTOR.[103] However, sustained activation of the MAPK/ERK pathway causes the accumulation of defective autolysosomes, selectively disrupts autophagy during the maturation step, and results in cytodestructive autophagy.[104]

p38 MAPK can either promote or inhibit autophagy.[105] p38α has been found to suppress both basal and starvation-induced autophagy by negatively regulating the interaction between mAtg9 and p38IP, a novel binding partner of mAtg9. [106] Additionally, p38α MAPK can also directly phosphorylate ULK1, inhibiting its activity and thereby reducing autophagic flux.[107] Conversely, sustained activation of p38α has been reported to enhance basal autophagic flux.[108] Furthermore, lysosomal p38 MAPK can activate chaperone-mediated autophagy by directly phosphorylates LAMP2A.[109]

JNK, initially identified as a stress-activated protein kinase, can be activated by various stressors such as irradiation and oxidative stress.[110] Upon activation, JNK phosphorylates serine residues at the N-terminal of its downstream target c-JUN, enhancing its transcriptional activity.[111] JNK/MAPK signaling appears to induce cytoprotective autophagy in cells under stress. In an oxidative stress model of Drosophila, JNK signaling was shown to control ATG gene expression and activate autophagy.[112] Another well-known mechanism by which the JNK/MAPK pathway regulates autophagy is through JNK's ability to phosphorylate Bcl-2. This phosphorylation interferes with the interaction between Bcl-2 and Beclin-1, freeing Beclin-1 to initiate autophagy.[113, 114] In these cases, JNK-induced autophagy can function as a survival mechanism; however, if the stress is prolonged or too severe, JNK signaling can shift towards promoting apoptosis, playing a vital role in determining cell fate. [115, 116]

1.5.3 The role of autophagy in liver diseases

Autophagy is an essential cellular mechanism responsible for the ongoing clearance and recycling of intracellular components. This process is especially important in the liver, given its central role in metabolism and detoxification. According to a previous study, the liver exhibits a high degree of amino acid responsiveness. Under basal condition, autophagic function degrades approximately 1.5% of total hepatic protein per hour. During starvation, this rate rises significantly, reaching approximately 4.5% of total liver proteins per hour.[117] Dysregulation of autophagy is associated with liver disorders like fatty liver disease (both alcoholic and non-alcoholic), drug-induced liver injury (DILI), viral hepatitis, and hepatocellular carcinoma.[118]

1.5.3.1 Autophagy in NAFLD

Autophagy is essential for lipid degradation in the liver. Impaired autophagic function in the liver has been linked to the development of NAFLD in humans and mouse models of obesity.[119, 120] Moreover, autophagy function decreases with age, and this may account for the age-dependent accumulation of lipids.[121] Inhibiting autophagy by agents such as chloroquine has been shown to exacerbate liver steatosis.[122] Conversely, activating autophagy shows therapeutic potential by restoring autophagic flux to mitigate these conditions. Singh et al.[123] were the first to highlight the critical role of autophagy in managing intracellular lipid storage. Subsequent studies have shown that activating lipophagy significantly reduces steatosis,[124-126] further emphasizing the therapeutic potential of restoring autophagic flux in addressing these conditions.

However, autophagy in the liver acts as a double-edged sword. While increased autophagy flux can be beneficial, it is also elevated during hepatic stellate cell activation, which is a crucial process driving the development of liver fibrosis. [127] In their quiescent state, HSCs contain cytoplasmic lipid droplets, and it is likely that autophagy promotes the breakdown

of these droplets into free fatty acids, which are subsequently processed through β-oxidation to generate ATP. This energy supply facilitates HSC activation and the deposition of extracellular matrix, thereby contributing to the progression of fibrosis. [128] Studies have demonstrated that inhibiting autophagy can block HSC activation (Figure).[128, 129] Therefore, while enhancing autophagy may help resolve steatosis, its role in HSC activation suggests that careful regulation is needed to prevent exacerbating fibrosis.

In addition to lipophagy, mitophagy and reticulophagy are vital in the progression of NAFLD, particularly in the transition from simple steatosis to NASH.[130] Lipotoxicity induces mitochondrial dysfunction and ER stress, resulting in hepatocyte injury and inflammation. In response, mitophagy and reticulophagy act as self-protective processes, clearing damaged organelles and misfolded proteins to mitigate cellular damage. Evidence shows that in response to lipid overload, both mitophagy and reticulophagy are activated, suggesting a protective role in the early stages of NAFLD.[131] However, mitophagy is impaired in patients with NAFLD and in mice during the later stages, indicating a failure of the adaptive response to lipid stress.[132, 133] Additionally, impaired mitophagy can trigger NLRP3 inflammasome activation in a mouse model of NASH.[132] These findings highlight that proper mitophagic function is essential for maintaining cellular homeostasis and preventing disease progression in NAFLD.

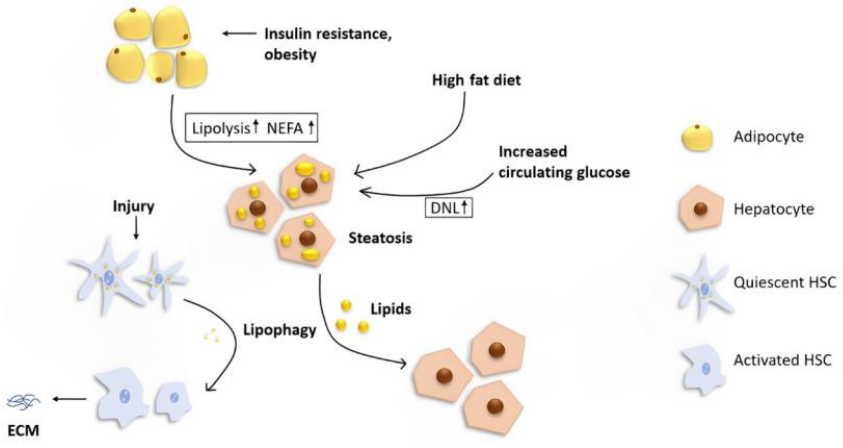


Figure 2. The dual role of lipophagy in the pathogenesis of NAFLD (adapted from An et al.[134]). In hepatocytes, lipophagy reduces intracellular lipid levels, thereby mitigating hepatic lipid accumulation. However, in contrast, lipophagy can also lead to the activation of hepatic stellate cells, triggering the secretion of extracellular matrix components, ultimately contributing to liver fibrosis. This illustrates the double-edged nature of lipophagy in NAFLD progression.

1.5.3.2 Autophagy in other liver diseases

DILI

The most common drugs that induce liver injury include acetaminophen (APAP), efavirenz (EFV), diclofenac and certain anticancer drugs like cisplatin. These drugs cause liver damage mainly by inducing mitochondrial dysfunction, which results in the accumulation of defective mitochondria, which further exacerbates liver injury. Autophagy serves a protective function in DILI by clearing damaged mitochondria, protein aggregates, and toxic adducts in hepatocytes. Among its processes, mitophagy, the selective degradation of damaged mitochondria, is particularly crucial in mitigating DILI. However, excessive activation of autophagy in response to severe drug-induced stress can result in cell death. Consequently, autophagy plays a dual role in DILI.

Alcohol-related liver diseases

Alcohol-related liver diseases encompass a spectrum of conditions, including liver injury, alcoholic fatty liver disease, and end-stage liver disease such as cirrhosis. Alcohol consumption has been shown to influence autophagy in different ways. In an alcohol binge model, autophagy is stimulated through ethanol metabolism, ROS production, and mTOR inhibition. In contrast, chronic alcohol consumption generally inhibits autophagy primarily due to a depletion of available lysosomes, which prevents autophagosomes from fusing and degrading their cargo effectively—a condition termed “insufficient autophagy.” Despite

these differences, the activation of autophagy serves a protective role by alleviating steatosis and liver injury caused by both binge and chronic alcohol exposure.[122, 135, 136]

Viral hepatitis

Both hepatitis B (HBV) and hepatitis C (HCV) have been observed to temporally regulate autophagic flux.[137, 138] These viruses hijack the autophagic machinery, utilizing autophagic membranes for the assembly of their replication complexes. This is evidenced by the accumulation of immature autophagosomes during early infection, indicating that the viruses induce incomplete autophagy.[139, 140] Autophagy facilitates the production of both HBV and HCV, and studies have demonstrated that inhibiting autophagy can reduce the replication of both HBV and HCV This makes manipulating autophagy a potential therapeutic target for treating HBV and HCV infections.

HCC

The function of autophagy in HCC is not yet fully understood. However, during the dysplastic stage of HCC initiation, autophagy functions as an anti-cancer mechanism, but as the disease progresses, it supports HCC development and contributes to treatment resistance. It is reported that mice with a deletion of ATG5 or ATG7 genes develop multiple liver tumors, highlighting autophagy's role in suppressing spontaneous tumorigenesis in the liver.[141] Conversely, other studies demonstrate that autophagy can promote HCC through various mechanisms.[142] This dual role of autophagy in HCC underscores the importance of understanding the specific circumstances under which autophagy contributes to or inhibits HCC progression, as this understanding is essential for developing effective therapeutic strategies. Further research is needed to clarify these roles and optimize autophagy-targeted therapies for HCC.

1.6 Insights into alkaline phosphatases

Alkaline phosphatase are ubiquitous ectoenzymes widely distributed in nature.[143, 144] There

are four distinct alkaline phosphatase isozymes: tissue-nonspecific alkaline phosphatase (TNAP), placental alkaline phosphatase (PLAP), germ cell alkaline phosphatase (GCALP), and intestinal alkaline phosphatase (IAP).[145] The gene encoding TNAP is located on chromosome 1 at band p36.1-p34, while the genes for the other three ALPs are clustered on chromosome 2, at bands q34.2-q37.[146, 147] These tissue specific ALPs are about 90% sequence homology with one another but are only about 50% identical to TNALP.

1.6.1 TNAP

Unlike the tissue-specific APs with highly restricted expression, TNAP is found in various tissues like liver, kidney, and bone, and constitute most of the circulating fraction in serum. This enzyme, located on the surface of mineralizing cells, is essential for proper skeletal mineralization. The nature substrate of TNAP is pyrophosphate (PPi), it is a potent inhibitor of mineralization. TNAP degrades PPi to inorganic phosphate (Pi) in bones and teeth and facilitates hard tissue formation.[148] Deficiency or malfunction of TNAP can lead to hypophosphatasia (HPP), an inborn metabolic bone disease that features rickets or osteomalacia.[149] TNAP knockout mice show defects in bone mineralization and vitamin B6 dependent epilepsy and die before weaning.[150] Conversely, elevated ALP levels are observed in bile duct obstruction, active bone formation, conditions affecting blood calcium levels (such as vitamin D deficiency or liver cell damage), and untreated celiac disease.

1.6.2 PLAP and GCAP

PLAP and GCAP exhibit a 98% identity at the protein level, highlighting their close similarity.[151] PLAP is a membrane-anchored, heat-stable enzyme predominantly localised in maternal-facing microvillous plasma membrane (MVM) of the placental syncytiotrophoblast. After the 12th week of normal pregnancy, its levels start to rise in the blood, suggesting a role in placental transport and metabolism, though its exact function remains unclear.[152-154] PLAP is also found in ovarian cancer, testicular seminoma, and

endometrial cancer, and the serum level of PLAP may be linked to the activity of seminoma [155-157] However, PLAP is not a reliable marker for seminoma due to its lack of specificity and sensitivity, as well as the frequent occurrence of nonspecific background signals. Additionally, smoking can significantly elevate PLAP levels in patients without the disease, further compromising its reliability. Furthermore, the high degree of similarity between PLAP and GCAP proteins, coupled with cross-reactive antibodies, has led to confusion regarding the distribution of these enzymes across various tissues.[155, 158]

GCAP (also known as PLAP-like enzyme) is mainly found in testis, cervix, as well as in the thymus.[159] It is commonly expressed in germ cell tumors and has also been identified in seminomas, where elevated serum GCAP levels are common.[160] Smoking can also elevate serum concentrations of the PLAP-like enzyme.[159] Both the placental and germ cell alkaline phosphatase genes are mapped to chromosome 2, and their encoded proteins are indistinguishable using standard enzymatic or immunohistochemical techniques.

1.6.3 IAP and its role as a regulator of autophagy

IAP is a gut brush border enzyme secreted by enterocytes in the proximal small intestine. It is released bilaterally into the gut lumen and the blood, where its primary function is to detoxify bacterial-derived proinflammatory factors such as LPS, CpG-DNA, and flagellin.[161] IAP maintains intestinal homeostasis through four major functions: regulating duodenal surface pH levels, controlling long-chain fatty acid absorption, alleviating intestinal inflammation, and modulating the gut microbiome. The functions of IAP have been thoroughly reviewed in various articles.[162, 163]

Due to its beneficial effects, IAP has been implicated in various diseases, particularly in inflammatory bowel disease (IBD), sepsis and metabolic conditions. In IBD patients, both protein and mRNA levels of IAP are reduced in the mucosa. Research has shown that exogenous oral administration of IAP can alleviate disease symptoms in mouse models of

chronic colitis. [164,165] In conditions such as ulcerative colitis (UC) and Crohn's disease, IAP has been explored as a gut mucosal defense factor and a potential biomarker for disease monitoring.[166, 167] IAP has been found to reduce inflammatory responses by detoxifying LPS during sepsis. Two phase II clinical trials have demonstrated that IAP can improve renal function in patients suffering from severe sepsis and septic shock.[168, 169] In mouse model of sepsis, IAP is shown to modify intestinal integrity, alleviate inflammation and bacterial translocation.[170] In human adults, fecal IAP levels have been shown to have an inverse relationship with type 2 diabetes mellitus (T2DM).[171, 172] In various metabolic conditions observed in mouse models, including obesity, glucose intolerance, insulin resistance, dyslipidemia, and fatty liver, IAP has been demonstrated to alleviate these conditions. The underlying mechanism may involve IAP reducing low-grade inflammation caused by gut-derived endotoxemia, which is linked to metabolic syndrome.[173, 174]

A study shows that IAP can induces autophagy in enterocytes and macrophages in a TLR4-dependent manner.[175] Additionally, numerous studies indicate that maintaining gut barrier function, exhibiting anti-inflammatory effects, preserving gut microbes, and promoting mucin secretion are related to autophagy.[176] These effects are closely linked to the functions of IAP. Although there is insufficient evidence to demonstrate that IAP achieves these effects through an autophagy-dependent manner, it can be hypothesized that IAP may act as an autophagy regulatory factor.

1.7 Aim of this study

NAFLD is associated with dysregulated autophagy. Research shows that inducing autophagy can alleviate hepatic lipid deposits. IAP, a gut brush border enzyme with multiple functions, has been shown to induce autophagy in gut epithelial cells and macrophages. This study aims to explore whether IAP, as a potential regulator of autophagy, can reduce lipid content in a fatty liver cell model via an autophagy-dependent mechanism.

2. Materials and Methods

2.1 Materials

2.1.1 Consumable items

Consumables	Company or source
6-well plates	Thermo Fisher Scientific, Roskilde,Denmark
96-well plates	Thermo Fisher Scientific, Roskilde,Denmark
5ml pipette	Costar, Maine, USA
10ml pipette	Costar, Maine, USA
25ml pipette	Costar, Maine, USA
50ml pipette	Costar, Maine, USA
1.5ml tubes	Eppendorf, Hamburg, Germany
2.0ml tubes	Eppendorf, Hamburg, Germany
15ml tube	Falcon, Reynosa, Mexico
20ml tube	Falcon, Reynosa, Mexico
Cell culture flask T75	Thermo Fisher Scientific, Roskilde,Denmark
Cell scraper TPP	Trasadingen, Switzerland
FACS tubes Falcon	New York, USA
Polyvinylidene difluoride membranes	Merck Group, Darmstadt, Germany
Western Blot paper	Bio-Rad, California, USA

2.1.2 Chemicals

Chemicals	Company or source
Ammonium persulfate (APS)	Serva, Heidelberg, Germany
Bafilomycin A1	Sigma-Aldrich, Germany

β-Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
BSA	Biomol, Plymouth Meeting, USA
calf IAP	Sigma-Aldrich, Germany
30%PolyAcrylamid	Carl Roth, Karlsruhe, Germany
DMSO	Sigma-Aldrich, Karlsruhe, Germany
ECLTM Western Blotting Detection System	Bio-Rad Laboratories, California, USA
80% Ethanol	Apotheke GH, Munich, Germany
>99% Ethanol	PanReac AppliChem, Germany
FBS	PAN-Biotech, Munich, Germany
Loading buffer 4x	Bio-Rad, California, USA
Methanol	Merck, Darmstadt, Germany
MTT powder	Thermo Fisher Scientific, Massachusetts, USA
MDC	Sigma-Aldrich, Germany
Mounting Medium with DAPI	Abcam, USA
Nile Red	Sigma-Aldrich, Germany
Oil Red O	Sigma-Aldrich, Germany
PBS	PAN-Biotech, Munich, Germany
Protein standards	Roche, Basel, Switzerland
Palmitic Acid	Sigma-Aldrich, Germany
Phosphatase, Alkaline, Calf Intestine	Sigma-Aldrich, Germany
10X Tris/Glycine/SDS buffer (Running buffer)	Bio-Rad Laboratories, California, USA
RPMI 1640 Medium	Gibco, New York, USA

RIPA lysis buffer 10X	Millipore, Darmstadt, Germany
SDS	Carl Roth, Karlsruhe, Germany
TEMED	Thermo Fisher Scientific, Massachusetts, USA
Transfer Buffer (20X)	Novex, Van Allen Way, Carlsbad, CA
Tris Base	Carl Roth, Karlsruhe, Germany
Trypsin/EDTA	Lonza, St Louis, USA
Tween 20	Sigma-Aldrich, Heidelberg, Germany
3-MA	Sigma-Aldrich, Germany

2.1.3 Antibodies

Antibodies	Company	Identifier
LC3B	Novus Biologicals	Cat#NBP2-46892
GAPDH	Santa Cruz Biotechnology	Cat#sc-25778
IgG HRP-linked Antibody	Cell Signaling Technology	Cat#70745

2.1.4 Apparatus

Apparatus	Company or source
Autoclave	Unisteri, Oberschleißheim, Germany
Centrifuge	Hettich, Ebersberg, Germany
Cool Centrifuge	Eppendorf, Hamburg, Germany
Micro centrifuge	Labtech, Ebersberg, Germany
CO2 Incubator	Binder, Tuttlingen, Germany
Drying cabinet	Thermo Fisher Scientific, Schwerte, Germany
Electronic pH meter	Knick Elektronische Messgeräte, Berlin, Germany

FACS Fortessa	BD Biosciences, Heidelberg, Germany
Fridge (4℃, -20℃ and -80℃)	Siemens, Munich, Germany
Fluorescence Microscopy	Zeiss, Goettingen, Germany
Liquid Nitrogen tank	MVE Goch, Germany
Lamina flow	Thermo Fisher Scientific, Schwerte, Germany
Microscope	Zeiss, Germany
Pipette boy	Eppendorf, Hamburg, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
ChemiDoc Imaging System	Bio-Rad Laboratories, California, USA
Shaker	Edmund Bühler, Bodelshausen, Germany
Vortex Mixer VF2 (Janke & Kunkel)	IKA, North Carolina, USA
Water bath	Memmert, Schwabach, Germany

2.1.5 Software

Software and version	**Company**
FlowJo Vesion 10.0	BD Biosciences
Graphpad Prism 7.04	GraphPad
ImageJ Version 1.50i	National Institutes of Health

2.2 Methods

2.2.1 Cell culture and treatments

The HepG2 carcinoma cell line was initially obtained from ATCC (Manassas, Virginia, USA) and kept in nitrogen-filled reservoirs within the laboratory of the Department of General, Visceral, and Transplantation Surgery at Ludwig Maximilians University. Cells were grown in RPMI Medium 1640 supplemented with 10% Fetal Bovine Serum (FBS) and incubated

in an incubator with humidity at 37°C and 5% CO2. The medium was replaced every 72-96 hours. No contamination was detected, and all cells tested negative for mycoplasma. Cells were cultured to approximately 70% confluency and subsequently treated for varying durations (6 to 48 hours) with different concentrations of calf intestinal alkaline phosphatase (5 U/ml to 50 U/ml; Sigma-Aldrich, cat. no. 524572). In certain conditions, bafilomycin A1 (Sigma-Aldrich, cat. no. SML1661), an autophagy flux inhibitor, was applied at a concentration of 100 nM during the last 3 hours of the 24-hour treatment. Cells treated with the vehicle only were used as negative controls. The cells were ruinously authenticated and checked negative for mycoplasma.

2.2.2 Establish a fatty liver cell model

To prepare a 15 mM fatty acid solution by mixing palmitic acid solution (10 mM) and oleic acid solution (20 mM), follow these steps:

One gram and two grams of fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich, cat. no. A8806) were placed into separate tubes. Deionized water was added, and the mixtures were shaken with a vortex shaker. After centrifuging at 4000 rpm for 5 minutes to remove foam, water was added to make 5 ml solutions, resulting in 20% and 40% BSA concentrations.

0.06 grams of sodium hydroxide (NaOH) was dissolved in 10 ml of deionized water to prepare a 0.15 mM NaOH solution, which was divided into two parts. 63.67 µl of oleic acid (Sigma-Aldrich, cat. no. O1008) was added to one part, and 25.64 mg of palmitic acid (Sigma-Aldrich, cat. no. P0500) to the other. These mixtures were heated at 75°C for 30 minutes to create 40 mM oleic acid (OA) and 20 mM palmitic acid (PA) solutions.

Immediately mix the 40 mM OA solution with the 40% BSA solution and the 20 mM PA solution with the 20% BSA solution, producing 20 mM OA and 10 mM PA solutions. Filter and sterilize the solutions using a 0.22 µm microporous membrane and store them in a

refrigerator at 4°C for later use. Before use, mix OA and PA solutions in a 1:1 volume ratio to obtain a 15 mM free fatty acid solution.

2.2.3 Cell viability assay

Cell viability was assessed using the MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide). Cells in the exponential growth phase were harvested using trypsin from each treatment group, and 8,000 cells/well were seeded into a 96-well plate with 200 μl of medium per well, allowing them to attach overnight. Subsequently, the medium was replaced, and the cells were exposed to varying concentrations of the FFA mixture. Following a 24h treatment, the cells were carefully washed with 50 μl of DPBS, after which 50 μl of MTT reagent (0.5 mg/ml) was added to each well. The plate was incubated at 37°C in a 5% CO2 atmosphere for 40 minutes to facilitate formazan crystal formation. To solubilize the crystals, 50 μl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was recorded at a wavelength of 570 nm with a background wavelength of 670 using a VersaMax microplate reader. Blank wells without cells were used as controls. The optical density (OD) correlated with the percentage of living cells, and viability (%) was calculated as follows: Cell viability (%) = (OD treated / OD control) × 100%.

2.2.4 Oil Red O staining

HepG2 cells were plated in a 6-well plate at a density of 1×10^6 per well and incubated overnight. After 24 hours of treatment with FFA, lipid accumulation was assessed using Oil Red O staining (Sigma-Aldrich, catalog number O0625). The culture medium was discarded, and the cells were gently washed with PBS. They were then fixed with 4% paraformaldehyde for 10 minutes at room temperature.

During the fixation process, the Oil Red O working solution was prepared by mixing a stock solution (0.5 g Oil Red O dissolved in 100 ml isopropanol) with deionized water in a 3:2

ratio. The mixture was thoroughly combined and filtered through a 0.22 μm syringe filter to obtain a clear, dark red solution. The cells were then gently rinsed three times with PBS at room temperature.

Next, 1 ml of 60% isopropanol was added to each well and incubated for 5 minutes at room temperature before being removed. Freshly prepared Oil Red O working solution (1 ml) was then applied to the cells, which were stained for 20 minutes in the dark. The cells were then rinsed three times with deionized water and immediately examined under a microscope (ZEISS Primovert).

2.2.5 Nile Red staining by flow cytometry

HepG2 cells were plated in a 6-well plate at a density of 1×10^6 per well and allowed to grow overnight. Following a 24-hour treatment with FFA, the cells were rinsed twice with FACS buffer, detached using trypsin, and spun down at 250×g for 5 minutes. Then resuspended and washed with cold PBS and centrifuged again, fixed in ice-cold 4% formaldehyde for 10 minutes at 4°C. The cells were then washed twice with PBS, centrifuged, resuspended in 200 μl of ice-cold methanol, and incubated for 15 minutes at 4°C. After washed twice with FACS buffer, the cell pellet was suspended in 200 μl of Nile Red staining solution (prepared by diluting 5 μl of Nile Red stock solution from Sigma-Aldrich, cat. no. 19123, into 5 ml of FACS buffer containing 0.05% Triton X-100 and 1% BSA). The staining process lasted 20 minutes in the dark at room temperature. The cells were then washed twice with FACS buffer containing 0.05% Triton X-100 and 1% BSA, and resuspended in 400 μl of FACS buffer for flow cytometry.

2.2.6 Protein isolation and western blot analysis

Cell lysates derived from HepG2 cells were prepared utilizing RIPA buffer supplemented with protease inhibitor cocktail tablets. The protein content was quantified using the Pierce™ BCA Protein Assay Kit. Protein samples were prepared by combining with 4x

Laemmli Sample Buffer and deionized water, then heated at 96°C for 10 minutes. 10 μg per lane of protein sample were loaded onto 12% SDS-PAGE gels (Bio-Rad Laboratories) and separated by electrophoresis.

The proteins were transferred onto Immun-Blot PVDF membranes, then subjected to blockade using 5% bovine serum albumin in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 hour at room temperature. The membranes were incubated overnight at 4°C with primary antibodies against LC3B (1:1,000, Novus Biologicals, cat. no. NBP2-46892) and GAPDH (1:5,000, Cell Signaling, cat. no. 14C10), with GAPDH used as a loading control.

After three washes with 1X TBST, the membranes were exposed to HRP-conjugated anti-rabbit IgG (1:5,000, Cell Signaling, cat. no. 7074S) for 1 hour at room temperature. The visualization of the protein bands was achieved using the Clarity™ Western ECL Substrate kit (Bio-Rad, cat. no. 170-5061) and the ChemiDoc™ Western blot imaging system (Bio-Rad).

2.2.7 MDC assay

HepG2 cells were seeded at a density of 1×10^6 cells per well in a 6-well plate with coverslips pre-placed in each well and incubated overnight. After a 24-hour treatment with calf IAP, the medium was discarded, and the cells grown on the coverslips were incubated with 0.1mM MDC (Sigma-Aldrich, cat. no. 30432) for 30 minutes at 37°C. The stock solution, consisting of 0.1 M MDC in DMSO, was diluted 1:1000 in PBS before incubation. Following incubation, the cells were washed twice with PBS, mounted using an antifade solution (Abcam, cat. no. 104139), and immediately observed under a fluorescence microscope (Zeiss Axio Imager.D2). MDC selectively stains autophagic vacuoles, which appear as punctate fluorescent spots. Fluorescence is typically excited at approximately 380 nm, with emission detected at 525 nm.

2.2.8 Statistical analysis

Data are presented as mean ± standard deviation and were analyzed using GraphPad Prism 6 software (version 7.04). Comparisons between two groups were performed using unpaired Student's t-tests, while analyses involving three or more groups were conducted using one-way ANOVA. A P-value of <0.05 was considered statistically significant.

3. Results

3.1 HepG2 cells after FFA mixture treatment showing a good fatty liver cell model by using Oil Red O staining

After treating HepG2 cells with a mixed FFA solution for 24 hours, Oil Red O staining was used to visualize intracellular lipid buildup. Under the microscope, the lipid deposits appeared as well-defined, round, orange-yellow droplets within the cytoplasm. These lipid droplets were prominent and clearly demarcated, with many droplets often present simultaneously. As the concentration of FFA increased, both the size and number of lipid droplets increased. In the control group, which was not treated with FFA, some small lipid droplets were naturally present in the HepG2 cells. Due to the lipotoxicity of FFA, as the concentration of FFA increased, the HepG2 cells progressively lost their original morphology. The cells transitioned from having a well-rounded, clearly defined fibrous appearance to a morphology characterized by indistinct boundaries, cytoplasmic contraction, nuclear condensation, and a darker color (Fig. 3).

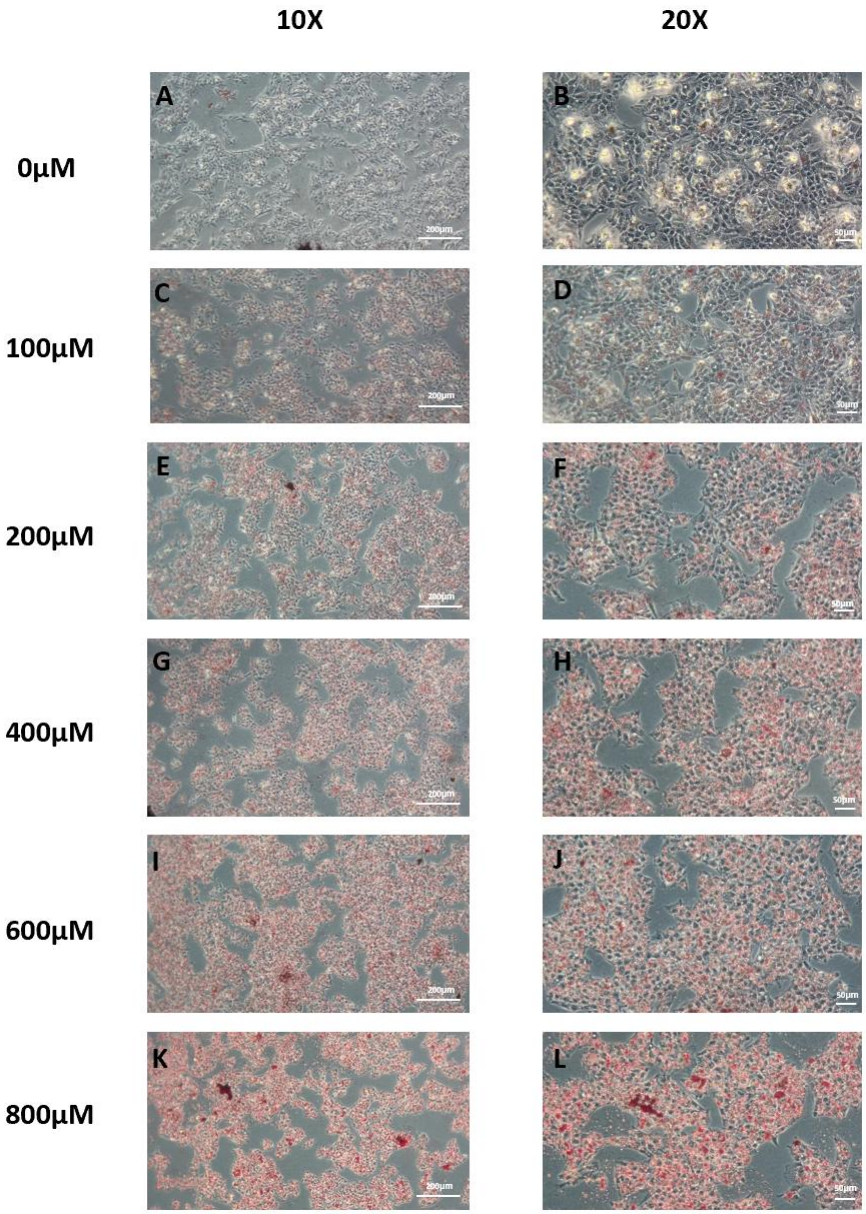
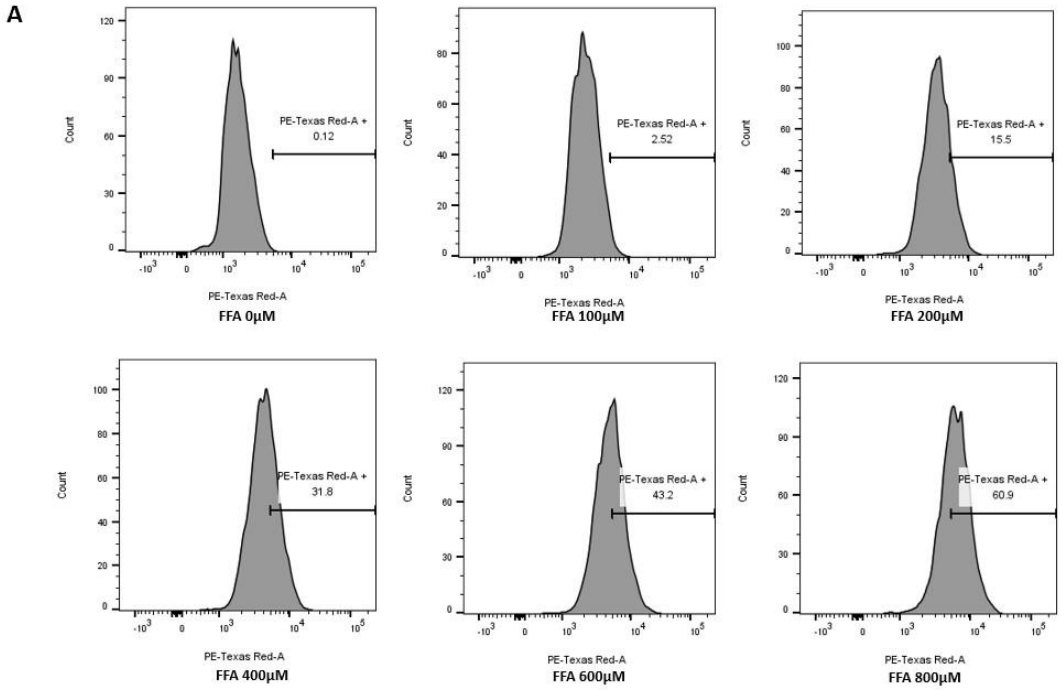


Figure 3. Oil Red O Staining of HepG2 Cells Treated with FFA Mixture. Orange-Red staining indicates intracellular lipid. (A-L) HepG2 cells treated with indicated concentrations of FFA mixture (0-800 μM).

3.2 Nile Red staining for quantification of intracellular lipid accumulation

To more accurately quantify intracellular lipid accumulation, we employed flow cytometry with fluorescence detection to analyze intracellular lipid droplets. Nile Red, a lipophilic fluorescent dye, binds to lipid substances such as wax esters, triglycerides, and various

fatty acids, emitting a fluorescent signal that allows for rapid, sensitive, and reliable detection of cellular lipid content. As the concentration of FFA increased, flow cytometry revealed a clear and progressive increase in intracellular lipid accumulation (Fig. 4A). Specifically, at an FFA concentration of 400 μM, we observed a significant and pronounced increase in lipid accumulation ($p = 0.0143$), confirming the substantial effect of high FFA concentrations on cellular lipid content (Fig. 4B).



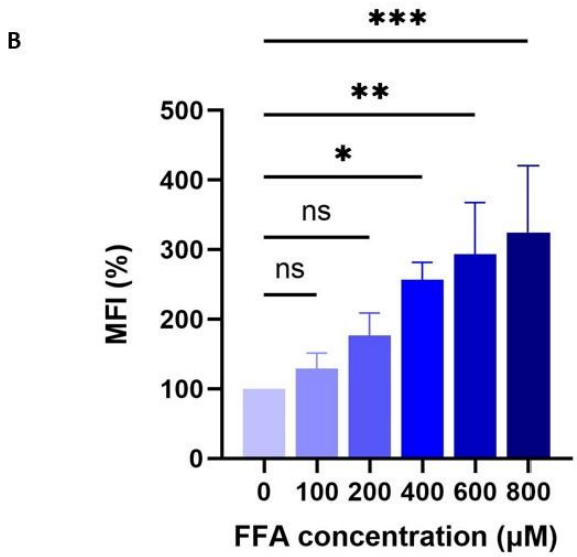


Figure 4. Flow cytometry analysis of Nile Red staining showing the effects of varying FFA mixture concentrations on lipid accumulation in HepG2 cells. (A) Illustrates the increase in Nile Red staining intensity with the rising concentration of the FFA mixture. (B) Bar graph representing the median fluorescence intensity (MFI%) of Nile Red in HepG2 cells after treatment with different concentrations of the FFA mixture (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

3.3 FFA has a significant cytotoxic effect on HepG2 cells

After successfully establishing a fatty liver cell model, we investigated the lipotoxic effects of FFA on HepG2 cells to find the best concentration for subsequent experiments. We used the MTT assay to measure the impact of FFA on cell growth. After 24 hours of FFA treatment, cell viability decreased as FFA concentration increased (Fig. 5). At concentrations above 400 μM, cell viability dropped to approximately 50%. Based on the results of flow cytometry analysis, lipid accumulation within the treated cells significantly increased when the FFA concentration exceeded 400 μM. In combination with Oil Red O staining results, noticeable morphological changes in the cells were observed at FFA concentrations above 400 μM. These findings indicate that the cytotoxic effects of FFA on HepG2 cells intensify with increasing FFA concentrations, especially beyond 400 μM. Therefore, for subsequent experiments, we chose 400 μM for future treatment.

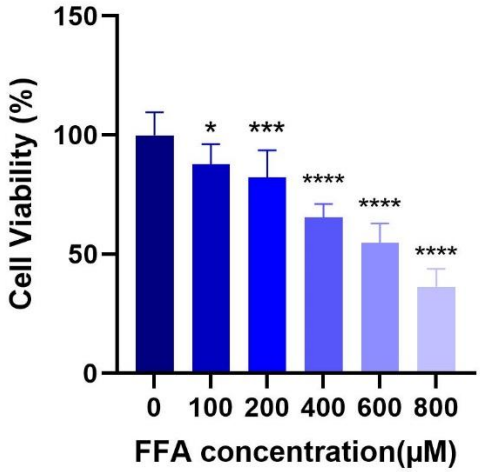
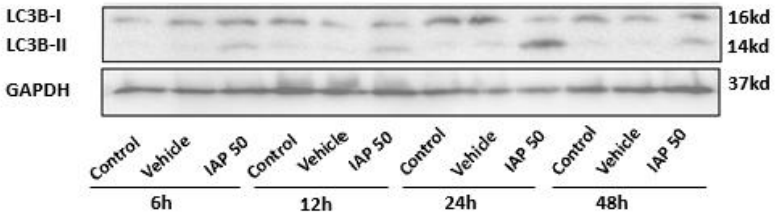


Figure 5. FFA mixture inhibits HepG2 cell proliferation. The evaluation of proliferation in HepG2 cells after FFA mixture treatment was conducted using the MTT assay. As the concentration of the FFA mixture increases, HepG2 cell proliferation decreases, with approximately 50% inhibition observed at an FFA mixture concentration of 400 μM (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

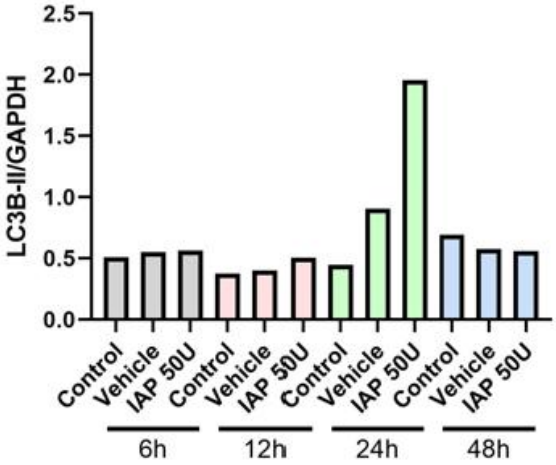
3.4 Optimal induction time and effects of IAP on autophagy in HepG2 cells

To explore whether IAP triggers autophagy in HepG2 cells and to identify the optimal time point for maximum autophagy induction, we incubated cells with IAP (50U/ml) for different timepoints (6h to 48h) and analyzed the expression of a key autophagy marker (LC3B-II) (Fig. 6A, B). The findings indicated that 50U/ml IAP effectively induces autophagy in HepG2 cells. The autophagy marker LC3B-II peaked at the 24-hour mark before exhibiting a decline at the 48-hour time point (Fig. 6C, D). This temporal pattern indicates a time-dependent induction of autophagy with a distinct peak in marker expression at 24 hours. (Control groups: without IAP; vehicle-treated cells solution: 6 mM $MgCl_2$, 6 mM TRIS-HCl, 0.12 mM $ZnCl_2$, 40% glycerin, pH 7.6.)

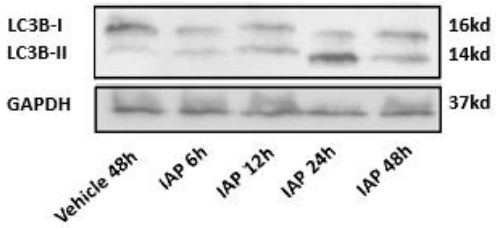
A


LC3B-I
LC3B-II
16kd
14kd
GAPDH
37kd
Control
Vehicle
IAP 50
6h
12h
24h
48h

B


LC3B-II/GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
Control
Vehicle
IAP 50U
6h
12h
24h
48h

C


LC3B-I
LC3B-II
16kd
14kd
GAPDH
37kd
Vehicle 48h
IAP 6h
IAP 12h
IAP 24h
IAP 48h

D

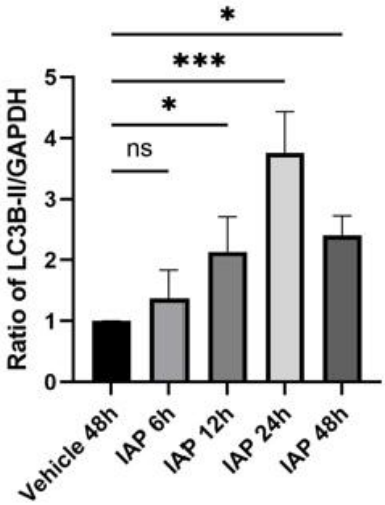

*
***
*
ns
Ratio of LC3B-II/GAPDH
5
4
3
2
1
0
Vehicle 48h
IAP 6h
IAP 12h
IAP 24h
IAP 48h

Figure 6. Western blot analysis shows the expression of LC3B-II in HepG2 cells after treatment with IAP 50 U/ml at various time points. (A, B) The images display the expression of LC3B-II protein at different time points in the control group, vehicle group, and IAP-treated group. (C, D) Compared to the vehicle group at 48 hours, LC3B expression in cells treated with IAP 50 U/ml initially increases and then decreases over time, with a significant rise at 12 hours ($p=0.0446$), peaking at 24 hours ($p=0.0001$), and declining by 48 hours ($p=0.0141$).

3.5 Dose-dependent effects of IAP on autophagy flux in HepG2 cells

To observe the effects of IAP on the autophagy signaling in HepG2 cells, we treated cells with different concentrations of IAP (5 U/ml, 10 U/ml, 25 U/ml, and 50 U/ml) and analyzed the LC3B-II/GAPDH processing. A western blot analysis of IAP-treated cells (for 24 hours) showed that the expression of autophagy marker proteins increased with higher concentrations of IAP, with a significant increase observed at 50 U/ml (Figure 7A, B). In this system, we introduced BafA1, a late-stage autophagy inhibitor, that block V-ATPase in lysosomes as well as the fusion of autophagosomes with lysosomes, effectively blocking degradation of proteins in the autolysosome, to monitor autophagic flux. The increase in autophagy marker protein expression could be due to enhanced autophagy or blockage of the autophagy flux. The purpose of introducing BafA1 was to evaluate whether the original treatment factor promotes the autophagy process. If the expression of autophagy markers increases after the addition of this late-stage autophagy inhibitor, it indicates that the original treatment factor promotes autophagy. If no change in autophagy marker expression is observed, it suggests that the original treatment factor has already blocked the autophagy pathway, and the addition of BafA1 cannot further enhance the expression of autophagy markers. In this part of the study, an increase in autophagy protein expression was observed in each group following the addition of BafA1, and the expression of

autophagy marker proteins increased with higher concentrations of IAP (Fig. 7C, D), indicating that IAP promotes autophagy in HepG2 cells.

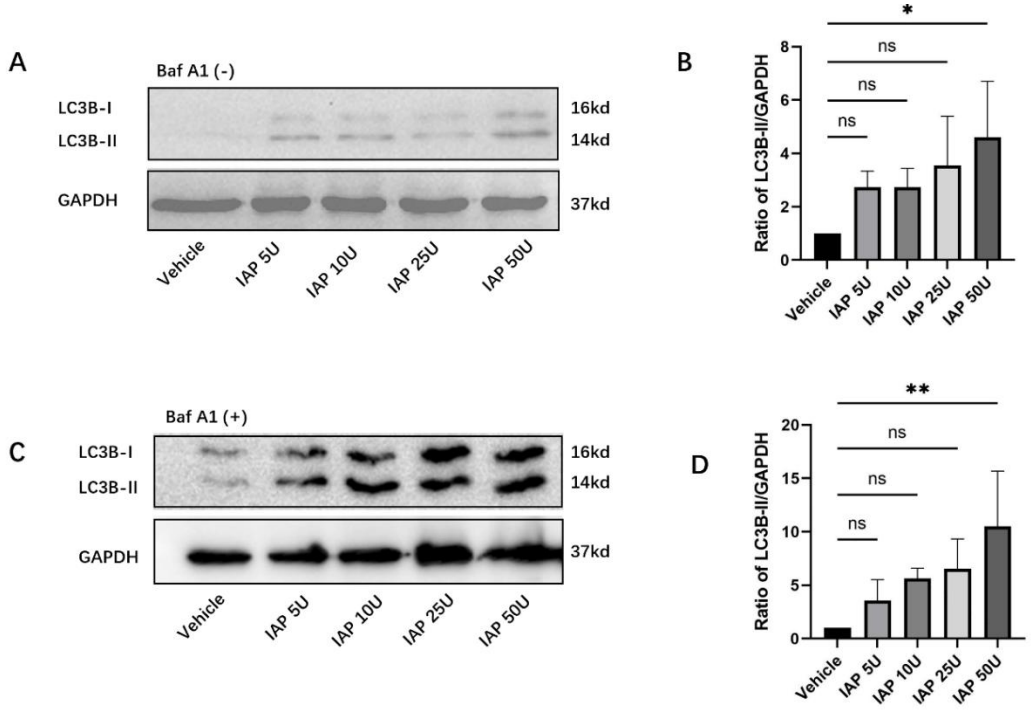


Figure 7. The expression levels of LC3B-II protein in HepG2 cells following 24-hour treatment with varying concentrations of IAP (5 U/ml, 10 U/ml, 25 U/ml, 50 U/ml). (A, B) In the absence of BafA1, the expression of LC3B-II in HepG2 cells exhibited an upward trend with increasing concentrations of IAP, showing a significant elevation at 50 U/ml of IAP (4.61±2.08 vs. 1.00±0.00, p = 0.0223). (C, D) After the addition of BafA1 (100nM for 3 hours at the end of the 24-hour treatment period), a similar pattern emerged, with the expression of LC3B-II in HepG2 cells increasing with higher IAP concentrations. Notably, the magnitude of this increase was greater, and it reached statistical significance at 50 U/ml of IAP (10.51±5.17 vs. 1.00±0.00, p = 0.0065).

3.6 Detection of autophagic vacuoles by MDC staining

The formation of autophagic vacuoles in IAP-treated HepG2 cells was analyzed using MDC staining. MDC, a specific autophagosome marker, was employed to detect the occurrence of autophagic vacuoles. Under fluorescence microscopy, a significant

increase in MDC-labeled puncta was detected in cells treated with 50 U/ml IAP for 24h (Fig. 8A, B), suggesting enhanced autophagic activity compared to the control group. This increase in autophagic vesicles aligns with the induction of autophagy observed in the western blot experiment.

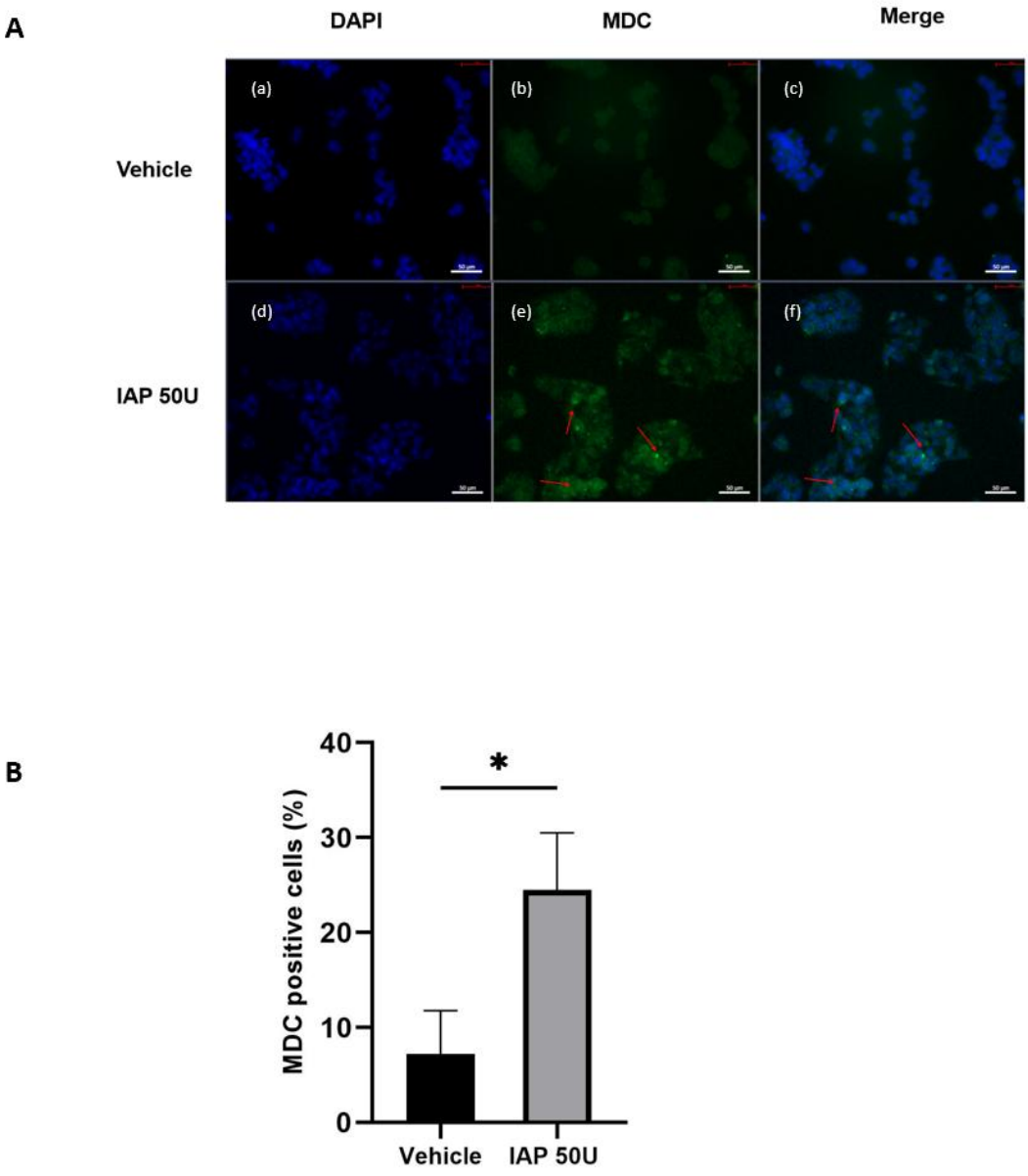


Figure 8. IAP induces the formation of autophagosomes in HepG2 cells. (A) Under MDC staining, autophagosomes within the cells are visualized as small, scattered punctate structures. (B) Bar graph shows that compared to the Vehicle group, a significant increase

in the percentage of MDC-positive cells was observed in the IAP 50U group ($p = 0.0169$).

3.7 IAP pretreatment reduces intracellular lipid accumulation via autophagy promotion

We investigated the effect of IAP pretreatment on intracellular lipid accumulation. Cells were first treated with or without IAP for 24 hours, followed by treatment with 400 μM FFA mixture for an additional 24 hours. Subsequently, intracellular lipid accumulation was detected using Nile Red staining with flow cytometry. Compared to the group without IAP pretreatment, cells in the IAP pretreatment group showed a significant reduction in lipid accumulation. We introduced 3-MA (Sigma-Aldrich, cat. no. 609263), an autophagy inhibitor, into this system and found that the group treated with 3-MA reversed the effect of IAP on reducing cellular lipid accumulation (Fig. 9A, B). These results suggest that the reduction in lipid accumulation by IAP may be mediated through the promotion of autophagy.

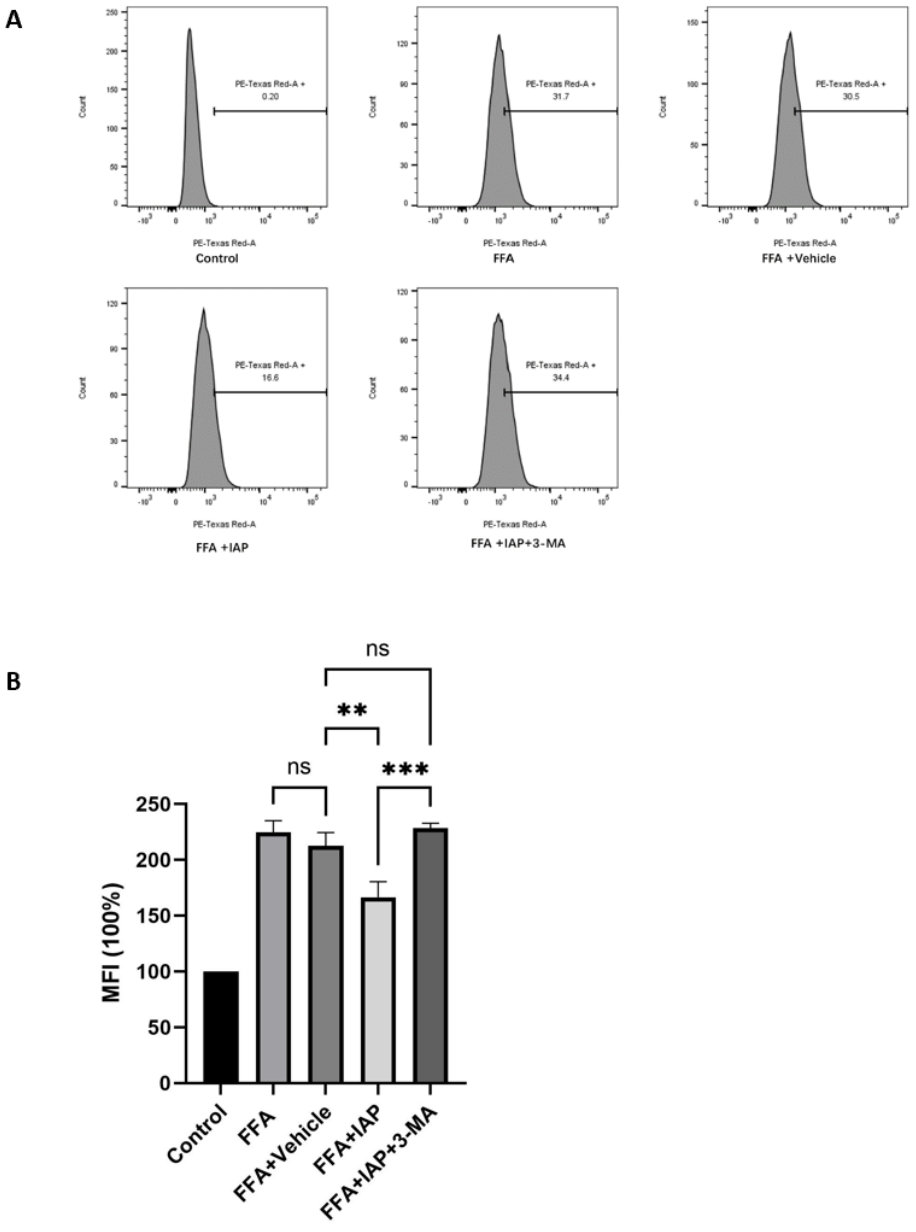


Figure 9. IAP pretreatment reduced intracellular lipid accumulation through an autophagy-dependent pathway. (A) and (B) demonstrate that IAP pretreatment significantly reduced lipid accumulation in fatty liver cell model ($p = 0.0012$). However, this lipid-reducing effect was notably abolished after the addition of 3-MA (cells were pretreated with 3-MA [2 mM] for 1 hour before treatment with IAP) compared to the IAP group ($p = 0.0001$).

4. Discussion

4.1 The potential role of IAP in NAFLD

The influence of IAP on autophagy has been scarcely studied. So far, only a single study has demonstrated that IAP can promote autophagy in intestinal epithelial cells and RAW264.7 macrophages. This finding is significant because it suggests that the autophagy-promoting effects of IAP might not be limited to these cells alone, but could potentially extend to other types of cells as well, such as hepatocytes, which are crucial in liver function and health. The autophagic effect of IAP in this study was shown to be dependent on the activation of the TLR4 signaling pathway. This was further confirmed when IAP induced autophagy was shown to be suppressed by the TLR4 inhibitor, TLR4INC34 (C34), demonstrating the critical role that the TLR4 pathway plays in this mechanism.[175] While the induction of autophagy by IAP is a relatively novel discovery, the most well-established and widely recognized function of IAP is its potent anti-inflammatory effect.[177] IAP exerts this effect primarily by detoxifying pro-inflammatory substances, such as LPS, a component of the outer membrane of gram-negative bacteria that can provoke strong inflammatory responses in the body. Through this detoxification process, IAP helps to mitigate inflammation, a major contributor to the onset of numerous diseases associated with both acute and chronic inflammatory processes. These diseases include sepsis, a life-threatening condition resulting from the body's extreme response to infection, as well as IBD and metabolic disorders, which can arise due to persistent low-grade inflammation.[178] Beyond its anti-inflammatory properties, IAP is vital for maintaining gut barrier integrity.[176] This barrier serves as a critical defense mechanism, preventing harmful substances from passing from the intestines into the bloodstream. Additionally, IAP has been demonstrated to have a regulatory effect on the composition of gut microbiota—the diverse community of microorganisms that reside in the digestive tract that is essential for overall health.[163]

NAFLD is another condition in which IAP may have therapeutic potential. NAFLD is also thought to result, at least in part, from chronic inflammation triggered by gut-derived pro-inflammatory substances that reach the liver.[179] The gut-liver axis is thought to be involved in the development and progression of NAFLD. As a beneficial regulator of the gut, IAP has shown promise in treating liver diseases. In fact, a previous study demonstrated that long-term IAP supplementation was able to decrease age-related gut permeability, which tends to increase with age and can lead to systemic inflammation.[180] Additionally, the same study found that IAP supplementation reduced endotoxemia in mice—a condition in which endotoxins like LPS enter the bloodstream and trigger inflammatory responses. Notably, the study also observed that IAP supplementation extended the lifespan of both mice and *Drosophila melanogaster*, further underscoring the broad protective effects of IAP on health and longevity. Furthermore, in patients with cirrhosis, a condition characterized by severe liver scarring due to chronic liver damage, lower fecal IAP activity has been observed. This suggests that reduced IAP activity may be linked to liver disease severity, and oral IAP supplementation has been shown to attenuate liver fibrosis in mice.[181]

4.2 The significance of autophagy in the treatment of NAFLD

NAFLD has quickly become the most prevalent liver condition globally, with no FDA-approved medications currently available for its treatment. Recently, there has been increasing interest in targeting autophagy as a promising therapeutic strategy for NAFLD.[182] Research has shown that enhancing autophagy can significantly improve NAFLD by promoting the breakdown of lipid droplets, thereby reducing hepatic steatosis.[124, 183, 184] This suggests that autophagy induction could serve as a promising and viable therapeutic approach to managing NAFLD.

Several autophagy-inducing compounds, such as rapamycin and carbamazepine, have been investigated for their potential application in treating NAFLD.[122] Berberine, a natural

plant compound, has also been demonstrated to enhance autophagic activity, leading to reduced hepatic lipid content in preclinical studies.[185] Despite the promising results from these autophagy-inducing drugs, it is important to investigate their safety and efficacy in clinical treatments. For a treatment to be helpful for long-term use in NAFLD, it must not only reduce lipid accumulation but also do so without causing harmful side effects. For example, while rapamycin can promote autophagy, it may also suppress the immune system, [186] highlighting the need for safer options that can target autophagy without causing unwanted problems. Within this context, we are exploring how IAP influences autophagy and lipid metabolism in liver cells. While IAP is well-known for its ability to detoxify harmful bacterial endotoxins, but its role in autophagy remains relatively unexplored.

Our current research focuses on two critical questions: First, can IAP induce autophagy in liver cells? This question is important because if IAP can trigger autophagy in liver cells, it could provide a novel mechanism through which the enzyme helps regulate lipid metabolism. Autophagy induction, particularly the degradation of lipid droplets via lipophagy, could prevent excessive accumulation of fat in the liver. If IAP is capable of triggering this autophagic response, it could be a valuable target for future therapeutic strategies aimed at reducing liver fat content. Second, help decrease the buildup of intracellular lipids in a fatty liver cell model? This question focuses on whether IAP can directly affect lipid metabolism in a fatty liver cell model, which mimics the pathological conditions of NAFLD. By treating hepatocytes with IAP and examining the intracellular lipid content, we aim to determine whether IAP can promote the clearance of lipid droplets. This reduction in lipid accumulation would indicate that IAP plays an active role in mitigating hepatic steatosis. These two questions are fundamental to understanding the implications of IAP in liver health. If IAP is found to both induce autophagy and reduce lipid

accumulation, it could represent a novel therapeutic agent for treating NAFLD. Moreover, unlike many other autophagy-inducing compounds, IAP is a naturally occurring enzyme with known beneficial effects in the gut and liver. Its favorable safety profile makes it a promising candidate for further study as a potential therapeutic agent. Therefore, by exploring whether IAP can induce autophagy in liver cells and reduce lipid accumulation in a fatty liver cell model, our work seeks to clarify IAP's role in this critical process.

4.3 The process of our experiments

Our experimental procedure began by establishing a reliable fatty liver cell model. In previous studies, it has been demonstrated that HepG2 cells are frequently employed as model cells for investigating the mechanisms underlying fatty liver disease.[187] To mimic the physiological conditions that lead to fatty liver formation, the HepG2 cells were exposed to a combination of oleic acid and palmitic acid for treatment. This combination of fatty acids was chosen because it closely mimics the fatty acid composition naturally present in the human body. Oleic acid, a monounsaturated fatty acid, is the most prevalent fatty acid in human adipose tissue and ranks as the second most abundant fatty acid across all human tissues, following palmitic acid. Palmitic acid, a saturated fatty acid, is the most abundant saturated fat in the body.[188] Together, these two fatty acids replicate the types of lipids commonly found in the liver, especially under conditions such as a high-fat diet.

Using this method, we successfully established a stable fatty liver cell model. We quantitatively assessed intracellular lipid deposition by employing flow cytometry, which allowed for a precise and detailed measurement of lipid accumulation within the cells. The mixture of oleic acid and palmitic acid effectively induced lipid buildup and at higher concentrations, the mixture was shown to lead to apoptosis. Based on the results obtained from both the MTT assay and flow cytometry analysis, we were able to select the optimal concentration of the fatty acid mixture that would be used in subsequent experiments.

To assess whether IAP induces autophagy in HepG2 cells, we employed western blot analysis to measure the expression levels of the autophagy-related protein LC3B-II in HepG2 cells treated with IAP. LC3B-II is a well-established marker for autophagy due to its relatively specifically associated with autophagosomes and autolysosomes.[189] While LC3B-I is found in the cytoplasm, and its levels can fluctuate based on factors such as cell type, experimental conditions, and the total cellular pool of LC3, making it a less reliable indicator of autophagic activity. Moreover, LC3B-I does not have the same functional relevance to autophagy as LC3B-II. Given these considerations, it is widely accepted that quantifying LC3B-II relative to a housekeeping protein like GAPDH is the preferred method for assessing autophagy through western blotting.[190] LC3B-II/GAPDH normalization provides a more consistent and reliable measure of autophagic activity, as it accurately reflects changes in LC3B-II levels. This approach allows us to directly evaluate the degree to which IAP induces autophagosome formation in HepG2 cells.

Based on previous studies, we chose an IAP concentration of 50 U/ml to study its impact on autophagy in HepG2 cells at different time points. We found that IAP at this concentration successfully induced autophagy, with the effect increasing over time. Specifically, autophagy was noticeably enhanced at 12 hours, peaked at 24 hours, and then declined by 48 hours. For future experiments, we decided to use 24 hours as the optimal treatment duration. In another set of experiments, we tested different concentrations of IAP on HepG2 cells and observed that higher concentrations further increased autophagy. This pattern aligns with previous research showing IAP's role in promoting autophagy in HCT116 epithelial cells and RAW264.7 macrophages.[175]

In addition to measuring LC3B-II expression, we also assessed autophagic flux, which is the dynamic process of autophagosome formation and degradation. We conducted experiments using lysosomal inhibitors, BafA1. The inhibitor blocks the degradation of

LC3B-II in the lysosomes, allowing us to monitor the accumulation of LC3B-II over time. By comparing the levels of LC3B-II in the presence and absence of these inhibitors, we can determine whether the increase in LC3B-II is due to enhanced autophagosome formation or impaired degradation. This combination of methods—quantifying LC3B-II relative to GAPDH and assessing autophagic flux with lysosomal inhibitors—provides a comprehensive and robust approach to evaluating whether IAP can induce autophagy in HepG2 cells.

In addition to conducting western blot analysis, we also employed MDC staining to visualize and detect intracellular autophagosomes. MDC staining is a well-established, classical method used for labeling autophagic vacuoles, and it has been widely used in a broad range of studies focused on assessing autophagic activity.[191] By using MDC, we were able to fluorescently label autophagosomes within the cells and complemented our western blot results, which focused on detecting the autophagy marker LC3-II. The MDC staining results were consistent with the findings from the western blot experiments, further confirming that IAP has the capability to promote the formation of autophagosomes in HepG2 cells. By integrating these two distinct but complementary methods we were able to strengthen the validity of our results. Both approaches indicated that IAP contributes to the activation of autophagic processes in HepG2 cells.

Finally, we investigated whether IAP could reduce lipid accumulation in the fatty liver cell model. Previous research has demonstrated that in certain diseases, such as inflammatory bowel disease or liver fibrosis, which compromise the intestinal barrier, patients exhibit reduced levels of IAP. In addition to its potential therapeutic effects, IAP may also have preventive benefits. We pre-treated the fatty liver cell model with IAP to observe whether it could reduce intracellular lipid accumulation. Flow cytometry results demonstrated a substantial decrease in intracellular lipids in the IAP-treated fatty liver cell model. At this

point, we introduced another autophagy inhibitor, 3-MA, a widely used compound that inhibits autophagy by specifically targeting PI3K, thereby blocking the early stages of autophagosome formation. This inhibitor is commonly used to study the role of autophagy by comparing the effects of autophagy inhibition with normal or enhanced autophagic conditions. The results showed that after autophagy inhibition, the lipid-reducing effect of IAP disappeared, further suggesting that IAP's ability to reduce intracellular lipid accumulation may be mediated through the promotion of autophagy.

4.4 Limitations and future directions

While our study offers valuable insights into the role of IAP in autophagy and lipid metabolism in an NAFLD cell model, there are several limitations that need further investigation. First, our study was conducted using the HepG2 cell line, which may not fully recapitulate the complexity of NAFLD in vivo. Future studies should aim to validate our findings in other cell lines especially human primary hepatocytes and animal models of NAFLD, to better understand the physiological relevance of IAP in liver metabolism and disease progression.

Second, while we demonstrated that IAP promotes autophagy and reduces lipid accumulation in HepG2 cells, the precise molecular pathways through which IAP induces autophagy remain unclear. Investigating the upstream signaling events involved in IAP-mediated autophagy, such as the potential involvement of the AMPK-mTOR pathway, will be essential for developing a deeper understanding of how IAP regulates autophagy in hepatocytes.

Additionally, the long-term effects of IAP treatment on NAFLD progression need to be explored. NAFLD is a chronic condition, and it is crucial to assess whether the beneficial effects of IAP observed in cell models can lead to long-term improvements in lipid metabolism and liver function. Longitudinal studies in animal models, followed by clinical

trials, will be necessary to determine the therapeutic efficacy of IAP in NAFLD patients. Finally, the safety profile of IAP needs to be thoroughly evaluated. Although IAP is a naturally occurring enzyme, its administration at therapeutic doses must be carefully studied to ensure that it does not cause unintended side effects or toxicity. This is particularly important for any future clinical applications of IAP as a treatment for NAFLD.

5. Conclusion

In conclusion, our study shows that IAP induces autophagy and reduces lipid accumulation in HepG2 cells, suggesting a potential mechanism for its therapeutic effects in NAFLD. By promoting lipophagy, IAP may help prevent disease progression and reduce the risk of fibrosis and liver failure. While further research is needed to uncover the molecular pathways involved and validate these findings in vivo, our results indicate that IAP could be a promising therapeutic option for managing NAFLD.

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Affidavit

An, Lingxuan

Surname, first name

I hereby declare, that the submitted thesis entitled:

Intestinal Alkaline Phosphatase Orchestrates Autophagy to Alleviate Hepatocellular Lipid Accumulation in Non-Alcoholic Fatty Liver Disease

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

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Surname, first name

I hereby declare that the electronic version of the submitted thesis, entitled:

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is congruent with the printed version both in content and format.

Munich, 24.08.2025

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List of publications

1. An, L.; Wirth, U.; Koch, D.; Schirren, M.; Drefs, M.; Koliogiannis, D.; Niess, H.; Andrassy, J.; Guba, M.; Bazhin, A. V.; Werner, J.; Kuhn, F., Metabolic Role of Autophagy in the Pathogenesis and Development of NAFLD. *Metabolites* **2023,** *13* (1).
2. An, L.; Wirth, U.; Koch, D.; Schirren, M.; Drefs, M.; Koliogiannis, D.; Niess, H.; Andrassy, J.; Guba, M.; Bazhin, A. V.; Werner, J.; Kuhn, F., The Role of Gut-Derived Lipopolysaccharides and the Intestinal Barrier in Fatty Liver Diseases. *J Gastrointest Surg* **2022,** *26* (3), 671-683.