Alcohol Plus Additional Risk Factors: Rodent Model of Liver Injury

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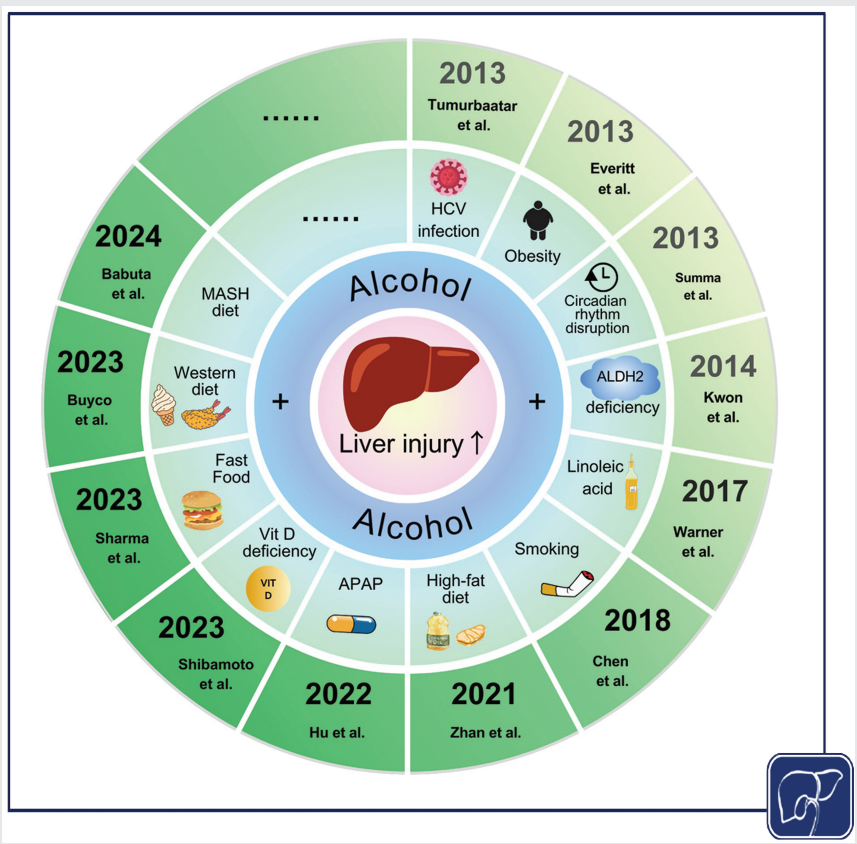
Graphical Abstract



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Abstract

Alcohol-associated liver disease (ALD), primarily caused by chronic excessive alcohol consumption, is a leading cause of chronic liver disease worldwide. ALD includes alcohol-associated steatotic liver, alcohol-associated hepatitis (AH), fibrosis, cirrhosis, and can even progress to hepatocellular carcinoma (HCC). Existing research indicates that the risk factors of ALD are quite numerous. In addition to drinking patterns, factors such as aldehyde dehydrogenase 2 (ALDH2) deficiency, smoking, medication administration, high-fat diet (HFD), hepatitis virus infection, and disruption of circadian rhythms can also increase susceptibility to ALD. However, there is limited understanding regarding the exacerbation of liver injury by alcohol plus additional risk factors. This review presents rodent models of EtOH + "X," which simulate the synergistic effects of alcohol and additional risk factors in causing liver injury. These models offer a further exploration of the interactions between alcohol and additional risk factors, advancing the simulation of human ALD and providing a more reliable platform for studying disease mechanisms and exploring therapeutic interventions. We summarize the modeling methods, relevant indicators of liver injury, and focus on the targets of the synergistic effects as well as the associated mechanisms.

Keywords

- alcohol consumption
- ALD
- MetALD
- animal model
- liver injury

Lay Summary

Drinking too much alcohol over a long time can lead to severe liver injury, causing a range of liver diseases. Existing research indicated that factors such as smoking, medication, high-fat diet, hepatitis virus infection, and circadian rhythm disruption can exacerbate alcohol-induced liver injury. This review summarized studies on novel alcohol plus risk factor rodent models, which more closely simulate human liver injury. These models are beneficial for further exploring the mechanisms of alcohol-induced liver injury in humans and guiding new treatment strategies.

Alcohol consumption is a significant risk factor threatening human health and has become a serious issue of social health. Between 1990 and 2017, the global average per capita alcohol consumption among adults increased from 5.9 L to 6.5 L, and it is projected to reach 7.6 L by the year 2030.[1] The World Health Organization (WHO) reported that harmful use of alcohol resulted in approximately 3 million deaths annually, with liver diseases being among the leading causes.[2] As one of the primary sites of alcohol metabolism, the liver is particularly susceptible to injury from excessive alcohol consumption.[3–5] Chronic excessive alcohol consumption often induces alcohol-associated liver disease (ALD), characterized by the pathological progression of the liver due to alcohol metabolism, including elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST), inflammatory cell infiltration, hepatocyte swelling, and apoptosis.[6]

Globally, the prevalence of ALD was approximately 4.8%, with a history of drinking for over 20 years accounting for 54.8% of ALD cases, and the average daily alcohol intake was 146.6 g.[7] Indeed, there were extensive studies on the liver injury induced by alcohol. Existing studies have elucidated the pathogenesis of ALD occurrence is closely related to alcohol and its genetic and epigenetic factors, metabolite-induced oxidative stress, metabolic reprogramming, immune damage, and dysbiosis of the gut microbiota.[8] However, in clinical practice, liver injury is rarely caused by alcohol alone but by a combination of multiple factors.[9] About 59.5% of ALD patients were current or former smokers, and 18.7% were complicated with hepatitis virus infection.[7] Growing evidence supports that genetic factor, unhealthy lifestyle, and environmental modifier effects are additional risk factors related to liver injury in ALD.[9,10] For example, an HFD can exacerbate the accumulation of fat in the liver, leading to steatosis, which is a precursor to ALD.[11] Simultaneously, an HFD increases the likelihood of developing obesity. Obesity is also recognized as one of the susceptibility factors of ALD and contributes to the progression of liver injury.[12,13] Moreover, smoking, as a common detrimental habit in daily life, has been identified as a significant risk factor for a wide range of diseases. Some studies have indicated that it can synergistically interact with alcohol, exacerbating liver injury.[14] Additionally, the combination of alcohol consumption with medication administration or viral infections, particularly hepatitis C virus (HCV), represents a significant risk factor for the development of ALD.[15,16] The aforementioned factors, combined with alcohol consumption, are very likely to occur in everyday life. Therefore, abstinence from alcohol and lifestyle adjustments are crucial for controlling and preventing the progression of ALD. Regarding treatment modalities, pharmacotherapy, nutritional support, and liver transplantation are extensively employed in ALD management.[6] Collectively, focusing on the synergistic effects of alcohol with additional risk factors is beneficial for further understanding the pathophysiological mechanisms of ALD, thus enabling better prevention and treatment of ALD.

Experimental animal models of ALD have been used extensively to simulate human ALD.[17] Among these ALD models, rodent models are the most commonly used animal models to study ALD.[18] In the past few decades, numerous rodent models have been established to investigate the impact of acute and chronic alcohol exposure on the occurrence and progression of ALD.[17] Commonly used rodent models include the Gao-binge model, the acute binge ethanol feeding model, the Lieber–DeCarli model, and the Tsukamoto–French model.[19–24] With the development of technology, more and more in vitro models for studying ALD have been established, such as hepatocyte culture models, liver organoid models, and Liver-on-chip models (►**Table 1**). These in vitro models can complement ALD models, thereby furthering our understanding of key developments and pathological mechanisms.[23,25] However, our current understanding of human liver disease still primarily comes from animal models. Traditional ALD models primarily aimed to simulate the diverse patterns of alcohol consumption and their effects on the liver. Often, they only considered alcohol as the single hepatotoxic factor, which clearly diverged from the multifactorial nature of most real-life cases of human liver injury. In recent years, the use of rodent models to research the synergistic effects of alcohol plus additional risk factors on liver injury has gained increasing interest. In this review, we discuss several representative studies that utilize rodent models to investigate the synergistic liver injury caused by alcohol plus additional risk factors.

Rodent Model of EtOH Plus Additional Risk Factors for ALD

In recent years, research on ALD has combined alcohol consumption with various additional risk factors to establish novel ALD models. These novel ALD models can emulate the conditions of the human liver in reality, allowing us to observe more pronounced liver injury caused by the combination of factors. Concurrently, these ALD models help

Table 1 ALD models

Models		Modeling method	Characteristic	Reference
In vivo model	Gao-binge model	Chronic ethanol feeding (10 days or longer) plus a single binge or multiple binges	Convenient and cost-effective Flexible application Marked elevation of ALT, AST, and steatosis No fibrosis and end-stage injuries	19
	Acute binge ethanol feeding model	Gavaged ethanol feeding by weight (4–6 g/kg)	Convenient and cost-effective Significantly affect liver mitochondrial function Only cause a mild increase in serum ALT and AST levels	20,21
	Lieber–DeCarli model	Chronic ethanol feeding (4–12 weeks)	Convenient and cost-effective Short-term feeding with no mortality rate Limited severity of ALD progression No circumvention of the animal's aversion to ethanol	22
	Tsukamoto–French model	Intragastric infusion (2–3 months)	Marked elevation of ALT, AST, and steatosis Overcoming the animal's aversion to ethanol Requirement for intensive medical care	23,24
In vitro model	Cell culture model	Hepatic parenchymal or nonparenchymal cells cultured in vitro and stimulated with ethanol	Convenient and cost-effective Lack of tissue microenvironment and cell–cell interactions	23
	Hepatic organoid model	Human pluripotent stem cells cultured in vitro and stimulated with ethanol	Provides a similar in vivo microenvironment	23
	Liver-on-chip model	Ethanol treatment on a biomimetic Liver-Chip	Reproduce hepatocellular environment and cell–cell interactions Achieve basic liver functions	23,25

Abbreviations: ALD, alcohol-associated liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

identify new targets for liver injury and enhance our understanding of the interaction mechanisms between alcohol and other risk factors. In this section, we review several selected rodent models of ALD induced by alcohol consumption plus additional risk factors, which we refer to as EtOH + "X." The modeling methods, liver injury indicators, and characteristics of these EtOH + "X" models are shown in ►**Table 2**.

EtOH Plus HCV Infection

HCV is a small, enveloped virus with a single-stranded, positive-sense RNA genome.[26] This virus can cause acute or chronic hepatitis, which can progress to cirrhosis and HCC, posing a life-threatening risk.[27] According to the WHO, it is estimated that there are approximately 50 million people

Table 2 EtOH↑ +↑ X models

Factors	Animals	Modeling method	Phenomena and indicators	Characteristic	Reference
EtOH ↑ +↑ HCV infection	9 months old male and female HCV/Sod2$^{+/-}$ mice	1. Ethanol feeding at 6 to 9 months old 2. Ethanol-containing Lieber–DeCarli diet for 3 weeks	• Hepatic steatosis • ↑ Hepatocyte ballooning • ↑ ALT • ↑ ICAM-1 • Necrosis and neutrophilic infiltration • ↑ TUNEL positivity	Simulates combined effects of ethanol and HCV infection exacerbates liver injury	29
EtOH ↑ +↑ Circadian rhythm disruption (Genetic)	7–9 weeks old male Clock$^{\Delta 19/\Delta 19}$ mutant mice (C57BL/6J coisogenic)	1. Constant 12:12 LD cycle 2. A gradual 2-week increase in ethanol (0–29% total calories) 3. 8 weeks on the full ethanol diet (29% total calories)	• ↑ Liver/body weight ratio • ↑ Intestinal permeability • ↑ LPS • ↑ Liver steatosis • ↑ Hepatocyte ballooning	Mutations in circadian rhythm–related genes exacerbate chronic ethanol-induced liver injury	35
EtOH ↑ +↑ Circadian rhythm disruption (Environmental)	7–9 weeks old male C57BL/6J mice	1. Once weekly 12-hour phase shift in LD cycle 2. A gradual 2-week increase in ethanol (0–29% total calories) 3. 8 weeks on the full alcohol diet (29% total calories)	• ↑ Liver/body weight ratio • ↑ Intestinal permeability • ↑ LPS • ↑ Hepatic steatosis • ↑ Hepatocyte ballooning • Lobular inflammation	The phase shift of the circadian rhythm cycle exacerbates chronic alcohol-induced liver injury	35
EtOH ↑ +↑ ALDH2 deficiency	8–10 weeks old male ALDH2$^{-/-}$ mice	1. Liquid diet containing 4% ethanol for 4 weeks 2. The administration of a liquid diet containing 4% ethanol and the intraperitoneal injection of CCl_4 (0.1↑ mL/kg body weigh) twice per week for 8 weeks	• ↑ MAA • ↓ Hepatic steatosis • ↓ Hepatic triglycerides • ↓ ALT and AST • Inflammatory cell infiltrate • ↑ α-SMA • ↑ TGF-β and TIMP-1	ALDH2 deficiency resists ethanol-induced steatosis and ALT/AST elevation but exacerbates liver inflammation and fibrosis	39
EtOH↑ +↑ LA	8 weeks old male C57BL/6J mice and Alox15 gene knockout male mice	1. USF-enriched diet with ethanol (5% w/v) for 10 days 2. A single dose of ethanol (20% v/v) gavaged by weight (5 g/kg)	• ↑ Liver/body weight ratio • Hepatic steatosis • ↑ ALT (lower in Alox15$^{-/-}$) • ↑ 9- and 13-HODEs (OXLAM levels)	Convenient and cost-effective Simulated combined effects of ethanol and high LA diet exacerbates liver injury	47
EtOH↑ +↑ Smoking	8–10 weeks old female C57BL/6 mice and cyp2a5$^{-/-}$ female mice	1. Liquid ethanol diet (gradual increase of ethanol every 3 days from 10% of total calories to 15, 20, 25, 30, and 35%)	• ↑ liver TG (lower in cyp2a5$^{-/-}$) • Macro-vesicular lipid droplets (lower in cyp2a5$^{-/-}$) • ↑ ALT	High relevance to human diseases Only nicotine is used to simulate smoking	54

Table 2 (*Continued*)

Factors	Animals	Modeling method	Phenomena and indicators	Characteristic	Reference
		2. Nicotine hydrogen tartrate salt (65 μM) or cotinine (52 μM) in diet Duration: 18 days	• ↓ Liver glutathione content • ↑ Oxidative stress (lower in cyp2a5$^{-/-}$)		
ETOH↑ +↑ APAP	8 weeks old male C57BL/6J mice and p38γ KD male mice	1. Liquid diet containing EtOH (5% v/v) for 10 days 2. Ethanol (25%,v/v) gavage according to weight (6 g/kg) 3. Intraperitoneal injection of APAP (200↑ mg/kg ip.)	• Hepatic steatosis (lower in p38γ KD) • ↑ Lipid accumulation • ↑ ALT and AST • Inflammatory cell infiltrate(lower in p38γ KD) • ↑ Oxidative stress (lower in p38γ KD) • ↑ Dlg1 expression (only in p38γ KD)	Highly relevant to human disease EtOH and APAP co-administration synergistically exacerbate liver injury	63
EtOH ↑ +↑ Vitamin D deficiency	10 weeks old female C57BL/6J mice	1. Fed with VtDD diet with ethanol (2.5%) 2. Intraperitoneal injection of CCl_4 twice a week by weight (1↑ mL/kg) Duration: 8 weeks	• Hemorrhagic liver necrosis • ↑ ALT and AST • Inflammatory cell infiltrate • ↑ Oxidative stress • ↑ TUNEL positivity • ↑ α-SMA • ↑ COL-1 matrix deposition	Vitamin D deficiency exacerbates ethanol-induced liver fibrosis	71

Abbreviations: ALDH2, aldehyde dehydrogenase 2; Alox15, arachidonate lipoxygenase 15; ALT, alanine aminotransferase; APAP, acetaminophen; HCV, hepatitis C virus; ICAM-1, intercellular cell adhesion molecule-1; LA, linoleic acid; LD, light–dark; LPS, lipopolysaccharide; MAA, malondialdehyde-acetaldehyde; TGF-β, transforming growth factor-β; TIMP-1, tissue inhibitor of metalloproteinases-1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; USF, unsaturated fat; VtDD, vitamin D-deficient diet; α-SMA, α smooth muscle actin.

worldwide with chronic HCV infection, with around 1 million new cases occurring each year. HCV infection and ALD, either alone or in combination, account for more than two-thirds of all liver disease patients in the Western world; hence, the relationship between alcohol consumption and HCV infection has always been a focal point of investigation.[28] In the study conducted by Tumurbaatar et al investigating the exacerbation of liver injury by ethanol plus HCV infection, HCV transgenic mice (SL-139 line) generated on a C57BL/6J background were used for modeling. These mice were a hemizygous model and expressed HCV structural proteins (core, E1, E2, and p7) in hepatocytes under control of the murine albumin enhancer/promoter. To maximize the chance of observing an oxidative liver injury, the mice were crossed with the superoxide dismutase$^{+/-}$ (Sod2$^{+/-}$) mice to produce the HCV/Sod2$^{+/-}$ genotype. HCV/Sod2$^{+/-}$ mice were used for ethanol feeding at 6 to 9 months old. And then the mice were fed an ethanol-containing Lieber–DeCarli diet. The ethanol concentration was increased stepwise to 1.6, 3.2, 4.8, and finally to 6.4% (v/v) at 3-day intervals and maintained. The ethanol concentration process lasted for 3 weeks to complete the model. As expected, histological analyses indicated exacerbated hepatocyte ballooning and serum biochemical analyses indicated increased ALT level. Further protein and gene expression analyses indicated elevated intercellular cell adhesion molecule-1 (ICAM-1) expression and heightened terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positivity, suggesting an exacerbation of inflammatory response and apoptosis, respectively.[29]

This study also verified whether forkhead box O 3 (FoxO3) has a protective effect against ethanol-induced liver injury. FoxO3 is a transcription factor known for its role in cellular metabolism, apoptosis, and response to stress that can be altered by HCV.[30] Under physiological conditions, hepatic exposure to ethanol triggers the phosphorylation of FoxO3 at S-574. Subsequently, approximately 10% of Kupffer cells undergo apoptosis. These apoptotic bodies interact with proinflammatory Ly6C+ infiltrating macrophages recruited to the liver due to alcohol exposure. This interaction induces delayed differentiation of infiltrating macrophages, thereby facilitating augmented tissue repair and a mitigated inflammatory phenotype.[31] The experiment conducted by Tumurbaatar et al indicated that both HCV infection and ethanol treatment activate FoxO3. However, the combination of HCV and ethanol suppressed this transcription factor, reducing the expression of cell-protective genes and leading to more severe liver injury. Complementary experiments using Huh7.5 cells, which support the entire life cycle of HCV infection and metabolize alcohol through alcohol

dehydrogenase (ADH), further confirmed that although HCV and alcohol individually activate FoxO3, their combination inhibits its function. In the EtOH plus HCV infection model, more pronounced liver injury was observed in FoxO3$^{-/-}$ mice compared with wild-type (WT) mice.[29] Additionally, in another study, acute ethanol-treated FoxO3$^{-/-}$ mice exhibited decreased expression of autophagy-related genes but increased steatosis and liver injury.[32] Given these findings, FoxO3-mediated autophagy-related gene expression has a protective effect against ethanol-induced liver injury. FoxO3 has the potential to become an important target for treating liver injury induced by ethanol plus HCV infection.

In summary, the EtOH plus HCV infection model successfully simulated the combined effects of ethanol and HCV infection on the liver. However, some more pronounced liver injury was only observed in Sod2 heterozygous knockout mice, which are sensitive to mitochondrial stress.[33] Therefore, the relevance of EtOH plus HCV infection model to human disease still needs further study.

EtOH Plus Circadian Rhythm Disruption

The circadian rhythm is an approximately 24-hour biological clock cycle within organisms, which influences various physiological behaviors such as sleep, wakefulness, and feeding, and is closely associated with metabolic balance.[34] Therefore, when the circadian rhythm is disrupted, this may lead to a series of health problems. For the purpose of further exploring whether disruption of circadian rhythms exacerbates ethanol-induced liver injury, experiments were conducted by Summa et al, who perturbed the circadian rhythm of mice genetically or environmentally. The study of genetic circadian rhythm disruption utilized homozygous mice with the Clock$^{\Delta 19}$ gene mutation, which were generated through chemical mutagenesis in C57BL/6J mice. These 7 to 9 weeks old male Clock$^{\Delta 19/\Delta 19}$ mutant mice were maintained on a constant 12:12 light–dark (LD) cycle for the duration of the experiment. The Clock$^{\Delta 19/\Delta 19}$ mutant mice were fed with gradually increasing amounts of ethanol (from 0 to 29% of total calories) for 2 weeks, and then maintained on a full ethanol diet (29% of total calories; 4.5% v/v) for an additional 8 weeks. Meanwhile, 7 to 9 weeks old male C57BL/6J mice underwent a once weekly shift in the LD cycle of 12 hours for 12 weeks to achieve environmental disruption of circadian rhythms. These mice were fed following the same regimen as Clock$^{\Delta 19/\Delta 19}$ mice. Upon completion of the model, subsequent results analysis indicated that both groups of experimental mice exhibited a higher liver/body weight ratio, more severe hepatocyte ballooning, steatosis, and increased hepatic inflammation.[35] Overall, both genetic and environmental disruption of circadian rhythms can exacerbate ethanol-induced liver injury in mice.

Notably, by administering sucralose to mice and measuring the sucralose content in their urine over 5 hours, it was found that mice with disrupted circadian rhythms exhibited increased intestinal permeability, particularly in the colon. Additionally, mice fed with ethanol exhibited significant endotoxemia and inflammation, which may be one of the reasons for exacerbating liver injury.[35] This model preliminarily confirmed that circadian rhythm disruption might be one of the risk factors for ALD. However, to discuss some mechanistic issues, such as the changes in the microbiome induced by circadian rhythm disruption and their impact on ethanol-induced liver injury, further research is needed.

EtOH Plus ALDH2 Deficiency

ALDH2 is one of the most crucial enzymes in the alcohol metabolism pathway. In this pathway, it catalyzes the conversion of acetaldehyde produced during alcohol metabolism into acetic acid.[36] Therefore, ALDH2 deficiency can lead to poor metabolism of acetaldehyde after alcohol consumption, resulting in its accumulation in the body and subsequent toxicity.[37] Studies have shown that ALDH2 deficiency is usually caused by the variant ALDH2*2 allele, which has been identified as one of the most common genetic enzymopathies in human. Approximately 8% of the global population exhibits a deficiency in ALDH2. In East Asia, this figure rises dramatically to between 40 and 50%.[38,39] Therefore, the impact of ALDH2 deficiency on ethanol-induced liver injury is worthy of attention. In the study by Kwon et al, 8 to 10 weeks old male ALDH2$^{-/-}$ mice were first fed a liquid diet containing 4% ethanol for 4 weeks. Subsequently, the mice were fed a liquid diet with 4% ethanol, and concurrently received intraperitoneal injections of carbon tetrachloride (CCl_4) (0.1 mL/kg body weight) twice a week for 8 weeks, to complete the model. Histological examination of the liver unsurprisingly revealed that compared with WT mice, the ALDH2$^{-/-}$ mice had higher levels of malondialdehyde-acetaldehyde (MAA) in the liver. Notably, ALDH2$^{-/-}$ mice exhibited lower hepatic levels of triglycerides and a lower degree of liver steatosis. However, after ethanol and CCl_4 treatment, ALDH2$^{-/-}$ mice show accelerated liver fibrosis. Serum biochemical analysis also indicated that ALDH2$^{-/-}$ mice had lower levels of ALT and AST. Additionally, immunological as well as protein and gene expression analyses showed that ALDH2$^{-/-}$ mice exhibit an intensified immune response, as evidenced by significantly elevated levels of cytokines such as interleukin-6 (IL-6).[39] Overall, the possible mechanism is that ethanol treatment leads to the accumulation of MAA adducts in the livers of ALDH2$^{-/-}$ mice. MAA stimulates Kupffer cells to produce proinflammatory cytokines, exacerbating inflammation and promoting liver fibrosis. IL-6 also activates signal transducer and activator of transcription 3 (STAT3) in hepatocytes, subsequently upregulating the expression of antioxidative stress genes and downregulating the expression of fatty acid synthesis genes, thereby reducing hepatocellular damage and steatosis.

It is worth noting that the EtOH plus ALDH2 deficiency model included the addition of an extra stimulus CCl_4, which is commonly used to induce liver fibrosis.[40] Because more than 95% of human heavy drinkers develop fatty liver, and up to 35% of them progress to more severe forms of ALD, including liver fibrosis.[41] So, the combined use of CCl_4 and ethanol can effectively accelerate the process of liver fibrosis

in mice, allowing researchers to observe significant fibrotic changes and more severe inflammatory responses in a shorter period of time. However, using ethanol alone may not be sufficient to induce significant liver fibrosis in a short period.[42] A study has shown similarities in terms of steatosis, inflammation, fibrosis patterns, and gene transcript correlation between ethanol-induced human liver injury and CCl_4 plus ethanol-induced liver injury in mice.[43] Additionally, a lower dose of CCl_4 (e.g., 0.08 mL/kg, twice a week for 8 weeks) can still induce significant liver fibrosis with a lower mortality rate.[44] In summary, after intraperitoneal injections of CCl_4, the model overcame the lack of obvious liver fibrosis in $ALDH2^{-/-}$ mice fed with ethanol alone, which might be due to the short treatment duration.

EtOH Plus Linoleic Acid

Linoleic acid (LA) is a polyunsaturated fatty acid that is widely found in vegetable oils. Although it is an essential fatty acid required by the body in moderate amounts, excessive intake may have adverse health effects.[45] Existing research indicates that the intake of unsaturated fatty acids in the diet has a significant impact on ethanol-induced liver injury.[46] Therefore, diets rich in LA are considered in conjunction with alcohol consumption to investigate the exacerbation of liver injury caused by the combination of alcohol and diets high in unsaturated fatty acids. In an experiment conducted by Warner et al, 8 weeks old male C57BL/6J mice were fed a diet rich in unsaturated fatty acids, which is supplemented with corn oil, a rich source of LA, along with a diet containing ethanol (5% w/v) for 10 days. On day 11, the mice were given a single dose of ethanol (20% v/v, 5 g/kg) by gavage. After 9 hours of gavage, the mice were sacrificed, and the liver tissue and blood were collected for subsequent analysis. Liver histological analysis revealed hepatic steatosis, and serum biochemical analysis showed elevated ALT levels. Meanwhile, protein and gene expression analyses showed that the levels of inflammatory factors, such as tumor necrosis factor-α (TNF-α), and oxidative stress markers, such as thiobarbituric acid reactive substance (TBARS), increased, confirming the exacerbation of inflammation and oxidative stress. The noteworthy observation was the increase in oxidized linoleic acid metabolite (OXLAM) levels, specifically 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODEs. These compounds are oxidized derivatives of LA and play a role in inflammation and oxidative stress.[47]

Further research in this study indicates that a potential therapeutic target through which LA exacerbates liver injury is arachidonate 15-lipoxygenase (Alox15), which is a member of the lipoxygenase family and is primarily responsible for catalyzing the oxidation of various fatty acids.[48] For example, arachidonic acid can be metabolized by Alox15 into various eicosanoids with either pro-inflammatory or anti-inflammatory effects; thus, Alox15 is associated with the pathogenesis of inflammatory diseases.[49,50] Furthermore, in human body, Alox15 is expressed in various cell types and organs and is associated with multiple diseases, including atherosclerosis, hypertension, diabetes, obesity, and neurodegenerative diseases.[50] In the EtOH plus LA model, Alox15 knockdown (KD) mice were used in the experiment and exhibited reduced liver injury. This could be associated with the role of Alox15 in oxidizing LA into OXLAMs.[47,51] OXLAMs can induce mitochondrial dysfunction in mice livers, leading to hepatocyte apoptosis and mediating macrophage pro-inflammatory responses.[52] However, currently there are no specific arachidonate lipoxygenase (Alox) inhibitors that lack nonspecific antioxidant properties.[50] Therefore, further research is needed to develop and test pharmacological inhibitors specific to Alox, to use them for therapeutic interventions in human diseases especially in ALD.

This model is convenient and cost-effective. It combines a high LA diet with the Gao-binge model to induce human-like moderately severe ethanol-induced liver injury. Additionally, the study tested the hypothesis that OXLAM-mediated pro-inflammatory responses in the liver exacerbate ethanol-induced liver injury and suggested that Alox15 could be a potential therapeutic target for ALD.

EtOH Plus Smoking

Like alcohol consumption, smoking is a global health issue that threatens human health. Approximately 40% of liver disease patients have a history of smoking, and an increasing number of studies are investigating the potential impact of smoking on chronic liver disease.[53] In the experiment conducted by Chen et al, 8 to 10 weeks old female C57BL/6 mice were fed the control liquid dextrose diet for 3 days to acclimate them to the liquid diet. Then these mice were fed liquid ethanol diet to induce ALD, and the ethanol concentration in the liquid diet was increased from 10% of the total calories (1.77% v/v) to 15% (2.65 v/v), 20% (3.53 v/v), 25% (4.42 v/v), 30% (5.31 v/v), and finally 35% (6.2% v/v) every 3 days. Nicotine hydrogen tartrate salt was mixed in the liquid at 65 μM (30 mg/L) or cotinine was mixed in the liquid diet at 52 μM (9 mg/L) because approximately 80% of nicotine is metabolized to cotinine. The entire feeding lasted 18 days. The subsequent histological examination of the liver revealed aggravated liver injury. Moreover, the increase in markers such as ALT and oxidative stress levels indicated by TBARS, malondialdehyde (MDA), and 3-nitrotyrosine (3-NT) also demonstrated this.[54] In summary, compared with mice fed only a liquid alcohol diet, the addition of nicotine or cotinine significantly exacerbated liver injury in the mice.

One of the key roles behind this exacerbation of liver injury involves the cytochrome P450 enzyme CYP2A5, a member of the P450 superfamily in mice, which shares a high degree of similarity with human CYP2A6. CYP2A5 is involved in various toxicological reactions, including the metabolism of nicotine, aflatoxin B1, and numerous other exogenous and endogenous substances.[55] Moreover, studies have shown that alcohol consumption can increase the levels of CYP2A5 in the liver of mice.[56] Therefore, CYP2A5 has been investigated for its potential role in mediating the effects of

alcohol consumption and the subsequent liver injury.[57] In the model of EtOH plus smoking, CYP2A5 gene of mice were knockout, resulting in cyp2a5$^{-/-}$mice, which showed reduced liver injury. This is because CYP2A5 metabolized nicotine or cotinine and produced reactive oxygen species (ROS). CYP2A5 was mainly located in the endoplasmic reticulum (ER), so the ROS produced in the ER could attack proteins, leading to the accumulation of unfolded and misfolded proteins in the ER, resulting in ER stress. Under oxidative stress and ER stress, misfolded apolipoprotein B could be retained in the ER, leading to the accumulation of fat in the liver. Taken together, nicotine-induced oxidative stress and ethanol-induced oxidative stress may synergize, exacerbating alcohol-associated steatotic liver disease.[54] Consequently, although CYP2A5 shows promise as a therapeutic target, a comprehensive understanding of its role and efficacy in treating ALD and liver injury induced by multiple factors needs further research.

This model is highly relevant to human disease and clinical research because smoking and alcohol consumption are major threats to human health and often occur in the same patients. In establishing this model, the main components of tobacco, nicotine and its metabolite cotinine, were used to simulate smoking. However, it might be better if other major toxic substances in cigarettes, such as polycyclic aromatic hydrocarbons (PAHs), including the potent carcinogen benzo[a]pyrene as well as tobacco-specific nitrosamines, were also considered.[58]

EtOH Plus Acetaminophen

Acetaminophen (APAP) is a widely used antipyretic and analgesic agent.[59] Although adverse reactions to APAP are typically mild, it possesses hepatotoxic properties and can cause severe liver injury, particularly in cases of overdose.[60,61] An overdose of APAP leads to 56,000 to 80,000 emergency room visits, 26,000 to 34,000 hospitalizations, and an estimated 500 deaths annually in the United States of America.[62] Hence, we incorporated APAP as a risk factor for liver injury in rodent models of ALD to simulate the exacerbation of liver injury induced by the combination of alcohol and APAP. In our experiment, 8 weeks old male C57BL/6J mice were used as model animals. After a 5-day liquid diet adaptation stage, mice were fed a Lieber–DeCarli liquid diet containing 5% ethanol for 10 days. Subsequently, these mice were gavaged 25% ethanol by weight (6 g/kg), along with an intraperitoneal injection of APAP (200 mg/kg). After 9 hours, the mice were sacrificed and processed into specimens for subsequent analysis and research purposes. Histological examination of the liver revealed increased levels of microsteatosis, macrosteatosis, and hepatocyte ballooning. Simultaneously, serum biochemical analysis revealed a marked increase in levels of AST and ALT, along with intensified oxidative stress, primarily reflected in the increased levels of MDA and decreased glutathione (GSH) and superoxide dismutase (SOD) expression. Furthermore, elevated levels of inflammatory factors such as TNF-α and IL-6 suggested an aggravation of inflammation.[63]

Next, we validated the role of p38γ in ethanol plus APAP-induced liver injury. p38γ is an encoded member of the p38 mitogen-activated protein kinases (MAPKs) and is crucial for various cellular processes, including the response to stress and inflammation.[64] Existing research has shown that p38γ can influence the activity of various transcription factors and other proteins involved in metabolic processes. For example, it influences the expression of genes associated with lipid metabolism, which is frequently disrupted in ALD.[65] In our EtOH plus APAP model, p38γ KD mice were made by slowly injecting AAV9-packaged p38γ KD plasmids via tail vein injection. In p38γ KD mice, reduced liver injury and elevated levels of discs large homolog 1 (Dlg1) were observed compared with WT mice.[63] Furthermore, we found that Dlg1 could be combined with p38γ. Therefore, p38γ plays a critical role in the regulation of Dlg1, affecting the severity of liver injury induced by ethanol plus APAP. Indeed, recent studies have identified therapeutic strategies involving the modulation of p38γ activity to mitigate ethanol-induced liver injury. For instance, compounds like scutellarin and boswellic acid can target this pathway, reducing liver injury due to alcohol.[66,67] With a further understanding of p38γ in the pathogenesis of ALD, the development of therapeutic strategies targeting this site has considerable prospects.

Based on the Gao-binge model, we prepared an EtOH plus APAP model by intraperitoneally injecting APAP into mice to increase clinical relevance. We also used p38γ KD mice to verify whether p38γ affects ethanol plus APAP-induced liver injury. This EtOH plus APAP model is convenient and cost-effective, but the severity of ALD induced is relatively limited, such as the inability to observe fibrosis. Therefore, the EtOH plus APAP model has limitations in simulating ALD progression and discussing deeper mechanisms.

EtOH Plus Vitamin D Deficiency

Vitamin D is a general term for cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2), which play a crucial role in regulating the metabolism of calcium and phosphate.[68] Moreover, studies have shown that vitamin D deficiency is common in patients with chronic liver disease. Severe vitamin D deficiency is strongly associated with liver dysfunction and the severity of the disease.[69] So, ethanol-induced liver injury is also considered to be associated with vitamin D deficiency.[70] For further study, Shibamoto et al adjusted the diet of mice to induce vitamin D deficiency and established a model of synergistic liver injury caused by vitamin D deficiency plus alcohol consumption. To establish an animal model of ALD-related liver fibrosis, 10 weeks old female C57BL/6J mice were fed a Lieber–DeCarli liquid diet containing 2.5% ethanol and administered CCl_4 intraperitoneally twice a week by weight (1 mL/kg). Simultaneously, the mice were fed a vitamin D–deficient diet (VtDD) for a total duration of 8 weeks. Upon examining the liver tissue of mice, researchers found that mice given a VtDD in addition to ethanol administration exhibited hemorrhagic liver necrosis and aggravated liver fibrosis compared with those given alcohol alone, as also reflected by increased α smooth muscle

actin (α-SMA) and collagen type I (COL-1) matrix deposition. Further analyses indicated that AST and ALT levels were elevated, and oxidative stress and inflammatory reactions were exacerbated. Meanwhile, it is worth noting that the combination of ethanol treatment and VtDD leads to a reduction in the expression of tight junction proteins in the intestines of mice. This indicates impaired intestinal barrier function, which may result in dysbiosis of the gut microbiota, leading to an increase in lipopolysaccharides (LPS) and exacerbating liver injury. In a nutshell, vitamin D deficiency may exacerbate ethanol plus CCl_4–induced liver fibrosis through hepatic oxidative stress and LPS-mediated pro-inflammatory responses. Additionally, vitamin D deficiency may worsen hepatic pathology due to gut barrier disruption and exacerbation of LPS portal translocation.[71]

This EtOH plus vitamin D deficiency model introduced the fibrosis inducer CCl_4 during the animal feeding stage, resulting in late-stage liver inflammation and fibrosis in mice. It was confirmed that vitamin D deficiency exacerbates the development of liver fibrosis in ALD mice. Additionally, studies on this model compellingly demonstrated that vitamin D deficiency–induced disruption of the intestinal barrier is closely related to ethanol-induced liver injury.

MetALD and Experimental Models

Metabolic and alcohol related/associated liver disease (MetALD) is a new term used to describe patients with metabolic dysfunction–associated steatotic liver disease (MASLD) with moderate to excessive alcohol consumption.[72] MASLD is often closely associated with obesity and other metabolic disorder risk factors.[73] Therefore, liver injury in MetALD patients is influenced by multiple factors, including alcohol consumption. Currently, many teams are actively developing experimental models for MetALD. Additionally, some previously established models also align with MetALD, and these models are reviewed in this section (►**Table 3**).

EtOH Plus Obesity

Obesity, as defined by the WHO and the National Center for Health Statistics, is a body mass index (BMI) greater than 30 kg/m^2.[74] Obesity is considered a global epidemic and overwhelming evidence indicates that it is a risk factor for numerous liver diseases, including MASLD, and even HCC.[75,76] Moreover, existing research has also already indicated that increased body weight is associated with histological liver injury in chronic alcohol-associated patients.[77] To further investigate the exacerbation of liver injury in obese individuals consuming alcohol, a rodent model of EtOH plus obesity was established by Everitt et al. In this 12 weeks old male C57BL/6-J-Rj-ob mice were used as the model animals.[78] This mouse strain carries a mutation in the gene responsible for producing leptin, a hormone that regulates appetite and energy balance. So, these mice typically exhibit characteristics such as weight gain, increased adipose tissue, and insulin resistance, making them an ideal model for studying obesity and its complications.[79] To establish the model, mice were provided with a polyunsaturated fat control diet (PUFA; 40% of calories from fat, primarily from corn oil) containing ethanol that made up 27.5% of their total caloric intake every day, continuing this regimen for 4 weeks. Subsequent histological analysis of liver tissue revealed exacerbated hepatic steatosis in the mice, and serum biochemical analysis detected elevated levels of AST and ALT. Furthermore, protein and gene expression analysis revealed a decrease in Sirtuin 1 (SIRT1) mRNA levels, and an increase in mammalian target of rapamycin (mTOR) levels.[78] Collectively, these indicators suggested that the genetically obese mice are more susceptible to liver injury from alcohol consumption.

This model uses genetically modified C57BL/6-J-Rj-ob mice fed with a polyunsaturated fatty acids (PUFA) control diet to simulate obesity-related metabolic disorders. It is also used to examine the effects of long-term ethanol administration on obese ob/ob mice and to study the liver lipid metabolism pathways affected by this combination. However, the severity of liver injury induced by alcohol feeding in this model is limited, and hepatitis, fibrosis, or cirrhosis cannot be observed.

EtOH Plus Fast Food

Fast food typically refers to food that is pre-prepared and quickly served to customers. Although fast food offers convenience, most fast food is detrimental to health due to its high fat, high salt, high sugar, and low fiber content.[80,81] A study from the Keck Medical Center of the University of Southern California indicated that among groups of obese or diabetic patients who consumed 20% or more of their daily calories from fast food, the fat content in the liver significantly increased.[82] Meanwhile, excessive alcohol consumption can also lead to abnormal fat accumulation in the liver.[83] Therefore, it is meaningful to study the exacerbation of liver injury by combining fast food diet as a risk factor with alcohol consumption. To gain deeper insights, Sharma et al conducted a study in which 5 to 6 weeks old male C57BL/6J mice were fed a diet designed to simulate fast food. The diet contained 20% fat, 2% cholesterol, and 34% sucrose, and were regularly given high fructose (42.2 g/L) in water. Meanwhile, these mice were orally administered ethanol diluted in water every other day, with the weekly dose exponentially rising from 10% v/v (7.9 g) to 50% v/v (39.5 g), over a period of 8 weeks to complete the modeling process. By recording and analyzing the food intake of the mice, researchers found that the mice in this model exhibited a lower average food intake (AFI) compared with mice consuming alcohol alone. Besides, histological examination of the liver revealed more severe hepatomegaly, microsteatosis, and macrosteatosis. Serum biochemical analysis revealed significant elevation in levels of AST and ALT. Further protein and gene expression analysis revealed higher levels of α smooth muscle actin (α-SMA), tissue inhibitor of metalloproteinases-1 (TIMP-1), IL-6,

Table 3 MetALD model

Factors	Animals	Modeling method	Phenomena and indicators	Characteristic	Reference
EtOH + Obesity	12 weeks old male ob/ob mice (C57BL/6-J-Rj-ob)	1. The PUFA diet supplement with ethanol (27.5% of total calories) for 4 weeks (once a day)	• ↑ Liver/body weight ratio • Hepatic steatosis • ↑ Hepatic fat accumulation • ↑ ALT and AST	Convenient and cost-effective The severity of liver injury is limited	78
ETOH + Fast food	5–6 weeks old male C57BL/6J mice	1. An FF diet (20% fat, 2% cholesterol, and sucrose 34% by weight) 2. High fructose (42.2 g/L) 3. Ethanol administered orally on alternate days (weekly exponential rise from 7.9 to 39.5 g) Duration: 8 weeks	• ↓ AFI • Hepatomegaly • ↑ Serum glucose • ↑ Total cholesterol • ↑ ALT and AST • Inflammatory cell infiltrate • ↑ Oxidative stress • ↑ α-SMA and TIMP-1	High relevance to human diseases Only mild liver fibrosis	84
Acute EtOH binge + HFD	8–10 weeks old male ICR mice	1. HFD (60 kcal% fat) for 12 weeks 2. A single dose of ethanol (5 g/kg body weight) on the last day of HFD feeding	• Hepatic steatosis • ↑ ALT and AST • Inflammatory cell infiltrate • ↑ Oxidative stress	Recapitulates major hepatic phenotypes of ALD Acute ethanol binge has limitations to ALD development	86
Chronic EtOH binge + Western diet	8 weeks old male C57BL/6J mice	1. Free access to a Western diet (40 kcal % fat, 20 kcal% fructose, and 2% cholesterol) for 4 weeks 2. 5% ethanol ad libitum, and weekly gavage with ethanol (2.5 g/kg body weight) for another 8 weeks	• Hepatic steatosis • ↑ Hepatic TG • ↑ ALT • Inflammatory cell infiltrate • ↑ Oxidative stress	Simulates human dietary habits and chronic drinking patterns High relevance to human diseases No histological inflammation or fibrosis	88
EtOH plus MASH diet	9–10 weeks old male C57BL/6J mice	1. A MASH diet (fat 33 g %, cholesterol 10 g%, and sucrose 208.4 g %) for 3 days 2. 5 g/kg ethanol gavage for another 3 days	• Hepatic steatosis • ↑ ALT and AST • Inflammatory cell infiltrate	Convenient and cost-effective Only early stages of Met ALD progression	90

Abbreviations: AFI, average food intake; ALD, alcohol-associated liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FF, fast food; HFD, high-fat diet; MASH, metabolic dysfunction-associated steatohepatitis; MetALD, metabolic and alcohol related/associated liver disease; PUFA, polyunsaturated fatty acids; TG, triglyceride; TIMP-1, tissue inhibitor of metalloproteinases-1; α-SMA, α smooth muscle actin.

TNF-α, and transforming growth factor-β (TGF-β), indicating a more severe occurrence of inflammation. Additionally, the increase in TBARS levels and the decrease in SOD-1 indicated an intensification of oxidative stress.[84]

This model gives mice a high-fat, high-sucrose, and high-fructose diet along with long-term alcohol feeding, causing them to develop progressive steatohepatitis, metabolic dyslipidemia, and molecular signaling disorders related to oxidative damage, inflammation, lipogenesis, fibrosis, DNA damage, and apoptosis. This model is helpful for studying the progression of MetALD and the development of therapeutic drugs.

Acute EtOH Binge Plus HFD

High-fat diet (HFD) is considered one of the common factors causing liver injury in humans and is often used in research to establish MASLD models.[85] Zhan et al established an HFD feeding plus acute EtOH binge model in a study. In this 8 to 10 weeks old male ICR mice were fed an HFD (60 kcal% fat) for 12 weeks, and on the last day of HFD feeding, the mice were given a single dose of ethanol (5 g/kg body weight). After 9 hours of gavage, the mice were sacrificed. Histological analysis of liver tissue revealed HFD feeding plus ethanol binge caused obvious hepatic steatosis and lipid droplets.

Serum biochemical analysis revealed significant elevation in levels of AST and ALT. Protein and gene expression analysis revealed the positive staining of sterol regulatory element-binding protein-1 (SREBP-1) around the central vein was elevated. Notably, under extra ethanol stimulation, the expression of peroxisome proliferators-activated receptor γ (PPARγ) in the liver of mice significantly decreased, whereas an HFD alone had no effect. Additionally, the expression of both neutrophil elastase (NE), a marker of the activated neutrophil, and F4/80, a marker of macrophages, increased, indicating an exacerbation of the inflammatory response.[86]

The model combines HFD and acute ethanol binge, simulating the condition of MetALD, which recapitulates major hepatic phenotypes of ALD, including steatosis and steatohepatitis characterized by neutrophil infiltration, oxidative stress, and ER stress. Meanwhile, the study explores the feasibility of regulating lipid synthesis by inhibiting SREBP-1 expression and upregulating PPARγ levels, as well as reducing inflammatory responses by inhibiting P2 × 7 receptor (P2 × 7R) expression.[86] However, although acute EtOH binge can simulate certain clinical conditions, its pathological mechanisms may differ from those of long-term chronic drinking, which may limit the broader applicability of the results.[23]

Chronic EtOH Binge Plus Western Diet

Both the Western diet and HFD contain high-fat components, but they differ in composition and health impacts. For example, the Western diet typically also includes high sugar, high salt, and high calories.[87] Therefore, the Western diet can better simulate certain aspects of modern human dietary habits, such as the intake of high fat, high sugar, and high calories, when constructing animal models. This makes the experimental results more relevant to real-world conditions. Buyco et al established a model with chronic EtOH binge combined with a high fructose, high fat, and high cholesterol diet. In this 8 weeks old male C57BL/6J mice were allowed free access to a diet containing 40 kcal% fat, 20 kcal% fructose, and 2% cholesterol. After 4 weeks, the mice were given ad libitum 5% ethanol in drinking water and were gavaged with ethanol (2.5 g/kg body weight) weekly for 8 weeks to complete the modeling. Histological analysis of liver tissue revealed chronic ethanol binge plus Western diet leads to more severe steatosis compared with alcohol consumption or a Western diet alone. Serum biochemical analysis revealed significant elevation of ALT activity and hepatic triglyceride (TG), but lower plasma TG, indicating increased de novo lipogenesis with TG flux shifted toward storage in hepatic lipid droplets instead of VLDL secretion during the early stage of injury. Although histological examination did not reveal discernible inflammation or fibrosis, some inflammatory markers, such as macrophage-specific protein Cd163, showed increased expression. Meanwhile, the increased expression of TGF-β indicates that chronic EtOH binge plus Western diet promotes more pro-fibrotic signaling because TGF-β can stimulate the production and deposition of extracellular matrix components such as collagen, which are crucial for the development of fibrosis. The examination of oxidative stress markers such as heme oxygenase 1 (Hmox1) indicates that oxidative stress in this model is primarily driven by the Western diet. Additionally, mice fed the combined diet showed more glucose intolerance compared with those fed either diet alone.[88]

In summary, this model closely mimics human dietary habits and chronic drinking patterns, effectively inducing a range of liver diseases, including steatosis, oxidative stress, inflammation, and pro-fibrotic signaling. It provides valuable insights for studying the lipid metabolism disorders commonly seen in metabolic syndrome induced by alcohol combined with a high-fat, high-sugar diet, as well as the development of glucose intolerance. However, this model also has its limitations, as it can only simulate the early development of ALD without histological inflammation or fibrosis.

EtOH Plus MASH Diet

Metabolic dysfunction-associated steatohepatitis (MASH) is a liver disease caused by metabolic disorders. MASH is a severe form of MASLD that can progress to advanced fibrosis, leading to an increased risk of cirrhosis and HCC, posing a global threat to human health.[89] Babuta et al established a model involving short-term feeding of a high-fat, high-cholesterol, and high-sucrose MASH diet combined with short-term alcohol binges to simulate the early stages of MetALD. In this 9 to 10 weeks old male C57BL/6J mice were given ad libitum access to a MASH diet (fat 33 g%, cholesterol 10 g%, and sucrose 208.4 g%) for 3 days. Then the mice received 5 g/kg ethanol gavage for 3 days. The mice were sacrificed 9 hours after the final binge. Subsequent histological analysis showed that the mice with ethanol binge plus the MASH diet had exacerbated liver steatosis compared with either treatment alone. Serum biochemical analysis revealed significant elevation in levels of AST and ALT. Protein and gene expression analysis revealed that ethanol binge plus the MASH diet led to the activation of the NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome. This process includes an increase in the transcript level of NLRP3 and other inflammasome components such as IL-1β, as well as an increase in the protein level of cleaved caspase-1 and cleaved IL-1β. Meanwhile, hepatic expression of C-X-C motif chemokine receptor 1 (Cxcr1), Cxcr2, CD11b, as well as the release of neutrophil extracellular traps (NETs) by activated neutrophils, all increased. This indicates that ethanol binge plus the MASH diet induces the neutrophil recruitment, infiltration, and NETs release in the liver, exacerbating inflammatory responses.[90]

In summary, this model is convenient and cost-effective, allowing for the observation of significant liver injury indicators in a short period. Further research emphasizes the novel role of the NLRP3-IL-1β axis in early MetALD by recruiting monocytes/macrophages and neutrophils to the liver to propagate inflammation. However, due to the short feeding duration, it remains limited to the early stages of MetALD progression.

Alcohol-Associated HCC (A-HCC) Model

Alcohol consumption is a well-established risk factor for HCC; research has shown that alcohol contributes to an estimated 19% of HCC deaths globally.[83] Compared with HCC from other causes, A-HCC is often diagnosed with more advanced stage and cirrhosis, partly due to the late diagnosis and lack of tumor screening in population with ALD. Currently, animal models for A-HCC research are extremely limited. Some A-HCC mice models use the carcinogen diethylnitrosamine (DEN) combined with long-term ethanol feeding, but the gene mutations induced by DEN in mice cancers are rarely found in human HCC. So, the clinical relevance of these models to human A-HCC is considered questionable.[84,85] However, an A-HCC model established by Seo et al using ALDH2-deficient mice is noteworthy. In this 10 to 12 weeks old male mice were injected intraperitoneally with CCl_4 (0.2 mL/kg in olive oil) twice a week for 28 weeks. For the last 10 weeks of the 28-week period, the mice were fed a 4% v/v ethanol-containing Lieber–DeCarli diet. Hepatic histopathology from these mice showed fibrosis, steatosis, inflammation, hepatocyte ballooning degeneration, and tumor nodules. Immunohistochemistry analyses detected the expression of hepatocyte paraffin 1 (HepPar-1), an immunohistochemical marker of HCC suggesting CCl_4 plus EtOH treatment induces HCC. Additionally, studies on ALDH2-deficient mice showed increased susceptibility to CCl_4 + EtOH-induced HCC. The results suggested that after chronic CCl_4 and ethanol exposure, ALDH2-deficient hepatocytes produced oxidized mtDNA-enriched extracellular vesicles (EVs). These oxidized mtDNA-enriched EVs were then transferred to HCC cells and activated multiple oncogenic pathways, such as c-Jun N-terminal kinase (JNK) and STAT3.[36] Collectively, this model induces liver fibrosis in ALDH2-deficient mice through CCl_4 administration followed by ethanol feeding, ultimately leading to HCC, serving as a mice model for studying A-HCC.

Developing better preclinical model is crucial for a deeper understanding of the characteristics, prevention, and personalized treatment of A-HCC. Although there is currently a lack of research in this area, A-HCC models such as the CCl_4 plus ethanol-induced A-HCC of ALDH2-deficient mice can provide inspiration for future studies.

Regulatory Mechanisms in the Model

In cell biology, signaling pathways are essential for transmitting information inside and among cells. These signaling pathways are involved in regulating a variety of biological processes such as cell death, proliferation, metabolism, and inflammatory responses, which are essential for maintaining the normal functions of an organism. This section introduces the representative signaling pathways involved in alcohol-induced liver injury and discusses how alcohol combines with other risk factors to influence these signaling pathways, thereby jointly exacerbating liver injury.

MAPK Signaling Pathway

The MAPK signaling pathway is a crucial set of signal transduction events associated with various biological processes. It primarily includes the extracellular signal-regulated kinase (ERK), JNK, and p38 signaling pathway.[91,92] Functionally, the ERK signaling pathway tends to promote cell survival and proliferation, while the JNK and p38 signaling pathway play more significant roles in cellular stress and apoptosis.[93] Studies have shown that the MAPK signaling pathway plays an important role in liver injury, particularly the p38 pathway, which has been extensively studied for its role in hepatic fibrosis.[94]

In the context of liver injury due to alcohol plus APAP administration, the MAPK signaling pathway has been shown to be a key player (►**Fig. 1**).[63] During the metabolism of alcohol in the liver, ROS and acetaldehyde are produced, which can activate the MAPK signaling pathway. Simultaneously, alcohol metabolites and ROS may trigger inflammatory responses, further activating the JNK and p38 subfamilies within the MAPK signaling pathway.[67,95] Similarly, APAP is metabolized in the liver into toxic intermediates, such as N-acetyl-p-benzoquinone imine (NAPQI), which induce oxidative stress and activate the MAPK signaling pathway.[96] Resultantly, the activation of certain members of the MAPK family, such as p38, can exacerbate alcohol-induced liver injury.[97] In summary, the combined hepatotoxic effects of alcohol and APAP are particularly concerning, primarily because both substances can independently activate the MAPK signaling pathway. Their combined effects can overwhelm the protective mechanisms of liver, leading to severe liver injury or even failure.

Nrf2 Signaling Pathway

The nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is a critical cellular protective mechanism closely related to oxidative stress resistance and inflammation regulation.[98] Increased levels of ROS stimulate Nrf2 signaling pathway, enhancing the activity of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase.[99]

In alcohol-induced liver injury, the Nrf2 signaling pathway is particularly important, especially the CYP2E1-ROS-Nrf2 signaling pathway.[57] Long-term alcohol consumption can generate a large amount of ROS through various pathways, including the induction of the CYP2E1 enzyme.[100] At this point, the Nrf2 signaling pathway helps to mitigate this injury by activating various antioxidant response elements (AREs) that leads to the production of detoxifying and antioxidant enzymes. Further studies have shown that natural Nrf2 activators can regulate lipid metabolism and oxidative stress in liver cells, thus helping to alleviate fatty liver disease caused by alcohol.[101] However, in some cases, the Nrf2 signaling pathway may not be sufficiently activated in response to alcohol-induced oxidative stress, leading to

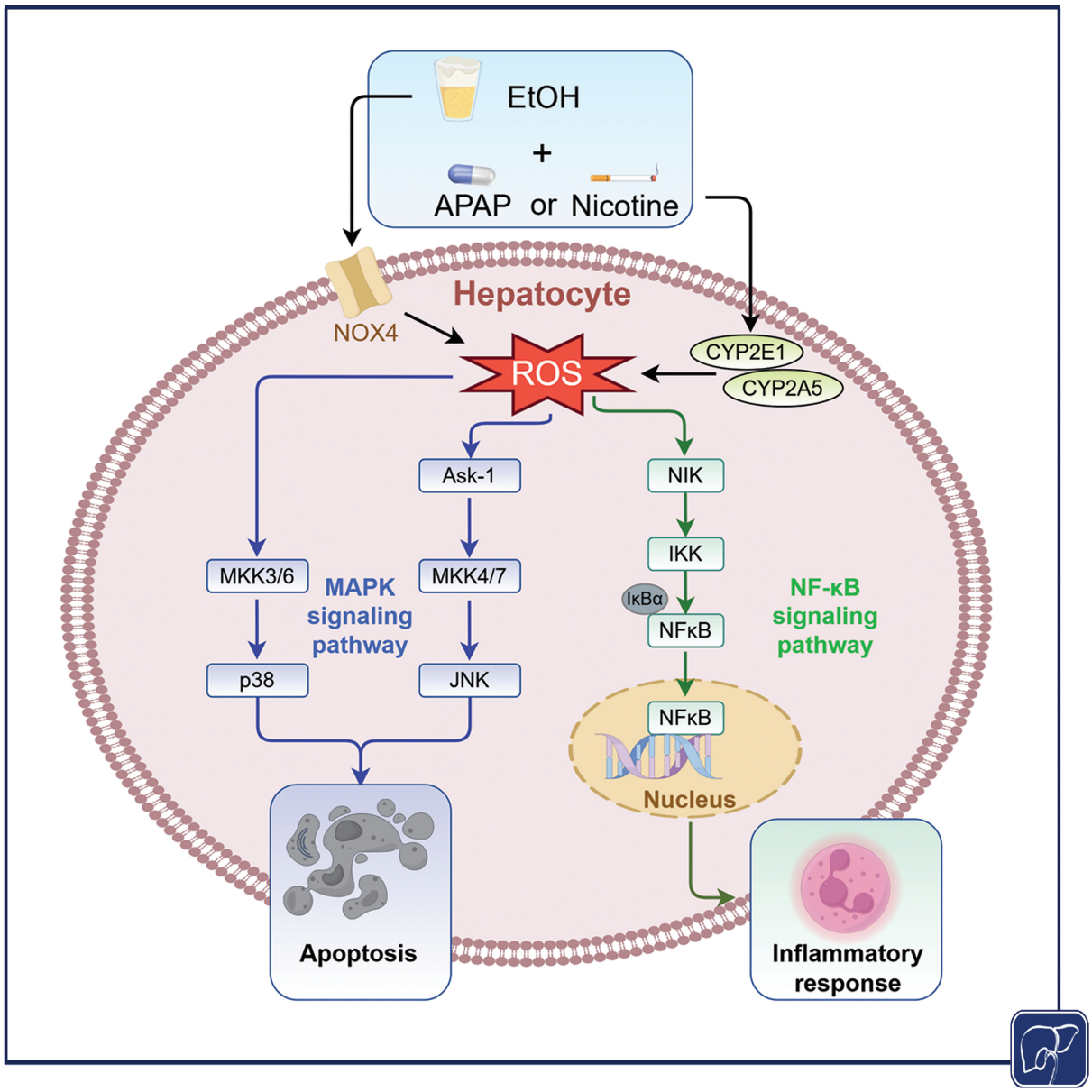

Fig. 1 **Alcohol plus acetaminophen (APAP) or nicotine exacerbates liver injury through the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) signaling pathway.** Alcohol exposure causes the liver cell membrane protein NOX4 to produce a large amount of reactive oxygen species (ROS). Meanwhile, alcohol, APAP, and nicotine can be metabolized by CYP450 enzymes (such as CYP2E1 and CYP2A5), resulting in the production of significant amounts of ROS. ROS induces Ask-1-JNK-MKK4/7, or induces p38 through activation of MKK3/6, collectively leading to apoptosis. In the NF-κB signaling pathway, ROS induces NF-κB-inducing kinase, which, through IκB kinase, causes the separation of NF-κB from IκB proteins, allowing NF-κB to enter the nucleus and initiate the transcription of target genes, thereby promoting the inflammatory response.

inadequate antioxidant defenses and more severe liver injury.[102] Nevertheless, the specific mechanisms require further study. In summary, the Nrf2 signaling pathway is a key player in protecting the liver from alcohol-induced oxidative stress, making it a potential signaling pathway for therapeutic interventions in ALD.

NF-κB Signaling Pathway

Nuclear factor kappa-B (NF-κB) signaling pathway is a crucial molecular cascade that regulates a wide range of biological processes, including innate and adaptive immunity, inflammation, and stress responses.[103] The NF-κB signaling pathway can be mainly classified into the canonical pathway and the non-canonical pathway. In the canonical pathway, NF-κB forms a complex with IκB proteins in the resting state, preventing its nuclear translocation. Upon stimulation, IκB proteins are phosphorylated and degraded, allowing NF-κB to enter the nucleus, activate gene transcription, and thereby regulate the expression of various pro-inflammatory genes, serving as a key mediator of inflammatory response. The activation of the non-canonical NF-κB signaling pathway occurs through a handful of members of the TNF receptor superfamily. The NF-κB precursor p100 is activated by

protein kinase cluster of differentiation 40 (CD40) and ultimately converted into its active form, p52.[103]

The molecular pathomechanism of ALD is primarily rooted in the innate immune system, particularly associated with the functional enhancement of the NF-κB signaling pathway.[104] Alcohol metabolism generates ROS and other byproducts that can activate the NF-κB signaling pathway. Concurrently, some additional risk factors can also activate this signaling pathway, thereby triggering an inflammatory response and exacerbating ALD. For instance, alcohol-induced endotoxins entering the portal venous circulation from the gut are believed to play a significant role in the activation of Kupffer cells, leading to an enhanced release of chemokines, and the deficiency of vitamin D can exacerbate this process.[71] In summary, these processes are associated with the involvement of the NF-κB signaling pathway, highlighting its importance in the pathogenesis of liver injury induced by alcohol and other risk factors.

AMPK Signaling Pathway

Adenosine 5′-monophosphate (AMP)–activated protein kinase (AMPK) is a crucial enzyme that acts as an energy sensor in cells, playing a significant role in maintaining energy homeostasis.[105] Therefore, the AMPK signaling pathway, in which this enzyme participates, emerges as a pivotal regulator of cellular energy balance. It has been shown to play a significant role in the pathogenesis of both alcohol-associated and non-alcohol-associated steatotic liver diseases.[106]

In the experiments conducted by Everitt et al, alcohol significantly increased the levels of mTOR in ob/ob mice, which is one of the key factors determining the nuclear retention of lipin-1 in hepatocytes. This, in turn, led to a decrease in SIRT1 and AMPK levels.[78] SIRT1 is a protein that belongs to the sirtuins family, which is well known for regulating cellular processes such as aging, transcription, and stress resistance through deacetylation of proteins.[107] Simultaneously, SIRT1 promotes the oxidation of fatty acids and reduces lipogenesis, while AMPK activation also favors fatty acid oxidation over synthesis. Furthermore, peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1α) and lipin-1, as downstream effects of the impaired SIRT1-AMPK signaling, showed respective decreases and increases in their levels.[78] Additionally, nuclear-localized lipin-1 forms a complex with PGC-1α/PPARα, leading to the induction of fatty acid oxidation genes. In conclusion, the aforementioned processes elucidate how the SIRT1-AMPK signaling pathway may exacerbate alcohol-associated steatotic liver disease (►**Fig. 2**). Therefore, given the role of AMPK signaling pathway in improving lipid metabolism, reducing inflammation, and enhancing autophagy, therapeutic strategies targeting the SIRT1-AMPK signaling pathway are actively under investigation.[105]

Future Prospects and Conclusion

Historically, rodent models of liver injury in ALD primarily simulated the early stages of the condition, such as steatosis and moderate steatohepatitis, with alcohol being the sole pathogenic factor.[16,23] This does not align well with the clinical presentation of ALD in humans, where multiple pathogenic factors are often intertwined. Currently, significant progress is being made in rodent models of alcohol-induced liver injury, particularly in the transition from traditional ALD models that focus solely on alcohol-induced liver injury to those EtOH plus X models that incorporate alcohol with additional risk factors. This shift in modeling approach is essential for a more comprehensive understanding of the multifactorial nature of liver injury. Future research should be dedicated to developing more complex models that not only simulate the intricate interactions between ethanol and other risk factors such as obesity, diet, metabolic syndrome, and viral infections, but also accurately reflect the severity and progression of liver injury in humans.[108]

In the future, research on liver organ tissues has the potential to become a hot topic. Organoid models represent an advanced in vitro approach that offers a unique three-dimensional platform, recapitulating the microenvironment of human organs and cell interactions, thereby approximating the complexity of the structure and function of the human liver.[109,110] Moreover, organoid models provide a more physiologically relevant model compared with traditional rodent models and address the limitations of traditional rodent models, such as species-specific responses that may not fully translate to human physiology.[111] Currently, a team from China has developed a human embryonic stem cell (ESC)-derived, expandable liver organoid model system that encapsulates the typical features of ALD pathophysiology.[112] Therefore, the integration of multiple risk factors including genetic predispositions, dietary habits, and environmental alternations into organoid models is a promising direction for future research.

Furthermore, new, single-cell sequencing technologies, such as spatial transcriptomics (ST), have shown great potential in liver injury model research.[113,114] ST, which combines gene expression data with spatial information, has rapidly developed in recent years. Applying this method to liver research has greatly enhanced our understanding of liver development, regeneration, and disease. Although the field is advancing, there are still various issues to be addressed, including sensitivity, the ability to obtain precise single cell information, and data processing methods.[115] In future liver injury research, as liver organoid technology matures, it can be combined with ST techniques to help us obtain liver injury models that are closer to reality and allow for more precise and in-depth analysis and study.

From a clinical standpoint, human ALD typically arises from a multitude of risk factors including alcohol. Therefore, rodent models induced with alcohol plus additional risk factors for liver injury can more accurately simulate the progression of human ALD. However, currently, there is a scarcity of such rodent models, making this a highly promising avenue for research. Simultaneously, improvements in detection techniques allow us to more accurately pinpoint targets and study signaling pathways. In conclusion, the

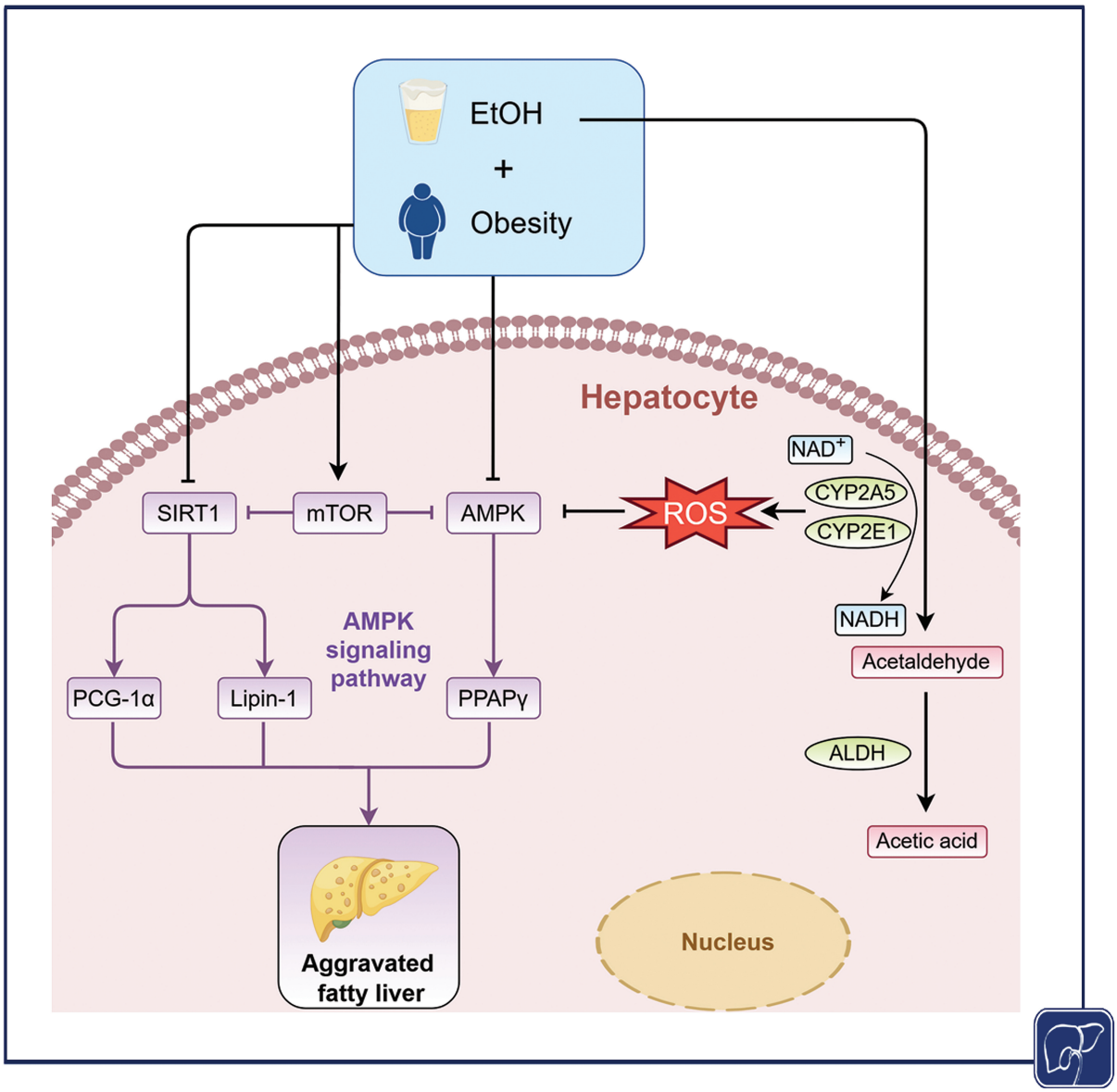

Fig. 2 **Alcohol plus obesity exacerbates liver injury through the adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) signaling pathway.** Alcohol combined with obesity directly inhibits SIRT1 and AMPK, or indirectly inhibits them by inducing mTOR, thereby activating downstream signals and exacerbating fatty liver disease (by Figdraw).

application of rodent models that combine alcohol plus additional risk factors for liver injury is a crucial method for in-depth investigation of the pathology of alcohol-induced liver injury and the identification of novel therapeutic targets and strategies. The development and refinement of these EtOH plus X models should be a focus of future research in this field.

Author Contributions

Q.W.: Conceptualization; writing—original draft, reviewing, and editing; visualization.
D.Y.: Conceptualization; writing—original draft, reviewing, and editing.
C.L.: Conceptualization; writing—original draft, reviewing, and editing.
T.X.: Conceptualization; supervision; funding acquisition; writing—original draft, reviewing, and editing.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship

Guarantor of the article: T.X. All authors approved the final version of the manuscript.

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Conflict of Interest

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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