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Invited Review

Animal Models of Alcoholic Liver Disease: Pathogenesis and Clinical Relevance

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Alcoholic liver disease (ALD), a leading cause of chronic liver injury worldwide, comprises a range of disorders including simple steatosis, steatohepatitis, cirrhosis, and hepatocellular carcinoma. Over the last five decades, many animal models for the study of ALD pathogenesis have been developed. Recently, a chronic-plus-binge ethanol feeding model was reported. This model induces significant steatosis, hepatic neutrophil infiltration, and liver injury. A clinically relevant model of high-fat diet feeding plus binge ethanol was also developed, which highlights the risk of excessive binge drinking in obese/overweight individuals. All of these models recapitulate some features of the different stages of ALD and have been widely used by many investigators to study the pathogenesis of ALD and to test for therapeutic drugs/components. However, these models are somewhat variable, depending on mouse genetic background, ethanol dose, and animal facility environment. This review focuses on these models and discusses these variations and some methods to improve the feeding protocol. The pathogenesis, clinical relevance, and translational studies of these models are also discussed.

Key words: Miscellaneous metabolic liver diseases; Liver diseases; Animal models; Alcoholic liver disease (ALD); Inflammation and injury; Molecular basis

INTRODUCTION

Excessive alcohol consumption is a leading cause of chronic liver diseases worldwide and causes a broad spectrum of liver disorders ranging from simple steatosis to severe forms of liver injury including steatohepatitis, fibrosis/cirrhosis, and hepatocellular carcinoma[1–3]. Over the last five decades, investigators have been actively exploring animal models for alcoholic liver disease (ALD), studying its pathogenesis and searching for therapeutic drugs for the treatment of ALD. However, currently, there are still no FDA-approved drugs for ALD treatment, and no animal models represent the full spectrum of human ALD[4,5]. Most animal models that involve chronic alcohol feeding develop steatosis but demonstrate low or little neutrophil infiltration, whereas hepatic neutrophil infiltration is a hallmark of alcoholic steatohepatitis in human patients.

Accumulating evidence suggests that drinking patterns and obesity have significant impacts on the progression of ALD. For example, recent drinking and binge drinking are associated with a high risk for the development of severe forms of ALD[6]. Obesity is another risk factor that exacerbates ALD as documented in clinical studies[7–9]. Furthermore, in a large cohort of patients with alcoholic hepatitis, body mass index is an independent predictor of the disease severity[10]. Based on these observations, we developed two murine models: chronic-plus-binge ethanol feeding and high-fat diet (HFD)-plus-binge ethanol challenge. Since it was published in 2010[11,12], the chronic-plus-binge model has been widely used by many

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investigators, and many novel mechanisms underlying liver inflammation and injury in this model have been revealed. Recently, the chronic-plus-binge ethanol mouse model has also been used to examine other organ damage such as the heart[13], pancreas[14], and adipose tissues[15]. In addition, this model was also established in rats[16,17], but has not been well characterized. The HFD-plus-binge ethanol model was published in 2015[18]. This model is clinically relevant and highlights the risk of liver injury in even one excessive binge drinking session in obese/overweight individuals. Using this clinically relevant model, we have identified several novel mechanisms that play important roles in inducing neutrophilia and liver injury after HFD-plus-binge ethanol challenge.

Both the chronic-plus-binge and HFD-plus-binge ethanol feeding models represent only the early stages of steatohepatitis, whereas a recently developed hybrid model induces a more severe form of steatohepatitis and liver injury. This hybrid model was established by allowing ad lib feeding of a solid Western diet followed by intragastric infusion of an ethanol liquid diet with addition of a weekly binge of ethanol[19]. This model reproduces some features of chronic alcoholic steatohepatitis and early alcoholic hepatitis (AH)[19]. The details of this hybrid model have previously been discussed[19,20] and will not be described here. In this article, we discuss several factors that affect chronic-plus-binge and HFD-plus-binge ethanol-induced liver injury and the mechanisms that underlie the liver injury in these models. We also discuss some translational studies of chronic-plus-recent binge drinking in patients with excessive alcohol use (EAU).

FACTORS THAT AFFECT CHRONIC-PLUS-ONE-BINGE ETHANOL FEEDING-INDUCED LIVER INJURY

A short-term (10-day)-plus-binge ethanol feeding model was first reported in 2010[11,12]. This protocol induces significant hepatic steatosis with substantial increase in the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Later, long-term (up to 12-week)-plus-one binge or multiple binges of ethanol feeding were also developed[21], which induce more severe phenotypes of steatohepatitis compared to the short-term (10-day)-plus-binge ethanol model. Over the last 5 years, we have noticed many factors that may affect the outcomes of liver injury and inflammation in the chronic-plus-binge ethanol feeding model. These factors are discussed below, and some potential improvements are also described.

Genetic Background of Mice

Inbred C57BL/6 mice are known to show higher voluntary alcohol consumption than other strains such as BALB/c and DBA/J[22]. Indeed, we found that chronic-plus-binge ethanol feeding works well in both C57BL/6J and C57BL/6N mice but not in BALB/c and DBA/J mice (both BALB/c and DBA/J mice are reluctant to eat the ethanol diet). Interestingly, chronic ethanol feeding for 4 weeks in C57BL/6J mice can result in a mortality rate up to 50%, but no mortality was observed in C57BL6/N mice (Fig. 1A). Therefore, we have often used C57BL/6J mice for the short-term (10-day)-plus-one binge ethanol feeding model. C57BL6/N mice are used for both short-term (10-day) and long-term (up to 12-week)-plus-one binge or multiple binges of ethanol feeding. If the genetically modified mice were not on a C57BL/6 background, we backcrossed them into either C57BL6/N or C57BL6/J background for up to 10 generations. Heterozygous breeding was often performed, and littermate wild-type controls were used for the control group.

Male and Female Mice

It was reported that female rodents (rats and mice) were more susceptible to chronic alcohol-induced liver injury than males[23]. A recent study also compared chronic-plus-binge ethanol-induced liver injury in male and female mice, and these data revealed that the chronic-plus-binge ethanol challenge induced a greater degree of adipose tissue inflammation and liver injury in female than in male mice despite lower levels of alcohol consumption[15]. However, by analyzing clinical data, we found that serum levels of ALT and AST were lower in female alcoholics than those in male alcoholics, despite the comparable alcohol amounts that were consumed in both genders[24]. Thus, further rigor studies are needed to clarify the gender differences on the susceptibility to ALD. In our laboratory, we have used both male and female C57BL/6 mice for chronic-plus-binge ethanol feeding but prefer to use female mice for the short-term (10-day)-plus-one binge ethanol feeding and male mice for the long-term (up to 12-week)-plus-one or multiple binges of ethanol feeding.

Animal Facility Environment

We observed that the C57BL/6J mice, which were purchased from The Jackson (Jax) Laboratory and housed in our facility for 1 or 2 weeks, were sensitive to chronic-plus-binge ethanol-induced liver injury (with the average peak of serum ALT levels ~250 IU/L)[25]. Interestingly, after being housed in the National Institute on Alcohol Abuse and Alcoholism (NIAAA) animal facility for a few months, the C57BL/6J mice became less sensitive to the chronic-plus-binge ethanol-induced liver injury (with average peak of serum ALT levels ~100 IU/L). The reasons for this difference are probably due to the differences in the facility environment and conditions between the Jax laboratory and the NIAAA animal facility. For

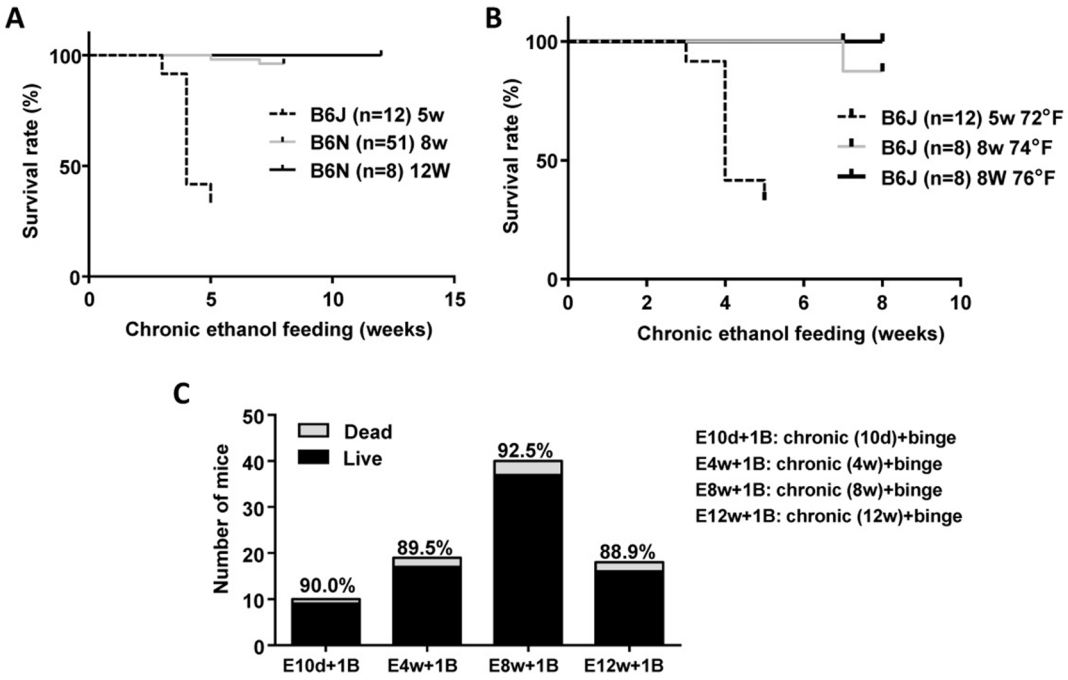

Figure 1. (A) Mortality rate of male C57BL/6J and C57BL/6N mice subjected to chronic ethanol feeding. Eight- to 12-week-old male mice were fed the Lieber–Decali diet containing 5% ethanol at room temperature of 72±3°F. (B) Mortality of male C57BL/6J mice subjected to chronic ethanol feeding under different room temperatures. Eight- to 12-week old male C57BL/6J mice were fed the Lieber–Decali diet containing 5% ethanol at different room temperatures (72±3°F, 74±3°F, and 76±3°F). (C) Mortality of male C57BL/6N mice subjected to chronic-plus-binge ethanol. Eight- to 12-week-old male C57BL/6N mice were fed the Lieber–Decali diet containing 5% ethanol up to 12 weeks, followed by one binge of ethanol (5 g/kg). Mice were fed at room temperature of 72±3°F. The percentage numbers on top of each bar indicate the corresponding survival rate.

example, the Jax laboratory animal facility is a "barrier" facility, whereas the NIAAA animal facility and most NIH intramural program animal facilities have a variety of "acceptable pathogens" (https://collab.niaaa.nih.gov/Intramural/OLAS/Shared%20Documents/Acceptable%20'Pathogen'%20List.pdf). Table 1 outlines the environmental differences between the two facilities.

Room Temperature

Many investigators have observed various percentages of mortality in C57BL/6J mice after chronic feeding with Lieber–Decali diet containing 5% ethanol for more than 4 weeks. However, this mortality was not observed in C57BL/6N mice. Alcohol consumption is known to cause hypothermia in mice. To determine whether a low room temperature contributes to the mortality in C57BL/6J mice, we chronically fed C57BL/6J mice in rooms with three different temperatures (72±3°F, 74±3°F, and 76±3°F). The mortality in C57BL/6J mice that were housed at 72±3°F room temperature (NIAAA animal facility holding rooms are set as alarm range is 72±3°F) after 4 weeks of ethanol feeding was 20%–50%. However, only one of eight mice died in the 8-week ethanol-fed C57BL/6J mouse group when the room temperature was set at 74±3°F, and no morality was observed when the room temperature was set at 76±3°F (Fig. 1B).

Table 1. Environmental Differences Between the Jax and NIAAA Facilities

Jax Lab Animal Facility	NIAAA Animal Facility
Sterilized autoclaved or irradiated feed	Nonsterilized or nonirradiated feed
Acidified drinking water (pH 2.5 to 3)	Tap water (pH 6.5 to 8.5)
Steam-sterilized bedding	Nonsterilized bedding
Mouse transfer by disinfected forceps	Mouse transfer by gloved hands
Strict barrier sanitation of caging equipment through tunnel washers	Caging equipment goes through the industrial washer; racks go through the rack washer
Restricted entry	Limited access

Interestingly, in the rooms with higher temperatures where no mortality was observed, the serum ALT and AST levels were lower than those in the rooms with lower temperatures (data not shown).

Doses of Ethanol

In our original protocol, we used a liquid diet containing 5% ethanol, and mice were gavaged with 5 g/kg ethanol[12]. As mentioned above, mice may become less sensitive to liver injury (as indicated by a less pronounced elevation in serum ALT and AST levels) when they are housed in the NIAAA facility or when they have a mixed background. To achieve a higher level of liver injury, we increased the concentration of ethanol in the liquid diet to 5.5%–6% or gavaged mice with 6 g/kg of ethanol.

Mortality

In addition to housing mice in the low-temperature environment, improper technique during the oral gavage is another leading cause of mortality. In general, mice should be gently restrained, and the head should be immobilized. The appropriate length of the gavage needle should be used and carefully passed along the side of the mouth. Improper technique may result in the injection of ethanol into the trachea and lungs during gavage. Furthermore, excessive force in advancing the gavage needle may cause esophageal or tracheal penetration[12]. The mortality rates in the chronic-plus-binge models in male C57BL/6N mice in our laboratory are shown in Figure 1C.

FACTORS THAT AFFECT HFD-PLUS-ONE BINGE ETHANOL FEEDING-INDUCED LIVER INJURY

Although obesity and alcohol drinking are known to synergistically exacerbate the progression of liver disease, there were no good models to mimic this condition. One of the major obstacles was that ad libitum feeding of mice with ethanol in drinking water or in liquid diet markedly reduced the HFD intake and body weight unless the mice were subjected to intragastric overfeeding with the HFD and alcohol[26]. Recently, we developed a model in which mice were fed with HFD first to induce overweight/obesity, which was then followed by oral gavage of a single dose of ethanol[18]. Our data revealed that chronic HFD feeding (3 months of HFD) in male C57BL/6J mice caused a moderate elevation of serum ALT (approximately 100–200 IU/L) (Fig. 2). While a single dose of ethanol (5 g/kg) gavage in chow-fed 12-week-old male C57BL/6J mice did not cause significant increases in the serum ALT and AST levels, a single-dose ethanol gavage (5 g/kg) caused a significant increase in the serum ALT levels to approximately 150 and 800 IU/L in male C57BL/6J mice that were fed with HFD for 3 days and 3 months, respectively[18]. In addition to highly elevated serum ALT and AST levels, significant neutrophilia, hepatic neutrophil infiltration, and liver inflammation were other important features that were observed in these mice with HFD-plus-binge ethanol challenge. In conclusion, an acute binge of a single dose of ethanol causes severe acute steatohepatitis in obese mice, and this is a clinically relevant model for studies of the interaction of obesity with binge alcohol drinking. During our study, we noticed several factors that affected the outcome of the HFD-plus-binge ethanol feeding model, which are discussed below.

Genetic Background and Gender of Mice

Male C57BL/6 mice are the most susceptible strain for the development of HFD-induced obesity and insulin resistance and are the most common strain used in the study of metabolic disease. We have tested the 3-month-HFD-plus-binge ethanol feeding model in several strains and found that HFD-plus-binge ethanol feeding induced higher levels of serum ALT and AST in male C57BL/6 mice than in male BALB/c and DBA mice. The underlying mechanisms probably involve the fact that male C57BL/6 mice gained the greatest body weight after HFD feeding among these strains. In addition, we found that female C57BL/6 mice were much less sensitive to the HFD-plus-binge ethanol feeding-induced liver injury than the male counterparts, probably because female C57BL/6 mice gained much less body weight after HFD feeding. Interestingly, we observed that the HFD-plus-one binge ethanol challenge induced greater serum ALT levels in male C57BL/6N mice than in C57BL/6J mice.

Animal Facility Environment

Any environmental factors that alter the weight gain may significantly affect the outcome of HFD-plus-binge ethanol-induced liver injury. For example, stress significantly affects food intake and weight gain during HFD feeding. Stresses to the mice should be avoided during HFD feeding. We tried not to disturb or only to minimally disturb these mice during the HFD feeding. Regrouping male mice from different litters in the same cage, which increases the stress level, is not recommended before HFD feeding. For example, 8- to 12-week-old male C57BL6 mice usually gained approximately 2–3 g after the first 3 days of HFD feeding (HFD: 60% fat), and the 3-day HFD-fed mice usually had serum ALT levels of approximately 150 U/L after one binge of 5 g/kg ethanol. However, mice under stress conditions such as being separated or placed in mixed housing before feeding gained less than 1 g after 3 days of HFD feeding, and they demonstrated minimally elevated serum ALT and AST after the ethanol binge.

Doses of Ethanol

In our original protocol, we gavaged the HFD-fed mice with a single dose of 5 g/kg ethanol[18] and observed

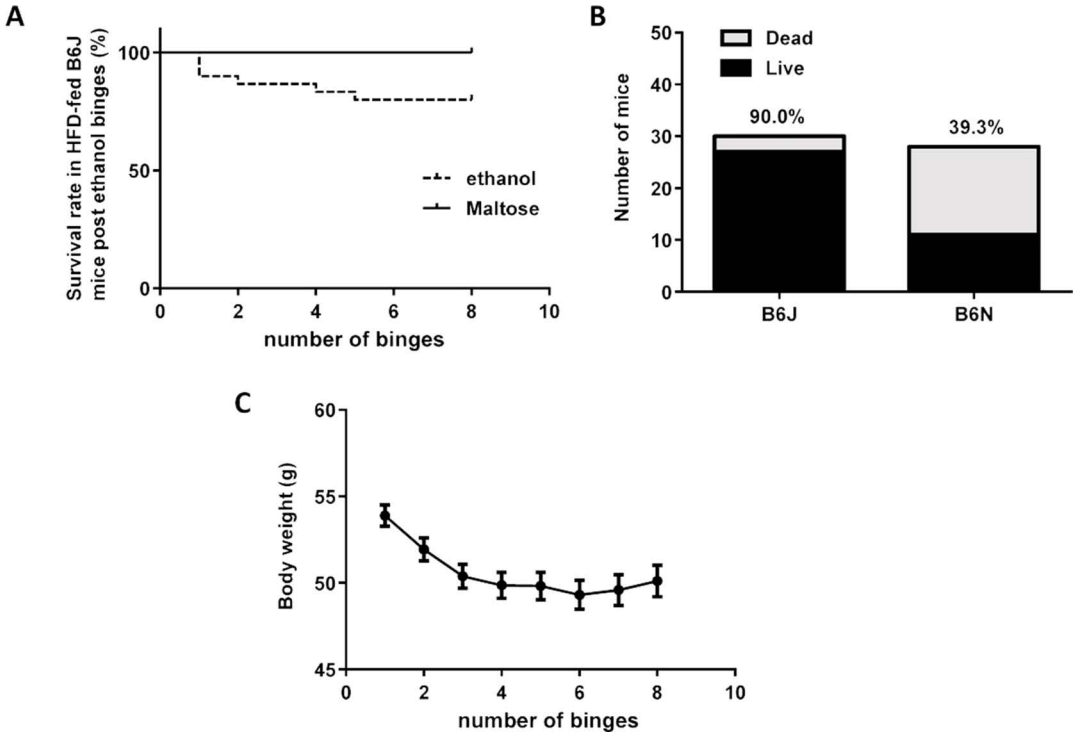

Figure 2. (A) Mortality rate of male C57BL/6J mice subjected to HFD-plus-multiple binges. Eight- to 12-week-old male mice were fed an HFD diet for 3 months. Then mice were continued to be fed with HFD for 4 weeks, plus binge ethanol (5 g/kg) twice a week for 4 weeks. The survival rate of the mice was determined at 24 h after the gavage. (B) Survival rate of male C57BL/6J and C57BL/6N mice subjected to HFD-plus-single ethanol binge. Eight- to 12-week-old male C57BL/6J and C57BL/6N mice were fed an HFD diet for 3 months and then subjected to one binge of ethanol (5 g/kg). The survival rate was determined 24 h after the gavage, and the percentage of survival is shown on the top of the bars. (C) Body weight change of male C57BL/6J mice subjected to HFD-plus-multiple binges. The mice ($n=27$) were treated as described in (A), and the body weight at the time of each gavage was examined and analyzed.

approximately 10%–15% mortality especially in the HFD-fed mice with body weights over 55 g. For mice over 60 g, we usually gavaged with a single dose of 4 g/kg ethanol. However, gavage with a lower dose of ethanol may cause less liver damage.

For the 3-day-HFD-plus-binge ethanol feeding model, we gavaged mice with a single dose of ethanol (5 g/kg body weight as a 31.25% solution in water), while for the 3-month-HFD-plus-bing ethanol feeding model, mice were given a single dose of ethanol (5 g/kg body weight as a 53% solution in water).

HFD Composition

The type of fat (saturated vs. unsaturated) may also affect the outcome of HFD-plus-binge ethanol feeding. Saturated fat has been shown to be protective against ALD, while unsaturated fat is deleterious for ALD[27]. We used an HFD in which 60% of the calories were derived from fat (Cat. No. D12492; Research Diet, New Brunswick, NJ, USA) in our HFD-plus-binge ethanol model, and this diet is working well in this model. We have not tested any diets with other types or proportions of fat in this model.

CHRONIC-PLUS-MULTIPLE BINGES OF ETHANOL MODEL

We performed two models of chronic-plus-multiple binges of ethanol. First, mice were subjected to chronic ethanol feeding up to 12 weeks, followed by three ethanol binges on consecutive days, and mice were euthanized at various time points after the last gavage. Second, mice were chronically fed an ethanol-diet-plus-binge ethanol twice a week throughout the chronic feeding period up to 12 weeks, and mice were euthanized at various time points after the last gavage. The mice that were subjected to these chronic-plus-multiple binges of ethanol models developed some degrees of hepatic inflammation and liver fibrosis but surprisingly had lower serum ALT and AST levels than those in the chronic (up to 12-week)-plus-one binge-challenged mice[21].

Chronic-plus-multiple binges caused approximately 10%–20% mortality, with most of the mortality being

observed after the second or third binge. One way to reduce the mortality is to reduce the dosage of ethanol during the initial binges (e.g., binge of 4 g/kg ethanol for the first three binges and 5 g/kg ethanol for the following binges).

HFD-PLUS-MULTIPLE BINGES OF ETHANOL FEEDING MODELS

We also performed HFD-plus-multiple binges of ethanol feeding and encountered two challenges, including loss of body weight and increase in mortality. Gavage with 4 or 5 g/kg ethanol significantly reduced the intake of HFD in mice, which resulted in significantly lower body weights in HFD-plus-multiple binge-fed mice than in the mice fed the HFD alone. Mortality is another problem with >50% death observed in HFD-plus-multiple binge-fed mice with binge of 5 g/kg ethanol. The C57BL/6N mice had >80% mortality after the first two binges, whereas the C57BL/6J mice had a better survival rate. After ethanol gavage, the HFD-fed C57BL/6J mice began to lose body weight but later regained body weight to the initial level. However, the mortality rate is also dependent on the skills of the person administering the gavage and animal care. Although we observed significant liver injury and inflammation with obvious fibrosis in mice subjected to the HFD-plus-multiple ethanol binges, this model is challenging to establish. Interestingly, the C57BL/6J mice had higher degrees of fibrosis than the C57BL/6N mice after the HFD-plus-multiple binge procedures.

NEW INSIGHTS INTO THE UNDERLYING MECHANISMS OF CHRONIC-PLUS-BINGE AND HFD-PLUS-BINGE ETHANOL FEEDING-INDUCED LIVER INJURY

Over the last 5 years, using the chronic-plus-binge or HFD-plus-binge ethanol feeding models, investigators have identified various mediators that regulate liver inflammation and injury in these models (see Fig. 3). These mediators are summarized as follows.

Neutrophils

One of the key features of alcoholic steatohepatitis in patients is the infiltration of neutrophils in the liver[28]. However, few neutrophils are found in the livers of mice that are chronically fed an ethanol diet. In contrast, significant neutrophils are observed in the livers of mice that

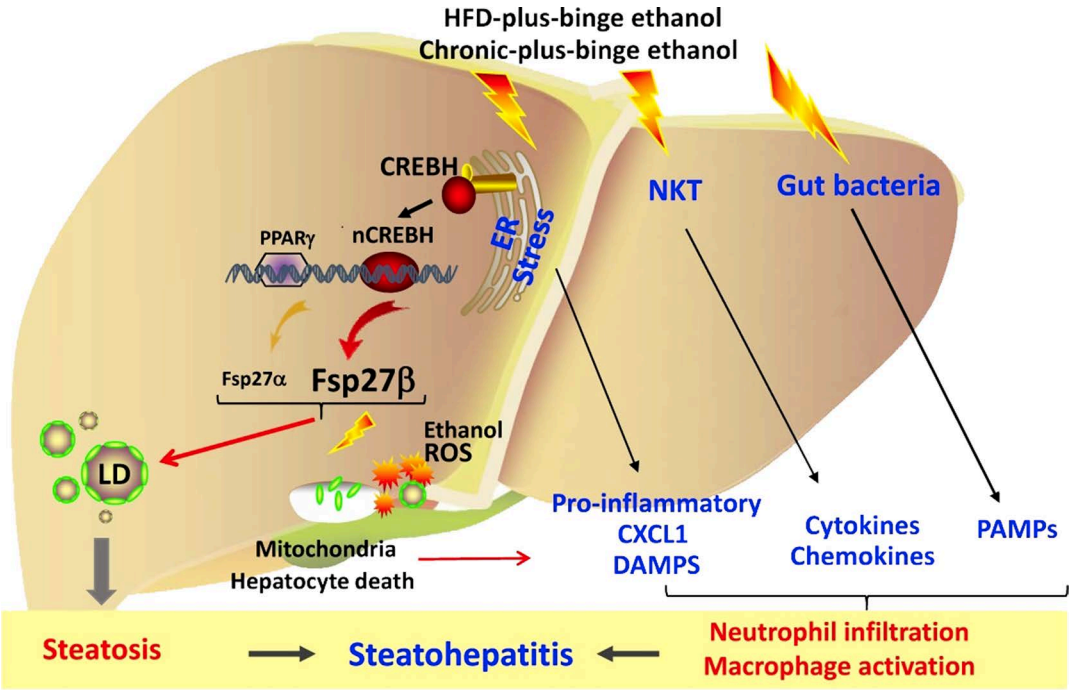

Figure 3. Multiple mechanisms underlying alcoholic steatohepatitis. Chronic-plus-binge or HFD-plus-binge ethanol consumption induces ER stress, followed by activation of the cyclic AMP-responsive element-binding protein H (CREBH) and nuclear translocation of nCREBH, and subsequent upregulation of the predominant form of FSP27β. FSP27 interacts with lipid droplet (LD) membrane proteins and subsequently promotes LD formation and steatosis. In addition, ethanol promotes FSP27 translocation into the mitochondria and subsequent mitochondrial injury and hepatocyte death. Ethanol also induces ER stress and subsequently produces proinflammatory mediators and danger-associated molecular patterns (DAMPs). Ethanol feeding activates natural killer T (NKT) cells to release proinflammatory mediators. Ethanol alters gut bacteria and elevates pathogen-associated molecular patterns (PAMPs). All of these factors lead to hepatic neutrophil infiltration and liver inflammation.

are challenged with chronic-plus-binge ethanol feeding or HFD-plus-binge ethanol feeding[18,21,29]. Such hepatic neutrophil infiltration is likely caused by several mediators including E-selectin, C-X-C motif ligand 1 (CXCL1), and TLR9. Real-time PCR analyses showed that among the various inflammatory mediators that promote neutrophil infiltration, E-selectin had the highest fold upregulation in the liver after chronic-plus-binge ethanol challenge[29]. Deletion of the E-selectin gene reduced the hepatic neutrophil infiltration and liver injury in this model, suggesting that E-selectin plays an important role in promoting hepatic neutrophil infiltration[29]. Interestingly, in the HFD-plus-binge ethanol feeding model, among the various inflammatory mediators that promote neutrophil infiltration, hepatic CXCL1 rather than E-selectin had the highest fold upregulation[18]. CXCL1 is one of the most important chemokines for neutrophil recruitment in mice. Indeed, blockade of CXCL1 via deletion of the *Cxcl1* gene or treatment with an anti-CXCL1 antibody or an interleukin-8 (IL-8) receptor inhibitor markedly prevented the hepatic neutrophil infiltration and liver injury after chronic-plus-binge or HFD-plus-binge ethanol challenge[18,30]. The dramatic upregulation of *Cxcl1* in the liver after HFD-plus-binge ethanol exposure is likely due to the significant elevation of the free fatty acids that directly induce hepatic *Cxcl1* gene expression[18]. Moreover, a recent study reported that activation of the TLR2- and TLR9-dependent MyD88-dependent pathway contributed to CXCL1 production by hepatocytes and hepatic stellate cells (HSCs), which subsequently promoted neutrophil infiltration and liver injury after chronic-plus-binge ethanol feeding[31]. In addition, lipocalin-2 seems to contribute to the hepatic neutrophil infiltration, steatosis, and liver injury induced by chronic alcohol feeding[32,33] or acute ethanol gavage[33]. Osteopontin (OPN), one of the most upregulated genes in human AH, contributes to the hepatic neutrophil infiltration and liver injury induced by short-term chronic-plus-binge ethanol feeding[34] but seems to have a protective role in a mouse model of severe AH induced by intragastric feeding of alcohol and an HFD diet with a weekly alcohol binge[19]. Another study suggested that OPN may protect against chronic alcoholic liver injury by blocking the gut-derived LPS and TNF-α in the liver[35]. These discrepancies could be explained, in part, by differences in the models being studied (i.e., varying degrees of severity, duration, and gender) and possibly by the different forms of OPNs [i.e., the intracellular OPN isoform (iOPN) and the extracellular/soluble cytokine (sOPN)][36].

Additional evidence for an important role of neutrophils in alcoholic liver injury is that the genetic deletion of miR-223, one of the most abundant miRNAs in neutrophils[37], enhanced neutrophil functions and liver injury, whereas deletion of phagocytic oxidase (phox) $p47^{phox}$ abolished reactive oxygen species (ROS) production by the neutrophils and ameliorated the liver injury induced by chronic-plus-binge ethanol feeding[24,38]. Mechanistic studies revealed that miR-223 directly inhibited IL-6 expression and subsequently inhibited $p47^{phox}$ expression in neutrophils, thereby attenuating hepatic neutrophil activation and liver injury[24,38].

Because blockade of E-selectin or CXCL1 not only prevented hepatic neutrophil infiltration but also ameliorated the liver injury induced by chronic-plus-binge or HFD-plus-binge ethanol exposure[18,29], it is plausible to speculate that neutrophils likely contribute to the hepatocellular damage in these models. Finally, more conclusive evidence for an important role of neutrophils in chronic-plus-binge ethanol-induced liver injury was given by the result that depletion of neutrophils using a neutralizing antibody ameliorates liver injury in this model[18,29]. Although we provided convincing evidence that neutrophils contribute to hepatocellular damage in the mild liver injury in these models, neutrophils likely play both detrimental and beneficial roles in the pathogenesis of severe forms of ALD, such as promoting hepatotoxicity, protecting against bacterial infection, and promoting liver regeneration[39–41].

Kupffer Cells/Macrophages

Hepatic Kupffer cells/macrophages are activated after chronic ethanol consumption in mice and play an important role in the pathogenesis of ALD[28,42]. Depletion of Kupffer cells/macrophages also reduced the chronic-plus-binge ethanol-induced liver injury and inflammation, which suggests that these cells also contribute to the pathogenesis in this model[43]. Mechanistic studies suggest that Kupffer cells/macrophages produce IL-1β and subsequently recruit/activate hepatic iNKT cells[43]. Activated natural killer T (NKT) cells promote hepatic neutrophil infiltration, liver inflammation, and alcoholic liver injury[43]. Interestingly, chronic ethanol feeding increases the number of hepatic $F4/80^+$ Kupffer cells/macrophages in mice, whereas an acute binge of ethanol reduces these cells in the liver[29]. Therefore, the changes in the number of hepatic $F4/80^+$ cells after chronic-plus-binge ethanol feeding are affected by the length of the ethanol feeding, the doses of ethanol feeding and binge, and the number of binges.

Invariant NKT (iNKT) Cells

iNKT cells are enriched among the liver lymphocytes and play important roles in the pathogenesis of liver diseases[44,45]. Several recent studies have revealed that chronic-plus-binge ethanol feeding synergistically increased the number of hepatic iNKT cells and induced their activation compared with chronic feeding or binges alone[43,46,47]. iNKT cell-deficient mice were protected

from the chronic-plus-binge ethanol-induced hepatic neutrophil infiltration and liver injury[43,46,47]. Mechanistic studies have suggested that chronic-plus-binge ethanol feeding activates hepatic iNKT cells, which play a critical role in the development of early alcoholic liver injury, in part by releasing mediators that recruit neutrophils to the liver[43,46,47], and thus iNKT cells represent a potential therapeutic target for the treatment of ALD.

Cell Death and Autophagy

Cell death through apoptosis as well as necrosis/necroptosis plays an important role in the pathogenesis of ALD[48–53]. Receptor-interacting protein kinase (RIP) 3 has emerged as a critical regulator of programmed necrosis/necroptosis and is implicated in a variety of pathological conditions, including chronic ethanol feeding-induced liver injury[53]. Recently, the roles of RIP3 in chronic-plus-binge ethanol-induced hepatotoxicity were also examined. Chronic-plus-binge ethanol feeding markedly upregulated hepatic RIP3 protein but not RIP1 protein expression[54]. This upregulation of hepatic RIP3 protein was due to impaired hepatic proteasome function after alcohol exposure[54]. Genetic deletion of the *Rip3* gene reduced the chronic-plus-binge ethanol-induced elevation of serum ALT activity and hepatic steatosis but did not affect hepatic neutrophil infiltration[54]. Thus, hepatic accumulation of RIP3 protein results in necroptosis and steatosis after chronic-plus-binge ethanol challenge[54].

Autophagy is a critical intracellular degradation pathway that promotes the trafficking of long-lived proteins and cellular organelles to the lysosome for degradation to maintain cellular homeostasis. Although autophagy has been generally believed to serve a beneficial function in alcoholic liver injury by removing damaged mitochondria and lipid droplets, the results regarding the role of autophagy in the pathogenesis of ALD have been controversial due to the complexity of the different ALD models, the lack of reliable markers, and the difficulty of monitoring autophagic flux[55–59]. Several recent studies have suggested that Parkin-mediated mitophagy and hypoxia-inducing factor-1α-dependent FoxO3a-mediated hepatic autophagy in hepatocytes protect against chronic-plus-binge ethanol-induced liver injury[60,61]. Autophagy in myeloid cells has also been suggested to play a protective role in mediating the anti-inflammatory and anti-steatogenic functions of cannabinoid receptor 2 in ALD induced by chronic-plus-binge ethanol feeding[62].

FSP27/CIDEC

Microarray analyses have revealed that various genes in the liver were highly upregulated in mice after chronic-plus-binge ethanol feeding, and many of these genes are also markedly elevated in human AH liver samples[21]. Among these highly upregulated genes, the *Fsp27* gene was upregulated by 13-fold in chronic-plus-binge ethanol-fed mice[21]. The mouse *Fsp27* gene is the homolog of the human DFF45-like effector-C (CIDEC), a member of the CIDE family of proteins, which includes CIDEA, CIDEB, and CIDEC (human)/FSP27 (mice). Interestingly, hepatic expression of *CIDEC* was also markedly upregulated in human AH but only slightly elevated in human alcoholic cirrhosis[21]. In mice, the *Fsp27* gene has two isoforms, *Fsp27α* and *Fsp27β*, and *Fsp27β* is the predominant form expressed in the liver[63,64]. The expression of both *Fsp27α* and *Fsp27β* mRNA in the liver is highly upregulated in mice after chronic-plus-binge ethanol feeding, and this upregulation is due to the activation of the endoplasmic reticulum (ER) by ethanol[21]. Inhibition of the *Fsp27* gene by Ad-*Fsp27* shRNA or hepatocyte-specific Fsp27 deletion markedly ameliorates chronic-plus-binge ethanol-induced liver injury, which suggests that FSP27 plays important roles in promoting alcoholic steatohepatitis by stimulating fat droplet formation and inducing mitochondrial injury[21]. In addition, clinical data have revealed that the upregulation of hepatic *CIDEC* is closely associated with the severity of hepatic steatosis as well as the disease severity [such as MELD (model of end-stage disease) and ABIC (age, serum bilirubin, INR, and serum creatinine) scores] in patients with AH. Hepatic *CIDEC* mRNA levels are also positively correlated with the hepatic venous pressure gradient and are an independent predictor for 90-day mortality in patients with AH[21], suggesting that CIEDC contributes to the pathogenesis of human AH.

FSP27/CIDEC is known to play an important role in promoting nonalcoholic steatohepatitis[64,65]. Hepatic Fsp27 expression is elevated in HFD-fed mice and is further upregulated in these mice after an ethanol binge[66]. Interestingly, the elevated FSP27 plays a critical role in inducing steatosis and liver fibrosis induced by HFD-plus-binge ethanol feeding but did not affect hepatic neutrophil infiltration in this model[66].

Sirtuin 1 (SIRT1)

SIRT1 is a NAD-dependent deacetylase that is able to catalyze lysine substrate deacetylation/deacylation at specific sites and thus plays critical roles in the control of metabolic stress, caloric restriction, and cancer. Using the chronic-plus-binge ethanol feeding model, several investigators have demonstrated that SIRT1 in hepatocytes ameliorates hepatic steatosis and injury[67] and that SIRT1 in HSCs attenuates HSC activation via the downregulation of platelet-derived growth factor receptor α (PDGFRα) and c-Myc[68]. Furthermore, SIRT1 expression in the liver is downregulated in aged and middle-aged mice, and such downregulation is probably one of the important mechanisms that contribute to the increased alcoholic liver injury in middle-aged and aged mice[68].

Finally, emerging evidence suggests that lipin-1 is a crucial downstream regulator of the SIRT1–AMPK signaling system that plays complex cell-specific roles in the pathogenesis of ALD[69–71].

Others

In addition to the mediators mentioned above, several other factors that regulate lipid metabolism have also been shown to play important roles in the pathogenesis of chronic-plus-binge ethanol-induced liver damage. For example, fibroblast growth factor 21 (FGF21) is an FGF family member that is produced by the liver and other metabolic tissues and plays an important role in regulating glucose and lipid metabolism[72–74]. Recent studies reported that chronic-plus-binge ethanol feeding or chronic ethanol feeding markedly upregulates FGF21 levels in the liver and serum, with much higher levels in chronic-plus-binge ethanol-fed groups than in chronically ethanol-fed mice[75,76]. Interestingly, FGF21 knockout mice were protected from chronic-plus-binge ethanol-induced liver injury but were more susceptible to chronic ethanol feeding-induced hepatotoxicity[75]. The detrimental roles of highly elevated FGF21 in the chronic-plus-binge ethanol-induced liver injury are likely due to FGF21-mediated induction of systemic catecholamines, which promote adipose tissue lipolysis and subsequently induce lipotoxicity in the liver[76]. The protective functions of FGF21 in chronically ethanol-fed mice were due to FGF-induced inhibition of the expression of genes associated with lipogenesis and stimulation of those associated with oxidation in the liver[75].

Stearoyl-CoA desaturase-1 (SCD1) is a δ-9 fatty acid desaturase that promotes the synthesis of monounsaturated fatty acids and has been found to play an important role in the development of steatosis and liver injury induced by chronic-plus-binge ethanol feeding[77]. Carboxylesterase 1 (CES1), a drug-metabolizing enzyme, has been shown to play an important role in the control of lipid metabolism[78]. Recent studies suggest that CES1 plays an important role in protecting against chronic-plus-binge ethanol-induced liver injury via the inhibition of acetaldehyde production and the induction of ROS and mitochondrial dysfunction[79]. Oxidized linoleic acid metabolites (OXLAMs), endogenous ligands for transient receptor potential vanilloid 1 (TRPV1), play a critical role in the development/progression of ALD. Using a chronic-plus-binge ethanol feeding model, Liu et al. recently demonstrated that TRPV1–OXLAM interactions play an important role in promoting alcoholic liver inflammation and injury, which suggests an important role for dietary lipids in the pathogenesis of ALD[80]. The small heterodimer partner (SHP and NROB2) is a member of the nuclear receptor superfamily that functions as a transcriptional repressor and is a critical regulator of lipid metabolism[81]. The role of SHP in the pathogenesis of ALD has been extensively investigated in the chronic-plus-binge ethanol feeding model by several groups[82–85]. The data from these studies suggest that SHP plays a complex role in the pathogenesis of ALD via the regulation of ER stress, homocysteine metabolism, hepatic circadian clock, and ethanol catabolism[82–85]. In addition to lipids, glycogen, which is another form of energy storage in the liver, also has an important role in the control of chronic-plus-binge ethanol-induced liver injury via the regulation of lipid metabolism[86].

The gut microbiota, which plays an important role in the pathogenesis of ALD[87–89], has been examined in mouse models of chronic ALD but not in the chronic-plus-binge ethanol feeding model. One recent study examined the role of immunoglobulin A (IgA) in chronic-plus-binge ethanol-induced liver injury[90]. Secretory IgA in the intestine prevents bacterial translocation by binding to the bacteria. However, surprisingly, the degree of liver damage in the IgA-deficient mice was similar to that of wild-type mice after chronic or chronic-plus-binge ethanol challenge[90]. IgA deficiency in mice is associated with an increased level of intestinal IgM that can limit bacterial translocation after ethanol challenge, which may compensate for the IgA deficiency and ameliorate ALD[90]. Spleen tyrosine kinase (SYK), a nonreceptor tyrosine kinase, plays a critical role in the pathogenesis of ALD by regulating multiple proinflammatory signaling pathways. Chronic-plus-binge ethanol exposure induces hepatic SYK activation in both hepatocytes and immune cells, and this activation seems to promote the inflammatory response and subsequently exacerbate liver injury[51]. FAT10, which belongs to the ubiquitin-like modifier (ULM) family, plays an important role in the control of protein degradation. Chronic-plus-binge ethanol feeding induces a reciprocal regulation of FAT10 and 4-hydroxynonenal (4-HNE) levels in hepatocytes, which stabilizes the oxidatively modified proteins and exacerbates liver injury[91].

APPLICATION OF CHRONIC-PLUS-BINGE ETHANOL MODEL IN TESTING THERAPEUTIC TARGETS IN PRECLINICAL STUDIES

Over the last several years, the chronic-plus-binge ethanol feeding model has been used to examine several potential therapeutic targets for the treatment of ALD. IL-22, a key cytokine for hepatocyte survival and regeneration[92], was the first drug to be tested in the chronic-plus-binge ethanol feeding model[11]. It was shown that treatment with IL-22 markedly ameliorated the steatosis and liver injury after chronic-plus-binge ethanol challenge[11]. Based on these promising preclinical data, IL-22 is currently being evaluated in a clinical trial for the treatment of patients with severe AH (Clinicaltrial.gov NCT02655510)[93]. IL-1 is an important constituent

of the inflammasome, which plays a critical role in promoting alcoholic liver injury, and IL-1R antagonists have been tested in a modified mouse model of chronic-plus-binge ethanol feeding[94,95]. The results from these studies revealed that blockade of IL-1 signaling ameliorated alcoholic liver inflammation and injury, and a clinical trial using an IL-1R blocker for the treatment of patients with severe AH is currently in progress (Clinicaltrial.gov NCT01809132)[94,95]. Heat shock protein 90 (hsp90) is activated in human ALD and animal models of ALD. Inhibition of hsp90 by 17-dimethylamino-ethylamino-17-demethoxygeldanamycin ameliorated the steatosis and liver inflammation induced by chronic-plus-binge ethanol feeding, which suggests that hsp90 is a promising therapeutic target for the treatment of ALD[96]. More recently, the chronic-plus-binge ethanol feeding model has also been used to test the therapeutic potential of many drugs/compounds, including a CB2 agonist[62], betulin (a triterpene from the bark of *Betula platyphylla* Suk)[97], luteolin (a flavonoid)[98], *Lactobacillus rhamnosus* GG[99], Korean Red Ginseng[100], glycycoumarin (a representative coumarin from traditional Chinese Medicine licorice)[101], flaxseed oil enriched in α-linolenic acid (a plant-derived n-3 polyunsaturated fatty acid)[102], thymoquinone (a biologically active compound isolated from the seeds of *Nigella sativa* L.)[103], and β-caryophyllene (BCP: a plant-derived FDA-approved food additive with anti-inflammatory properties)[104]. The data from these studies revealed that treatment with all of these components ameliorated the chronic-plus-binge alcohol-induced liver inflammation and steatosis in mice. Because the chronic-plus-binge ethanol feeding model represents the early stages of alcoholic steatohepatitis, further studies will be required to determine whether these components have beneficial effects for treatment of patients with severe ALD.

TRANSLATIONAL STUDIES OF CHRONIC-PLUS-BINGE AND HFD-PLUS-BINGE ETHANOL FEEDING-INDUCED LIVER INJURY IN PATIENTS

The data from the studies of chronic-plus-binge ethanol feeding models suggest that drinking patterns (e.g., chronic, binge, and chronic-plus-binge) markedly affect liver injury and inflammation. For example, only chronic-plus-binge ethanol feeding markedly induced neutrophilia, hepatic neutrophil infiltration, and injury, whereas chronic or binge ethanol challenge alone had less effects[24,29]. The drinking patterns in humans are much more complex, which likely has a significant impact on the progression of ALD, but how the effects occur remains largely unknown. Recently, we studied 300 excessive alcohol users (EAUs) and stratified them into two groups including EAUs with recent drinking and EAUs without recent drinking, which are similar to chronic-plus-binge and chronic ethanol feeding in mice, respectively. Our data revealed that the serum ALT and AST levels were much higher in the EAUs with recent drinking than in those without recent drinking[24]. These results are consistent with the mouse data that chronic-plus-binge feeding induced much greater liver damage than chronic ethanol feeding[29]. Another key feature from the chronic-plus-binge feeding mouse model is the elevation of circulating and hepatic neutrophils[29]. Remarkably, we also found that circulating neutrophils were highly elevated in EAUs with recent drinking, accounting for approximately 80% of the total WBCs, which was much higher than the numbers in those without recent drinking (approximately 60%–67% of total WBCs)[24]. Moreover, there was a strong positive correlation between circulating neutrophil counts and the serum ALT and AST levels in the EAUs with recent drinking, which suggested that excessive drinking likely causes the increase in hepatic neutrophil infiltration and liver injury in these subjects. Physicians should pay attention to recent drinking history when reviewing complete blood count results from alcoholics. In addition to other known causes of neutrophilia such as bacterial infection, a significant elevation of circulating neutrophils may indicate recent drinking in these subjects.

Obesity and binge drinking in humans are very common. Recent data that showed that a single oral binge exposure to ethanol can trigger acute steatohepatitis in HFD-fed mice suggested that binge drinking may cause acute liver injury in obese/overweight individuals and that obese individuals should avoid binge drinking on the basis that this would be likely to cause significant acute liver damage[18]. Future prospective cohort studies are urgently needed to investigate the effects of acute ethanol binging on liver damage in obese/overweight individuals.

PERSPECTIVE

Over the last several years, the addition of ethanol binging to the models of chronically ethanol-fed mice or HFD-fed mice has been one of the major advances in the field of ALD research. These clinically relevant models induce more severe steatosis, hepatocellular damage, and hepatic neutrophil infiltration, compared with the ad libitum chronic ethanol feeding models. However, these models still represent only early alcoholic steatohepatitis. Long-term chronic feeding and multiple binges of ethanol feeding induce more severe steatohepatitis with mild fibrosis than the chronic-plus-one binge ethanol feeding model. The model of long-term-HFD-plus-multiple binges of ethanol feeding is challenging to establish because of body weight loss and high mortality. The hybrid model developed by Dr. Tsukamoto's group induces much more severe liver damage and hepatic neutrophil infiltration. This model involves feeding mice with a Western diet that is high in cholesterol and saturated fat plus

intragastric alcohol delivery with weekly binges of alcohol and represents a model for early AH. However, the hybrid model also presents technical challenges. In conclusion, the chronic-plus-binge model represents moderate alcoholic steatohepatitis, whereas the hybrid model represents severe alcoholic steatohepatitis. The HFD-plus-binge model represents acute steatohepatitis caused by fat and binge ethanol. All of these models are clinically relevant. Mechanistically, they can be used to study the pathogenesis of ALD at different stages or with different severities. Therapeutically, they can be used to test compounds or small molecules for the treatment of ALD. Future prospective studies of human subjects with various drinking patterns and their interaction with body weight/obesity on the development of ALD are urgently required. These studies may help physicians better manage patients with ALD.

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