Chapter

Models of Hepatotoxicity for the Study of Chronic Liver Disease

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Abstract

Chronic liver disease affects globally and has a high morbidity and mortality rate. It is histopathologically characterized by the presence of inflammation, and the progressive destruction and regeneration of the hepatic parenchyma, which can lead to the development of fibrosis, cirrhosis, and hepatocellular carcinoma. Most liver diseases tend to become chronic and can be therefore studied in animal models, as it is possible to quickly develop pathological processes in animals with a high degree of reproducibility and obtain predictive data regarding the different hepatopathies. The development of animal models in the field of hepatology has been geared toward the search for new knowledge meant to favor human well-being and proved useful in translational medicine focused on liver disease. Like any other methodological tool, animal models provide valuable. Obviously, a single model cannot reproduce the complexity and spectrum of all liver diseases, which is why a wide variety are currently employed: they include chemically, immune, diet, surgically, and genetically modified damage in animals and involve biological agents or the use of humanized livers in rodents. This chapter surveys some of the main animal models used in the study of chronic liver disease and the disease characteristics they mimic.

Keywords: chronic liver disease, fibrosis, cirrhosis, hepatocellular carcinoma

1. Introduction

Chronic liver disease (CLD) is a major global health problem [1, 2]. Recent studies have shown a global increase in the morbidity and mortality of chronic liver diseases during the past decade [3, 4]. CLD was the cause of an estimated 1.32 million deaths in 2017. Around 1.5 billion people globally are thought to suffer from at least one CLD. The main problem with CLDs is that diagnosis takes place once the disease is already advanced and therapy is no longer as effective [5]. Their treatment also continues to lag behind despite available drug therapies because of three key issues: (1) costly treatments are not accessible to all sectors of the population; (2) the presence of

comorbidities such as obesity, hyperlipidemias, or the increase of intravenous drug use and nosocomial spread, just to mention a few, can promote and accelerate the disease, and (3) lack of treatment continuity by either the patient or the health system once the diagnosis has been made. All of these factors play a role in the increase of morbidity and mortality.

Liver cirrhosis is largely due to (1) chronic infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV), (2) alcohol-related liver disease (ALD), and (3) metabolic-associated fatty liver disease (MAFLD) [6–9]. The development of chronic liver disease occurs in different stages: acute hepatic decompensation, multiorgan failure, and compensated and decompensated cirrhosis resulting in higher mortality risks. Decompensated chronic liver disease is associated with the development of hepatocellular carcinoma [10]. Patients with end-stage chronic liver disease or in the hepatocellular carcinoma stage are admitted to hospitals more often, stay in there longer, and are readmitted more often compared with patients suffering from other serious chronic diseases [11]. CLD is a serious illness that entails high medical costs and impacts global public health [12]. It is characterized by extensive production of inflammatory mediators that include cytokines, chemokines, growth factors, bioactive lipid mediators, and immune-mediated tissue damage, all leading to subsequent liver failure [13–17]. The histopathological common denominator, independently of the CLD's origin, is liver inflammation as a mechanism of immune response to hepatocyte injury. Progressive destruction and regeneration of the hepatic parenchyma can lead to the development of fibrosis, cirrhosis, and hepatocellular carcinoma, causing both morbidity and mortality (**Figure 1**) [18–20]. Knowing each of these processes in detail is critical to our understanding of the disease and its therapeutic approaches. Animal models have played an important role in the study of the molecular mechanisms leading to the disease, data collection for early diagnosis, and the evaluation of most of the drugs currently employed in the clinic. Furthermore, they enable the study of new therapeutic alternatives for the prevention and treatment of this group of diseases. This chapter will review the different models employed for the study of the main histopathological and functional alterations that characterize the chronic liver disease. We also include some examples of drugs that have been evaluated using these models.

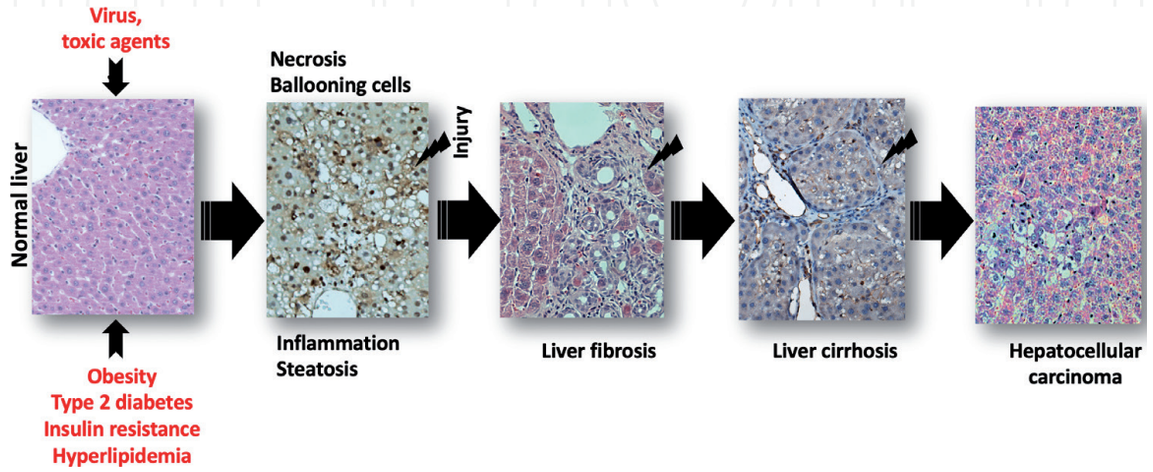

Figure 1.
Progressive necrosis, inflammation, and steatosis of the hepatic parenchyma can lead to the development of fibrosis, cirrhosis, and hepatocellular carcinoma.

2. The importance of animal models in the study of CLD

Throughout history, experimental animals have played a key role in the research of diseases affecting human beings. Rodents are similar to humans in their anatomical, physiological, genetic, molecular, and biochemical conditions, which facilitates studies involving certain diseases. They also incorporate complex factors, such as the environmental and background genetic and molecular changes within a cell under pathological conditions, making them an ideal research tool. From the perspective of scientific research, animal experimentation has contributed considerably to the growth of biomedical science, from the development of prophylactic measures and methods of diagnosis to disease treatments for humans [21, 22]. It can additionally provide information on mechanisms of injury, drug target identification, and characterization of the pharmacological and toxicological profile of innovative drug development.

The best animal model must be easily performed in the laboratory, be reproducible, have no features unrelated to the disease, have minimal limitations, and, most importantly, reproduce both the histopathological and clinical characteristics of the human disease. Poor or inadequate models will result in limited or erroneous information, perhaps even data that cannot be extrapolated to humans. The choice of an experimental model must be precise, as it is the most essential piece in the experimental strategy for the study of liver disease. The most pertinent animal models for CLD research are rodents (rats, mice, and hamsters), but rabbits have been used as well [23]. These animals meet all the basic conditions needed to induce, manipulate, and obtain biological samples for the study of liver disease. Although the choice of the appropriate animal model appears easy, one must take into consideration that it will not completely mimic the human disease because each patient has different and diverse clinical signs and symptoms, comorbidities, genetics, and the complications that might occur from the disease. These conditions cannot be identically mimicked in experimental animals, but it is possible to reproduce the histopathological and functional alterations occurring in the liver tissue. Therefore, the success of CLD studies in animals will depend on the choice of an experimental model that can represent those changes in the liver. Generally, CLD study involves experimentally inducing the disease in the animals, either *via* chemical agents, surgery, genetic modifications, or diet [24]. If the research, however, is focused on mechanisms of injury, specific pathophysiological processes of the disease, molecular targets, pharmacokinetics, or pharmacodynamics, it is important to accurately select species, gender, age, size, number of animals, etc. Said choice will depend on the desired number of samples, organ size, blood volume for the quantification of biochemical parameters and liver enzymes, or obtaining DNA, RNA, etc., for molecular studies. An appropriate experimental animal model enables understanding of the disease, including identification and stage differentiation.

3. Most commonly used models for the study of CLD

CLD is associated with a wide pathological spectrum of alterations, from inactive liver fat storage, associated with an asymptomatic benign clinical course, to progressive cardiovascular, metabolic, and/or liver and kidney diseases with higher cancer risks [25–27]. CLD results from the persistent action of various harmful agents on the liver tissue, exceeding the liver's capacity for defense and repair. The perpetuation of the damage and the liver's response to the damage can produce fat hepatic accumulation without inflammation—an excessive lipid accumulation that induces lipotoxicity

accompanied by necrosis and fibrosis development. A constant liver injury might lead more easily to chronicity, as well as a gradual decrease in the hepatocellular mass with subsequent progressive anatomical and functional distortion. From an anatomical point of view, this results in cirrhosis; from a functional point of view, it leads to chronic liver failure. CLD is histologically characterized by the presence of inflammation, steatosis, fibrosis, cirrhosis, and the development of carcinoma (**Table 1**). Therefore, most animal models for the study of liver disease mimic one or more of these histopathological features. We will now address some of the histopathological and functional characteristics of CLD and the animal models that best mimic them.

3.1 Models for the study of inflammation, steatosis, and liver fibrosis

An imbalance between lipid deposition and elimination may lead to hepatic steatosis. There are several cardinal features of hepatic steatosis such as synthesis of triglycerides (TG) and accumulation of free fatty acid (FFA) in the liver. FFA can be processed *via* two different pathways: The first pathway is β-oxidation to generate ATP, and the second is esterification of FFA to produce TG that are either stored within hepatic cells or incorporated into very low-density lipoprotein (VLDL) for release from hepatic cells, thus stimulating an imbalance in fat input/fat output that in turn leads to hepatic steatosis [58].

Histologically, the accumulation of fat in the liver is combined with lobular and periportal inflammation and cell damage (necrosis and ballooning) [59]. Hepatocytes are the main cells involved in the metabolism and mobilization of lipids in the liver. When there is lipid accumulation in hepatocytes, mitochondrial function is overloaded, leading to mitochondrial and peroxisomal dysfunction and increased oxidative stress. Uncontrolled and incomplete lipid oxidation generates toxic lipid products that cause hepatocyte damage and ultimately lead to lipoapoptosis [60, 61]. Apoptosis of hepatocytes can be considered a major cause of liver inflammation, persistent liver damage, and liver regeneration [61, 62]. Chronic inflammation of the liver can induce activation, expression, and signaling of growth factors [63, 64]. As a consequence, the hepatic stellate cells (HSCs) are activated and differentiate from the quiescent phenotype to proliferative and contractile myofibroblasts. Additionally, cells will have the ability to synthesize extracellular matrix [65, 66]. Therefore, a complex network of cytokine-induced pathways arises to coordinate the pro-fibrogenic cell interactions leading to the progression of fibrosis [67–69]. Liver fibrosis is considered a highly complex tissue repair process that appears in the face of sustained hepatocellular damage and that will go through the stages of steatosis, inflammation, regeneration, and liver fibrosis.

Several cells, growth factors, interleukins, receptors, and their signaling pathways participate in the development of CLD and must therefore be analyzed. The study of inflammation, steatosis, and fibrosis can be currently carried out using models that allow for the identification of the typical characteristics of said processes. Models developed for this purpose include diet-induced models, chemical/pharmacologically induced models, and genetic models. Some of the most important features of these models are described below.

3.1.1 Diet-induced models

The diet-induced models include a variety of dietary regimens that range from the administration of substances and fat supplies to caloric intake and additional supplements to facilitate the development of a NASH-like phenotype animal model.

Models	Inductor	Pathology	Advantages
Dietary [28–37]	HFD + CD	Inflammation, steatosis, fibrosis	Resembles human disease and does not require manipulation
	Fructose		
	Cholesterol + fructose		
	Cholesterol + trans fats + fructose		
	MCD	Inflammation, steatosis, fibrosis, cirrhosis, HCC	
	HFD + chemical	Inflammation, steatosis, fibrosis	Fast
Chemical Lee et al., [24, 38–46]	CCl_4	Inflammation, steatosis, fibrosis, cirrhosis, HCC	Fast
	CCl_4 + D-GH + LPS		Advanced fibrosis
	TAA + LPS		Fast
	CCl_4 + DEN		Fast
	DEN		Well tolerated and high HCC incidence
	DEN + TAA		Fast
	DEN + PB	Inflammation, steatosis, fibrosis, HCC	High HCC incidence
	DEN + 2-acetylaminofluorene + PB	Inflammation, steatosis, fibrosis, cirrhosis	Fast
	AS or PS		Lower mortality rates
Genetic [36, 47–54]	*ob*/*ob* mice	steatosis	Well reproducible
	db/*db* mice		
	Zucker *fa*/*fa*		
	SREBP-1 transgenic mice	Inflammation, steatosis, fibrosis	
	PPAR-α Knockout mice	Steatosis	
Surgical [55–57]	BDL	Inflammation, steatosis, fibrosis, cirrhosis	Fast and highly reproducible

HFD, high-fat diet; CD, choline deficiency; MCD, methionine and choline deficiency; CCL_4, AS, albumin serum; PS, porcine serum; carbon tetrachloride; D-GH, D-galactosamine hydrochloride; LPS, lipopolysaccharide; TAA, thioacetamide; DEN, diethylnitrosamine; PB, phenobarbital; BDL, bile duct ligation; HCC, hepatocellular carcinoma.

Table 1.
Models for the study of chronic liver disease.

1. *High-fat animal models.* This group includes the diets characterized by fat intake (up to 70%) with some other combinations such as a diet rich in fat but deficient in choline; a diet rich in fructose intake; a diet rich in cholesterol; a diet rich in cholesterol and fructose; a diet rich in cholesterol, fructose, and trans fats; or a diet rich in fat plus chemicals [28–34, 70]. The advantages are an oral route of

administration, small specimens, and an induction disease time that generally ranges between 12 and 30 weeks. However, the development of some models can be extended for longer periods. This includes a diet deficient in methionine and choline (up to 84 weeks), the high-cholesterol diet (up to 52 weeks), or the chemical diet (up to 52 weeks). The presence of cholesterol in these diets has been reported to increase the severity of the disease [35]. These diets generally lead to the development of steatosis (grade +++), the presence of inflammation (grade ++ or +++), and the development of metabolic syndrome; they are usually employed to address drug discovery or omics data. One advantage of these models is that most can reach the fibrosis stage (grade ++) and some, such as the high-fat diet combined with chemicals, achieve more advanced fibrosis (grade +++) [71, 72]. The development of hepatocellular carcinoma has only been reported with the choline-deficient diet, the high-fat, choline-deficient diet, and the high-fat, high-cholesterol diet [30–32, 70].

2. *The Western/cafeteria diet model.* Other diets try to imitate the bad eating habits that take place among people from big urban centers and with fast food ingestion [73]. These diets are made from a mixture of fat, sugar, and cholesterol. The disadvantage is that they are not standardized and vary greatly across different studies, which makes comparison and reproducibility difficult. There are several combinations that have produced NAFLD using the Western diet method. For example, rats have been fed a 40% fat, 40% sugar, and 2% cholesterol diet for 16 weeks. This diet induced the development of periportal steatosis and inflammation, but there was no development of fibrosis [74]. Another study using hamsters employed a diet containing 40.8% fat, 0.5% cholesterol, and sugar water for 12–16 weeks, leading to the development of microvesicular steatosis, fibrosis, obesity, and insulin resistance. A third study reported a diet based on 40% calories from fat, added sucrose/fructose, and 0.15–2% cholesterol (with and without sugar water) for 24–50 weeks and administered to mice. This study found the presence of steatosis, inflammation, and hepatic fibrosis, as well as obesity and insulin resistance [75]. Although these westernized diets show that the histopathological characteristics of NAFLD can be induced, an analysis of histological patterns, cytokine profile, activation of metabolic pathways, and the degree of damage shows differences in the metabolic profile and hepatic histopathological phenotype [76–78]. Therefore, the extrapolation of these findings to humans is difficult, and these models are not considered adequate for the study of new drugs or for obtaining omics data.

3.1.2 Chemical-induced models

Several studies have reported the use of hepatotoxic agents such as CCL_4, thioacetamide, and diethylnitrosamine to induce NAFLD in rodents. The degree of liver damage varies with the type of agent, the route, and the time of administration. However, said agents have been used for this purpose for over 50 years. CCl_4 is one of the toxic agents that has been widely employed in the research of the liver disease. It can be used by itself or in combination with other agents, either intraperitoneally, orally, by inhalation, or subcutaneously. Its administration ranges from 6 to 12 weeks. The advantage of using this agent is that the histopathological and functional characteristics it produces in experimental animals are already well established. This is one of the models that produces a high degree of steatosis (grade +), as well as a high

grade of inflammation and fibrosis (++ or +++), and can even cause portal hypertension and ascites in rodents [38, 39]. In combination with other hepatotoxic, it can lead to hepatocellular carcinoma. Differences in route of administration and dose are associated with variations between research groups. Thioacetamide is a toxic agent that is administered intraperitoneally or in drinking water, achieving liver damage in 10–12 weeks. This agent does not attain a high degree of steatosis, but it produces a high degree of inflammation and fibrosis (grade +++), and even portal hypertension in rodents. However, there have been discrepancies regarding the characteristics of the resulting liver injury. Hepatocellular carcinoma may develop if combined with other agents [40, 41]. Diethylnitrosamine (DEN) is a highly hepatotoxic agent that can be administered orally, intraperitoneally, and in drinking water. It achieves liver damage in 7–18 weeks. The degree of steatosis and fibrosis is very low, but there is a significant inflammatory response [42–44]. Due to its carcinogenic potential, it can lead to hepatocellular carcinoma. These toxic agents can be used as steatosis models for the study of new drugs and to obtain omics data.

3.1.3 Genetic models

Genetically modified animals, by insertion, modification, or deletion of certain DNA sequences, have been widely used for the study of NAFLD and new drug discovery. Genetic models used to study this pathological condition include the following:

1. The *ob*/*ob* mice. These mice do not express leptin and develop hyperphagia, reduced energy expenditure, obesity, hypothermia, and elevated plasma insulin. Obesity in these animals can be observed *via* an increase in adipocyte number and size. Adipose tissue transplants in Lep^{ob} homozygotes guard against obesity, normalize insulin sensitivity, and restore fertility. The main hepatic alterations manifested by these mice include elevations in hepatic mRNA expression for PGC-1α, PPAR-α, an impairment in hepatic mitochondrial function, and a dramatic upregulation in markers of hepatic *de novo* lipogenesis. They show an increase in fatty acid uptake proteins and downregulation of efflux lipid transporters, both of which promote lipid accumulation in the hepatocyte. There is also the recruitment of inflammatory cells, activation of inflammatory signaling pathways, oxidative stress, and lipotoxicity. Animals feeding on a high-fat diet can develop mild perisinusoidal fibrosis in portal areas (week 12) and bridging fibrosis (week 12). The presence of altered hepatic metabolism contributes to the development of NAFLD [47, 48].

2. The *db*/*db* mice are used to model phases 1 to 3 of diabetes type II and obesity. They have polyphagia, are polydipsic and polyuric, and suffer from chronic hyperglycemia, peripheral neuropathy, and myocardial disease. The liver abnormalities observed in these animals include accumulation of fat in the liver (steatosis) and inflammation. Their lipidomic feature indicates the presence of lipid species related to inflammation, energy, and lipid metabolism (FAS, SCD1, LXR β, SREBP-1, and DGAT-1), which leads to lipogenesis and loss of lipid homeostasis. The development of fibrosis in these animals is very low [36, 49]. This model develops neither steatohepatitis nor fibrosis.

3. Zucker *fa*/*fa* rats are the most known and widely used model for the study of obesity. Animals homozygous for the fa allele become noticeably obese by 3 to

5 weeks of age, and by 14 weeks of age, their body composition is over 40 percent lipid. Many metabolic syndrome features, such as hyperphagia, hyperglycemia, hyperinsulinemia, hypercholesterolemia, adipocyte hypertrophy, hyperplasia, and muscle atrophy, can also be observed in this animal model. The presence of an excess in adipocyte mass in these rats, along with insulin resistance, leads to *de novo* lipogenesis and an increased release of free fatty acids up taken by hepatic cells, leading to moderate hepatic steatosis. There is also documented presence of pro-inflammation markers such as TNF-alpha, IL-1beta, and IL-6. This model is more often employed to research inflammation and liver steatosis as associated with obesity [37, 50, 51].

4. SREBP-1 transgenic mice are characterized by the overexpression of the human nuclear sterol regulatory element-binding protein-1c in adipose tissue under the control of the adipocyte-specific aP2 promoter. These genetic alterations lead to an insufficiency of adipose tissue that is evident at birth and is accompanied by severe insulin resistance, leading to symptoms such as hyperinsulinemia and hyperglycemia. These mice exhibit similar 3–4-fold elevations in hepatic nSREBP-1c, which is associated with an increase in mRNAs for several lipogenic enzymes, an increase in the rate of fatty acid synthesis, and triglyceride accumulation in the liver. Liver steatosis is due to endoplasmic reticulum stress induced by SREBP-1 [52, 53, 79]. SREBP-1 transgenic mice are a good model for studying steatosis and steatohepatitis, but not for fibrosis.

5. PPAR-α knockout mice (PPARα$^{-/-}$) are characterized by high levels of serum triglycerides and extensive hepatic lipid accumulation and plasma fatty acid in plasma. PPAR-α is expressed in the adipose tissue of humans and rodents, stimulating lipolysis in adipocytes. It may also play an important role in regulating whole-body energy metabolism. Contrary to other genetic models, these animals suffer from hypoketonemia, hypothermia, and hypoglycemia but do not present obesity or fibrosis. PPAR-α Knockout mice are the best model for the study of liver steatosis. [54, 80, 81].

3.2 Models of cirrhosis and hepatocellular carcinoma

The main complication of CLD is cirrhosis [82]. Liver cirrhosis is a chronic, diffuse, and irreversible liver disease characterized by the existence of fibrosis, portal hypertension, and regenerative nodules. As a consequence, there are fewer liver cells and the liver stops carrying out its usual functions, including the synthesis of proteins (especially those that act in blood coagulation), the production of bile, the neutralization and elimination of foreign substances from the body, and the production of defenses against infection [83]. Although their clinical, biological, and laboratory manifestations can often suggest what the diagnosis is, this can only be confirmed *via* morphological study (biopsy). The prognosis is poor, and patients die from gastrointestinal bleeding, hepatocellular failure, neoplastic degeneration, or metastasis [84].

The development of hepatocellular carcinoma is common in the evolution of patients with liver cirrhosis [85]. Once cirrhosis is diagnosed, the chance of developing hepatocellular carcinoma is of 20% during the following 5 years. Since this type of carcinoma is frequently derived from cirrhosis, its clinical manifestations are often codependent. Prognosis depends on the evolution of cirrhosis at the time the cancer is

diagnosed. If hepatic functional reserve is good and hepatocellular carcinoma is asymptomatic, the patient may survive for several years. If the cirrhosis is very advanced and the carcinoma is very developed, the patient will die in a matter of weeks [86].

Chemically induced animal models are most often used for the study of cirrhosis and hepatocellular carcinoma, and some have already been mentioned above. However, to achieve the development of cirrhosis, portal hypertension, or liver cancer, it is necessary to extend the exposure time to the toxic agent, and there are other models specifically focused on the development of liver cirrhosis or hepatocellular carcinoma (**Figure 2**).

3.2.1 Chemical-induced models

CCl_4 in combination with D-galactosamine hydrochloride (GaIN) with or without lipopolysaccharides (LPS) is one of the more often employed models for developing liver cirrhosis. LPS is added to activate Kupffer cells and stimulate TNF-α, as well as an immune response *via* NF-κB pathway activation. D-galactosamine is used to potentiate this response by depleting the uridine nucleotides and interfering in protein synthesis, leading to acute lesions as a precipitating event [87]. Liver cirrhosis is developed in 10 weeks. In this model, we see an increase in aspartate amino transferase (AST) and alanine amino transferase (ALT), as well as the presence of necrosis, and excessive fibrosis and regenerative nodules. Thioacetamide combined with LPS has been used to induce decompensated cirrhosis. After 10 weeks of its administration in the drinking water, increases in liver enzymes, portal hypertension, fibrosis, and cirrhosis can be observed.

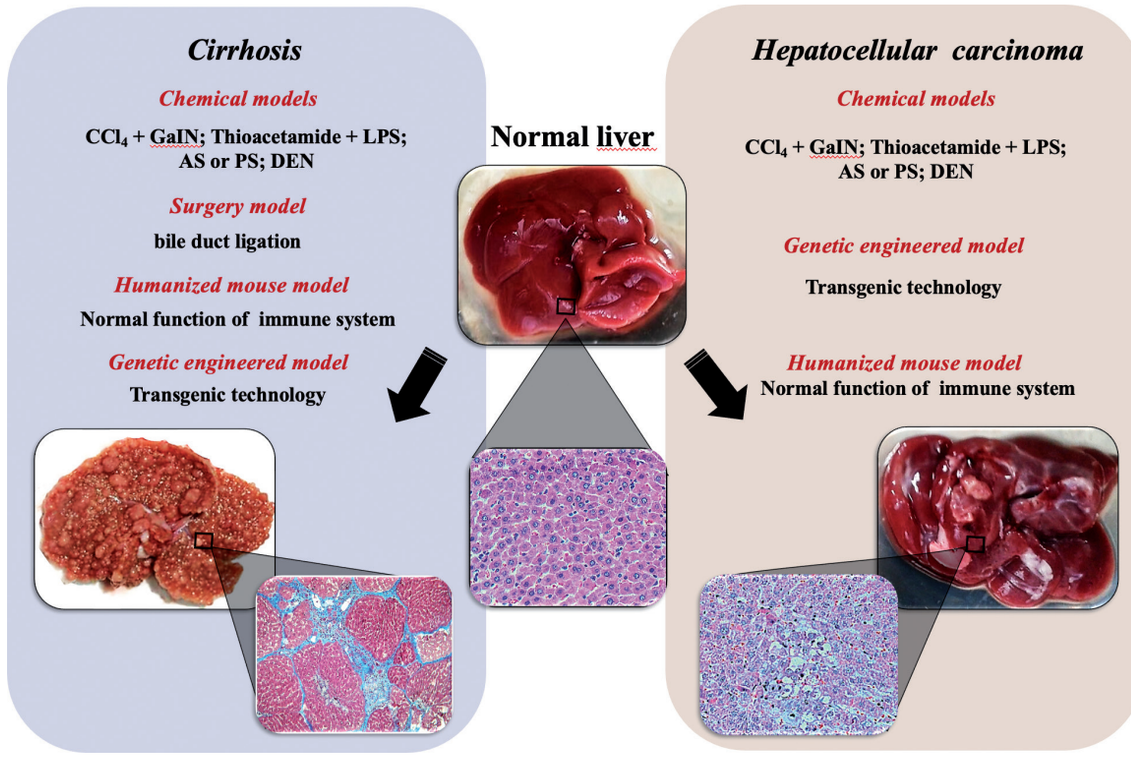

Figure 2.
Models of cirrhosis and hepatocellular carcinoma. Examples of animal models to induce cirrhosis and hepatocellular carcinoma: chemicals, surgery, genetic engineering, and humanized mice. CCl_4: carbon tetrachloride; GaIN: D-galactosamine hydrochloride; LPS: lipopolysaccharides; AS: albumin serum; PS; porcine serum; DEN: diethylnitrosamine.

Albumin serum (AS) and porcine serum (PS) administrations are two other potential models for inducing immune cirrhosis. Albumin becomes hepatotoxic when there is an imbalance of it in the liver [88]. Albumin or PS may induce cirrhosis when interacting with LPS or when undergoing irreversible alkalization *via* drug metabolization. The administration of albumin or PS twice a week for 6 weeks leads to CLD decompensation and subsequent histological and functional liver changes. There is also an increase in several inflammatory markers, such as IL-6, IL-18, and HMGB-1 [89]. The PS model may be more adequate for the induction of immune cirrhosis. It induces a response that is closer to human liver disease, is easier to manipulate, and results in lower mortality rates for animals when compared with the other mentioned models.

DEN is the most important and widely used toxic agent for the development of chemically induced HCC in rodents. It is a carcinogen, and DEN bioactivation occurs in the liver *via* cytochrome P450 (CYP 2E1) [90]. DEN is metabolized *via* alpha-hydroxylation and dealkylation reactions, producing an unstable ethyldiazonium hydroxide molecule that can generate highly reactive carbon, oxygen (ROS), and nitrogen (RNS) ion species. These reactive molecules bind to DNA and proteins and can contribute to genomic instability, DNA damage, mutation, and tumor initiation [91]. DEN "initiates" the hepatocarcinogenesis process and produces a stable and heritable mutational change in hepatocytes, inducing genomic alterations in oncogenes and tumor suppressor genes. HCC can clonally expand from this single DEN-"initiated" hepatocyte and result in HCC.

There are different protocols where DEN is used to induce HCC in rodents. For example, it can be administered alone and in single intraperitoneal doses ranging from 1 to 5 mg, resulting in adenomas (0–20%) in 24 weeks or carcinoma in 36 to 52 weeks [92–94]. When administered in multiple doses, it is used in doses of 25 to 50 mg for 4–8 weeks, resulting in preneoplastic lesions, adenoma, or carcinoma depending on the total administration time. DEN may be used in single or multiple doses in combination with CCl_4 0.5 mg/mL to obtain preneoplastic lesions, adenomas, or carcinomas, also depending on the total time of administration [95, 96]. In another rat protocol, a single dose of DEN plus 0.03% thioacetamide is administered, leading to adenomas or carcinomas between 24 and 52 weeks [97, 98]. A rat model employs DEN in a single dose (80–200 mg) in combination with phenobarbital (PB) (0.025–0.1%) to obtain the desired preneoplastic lesions, adenomas, or carcinomas [99]. DEN has also been combined with 2-acetylaminofluorene (0.02%) and PB (67%) to cause carcinoma and metastasis [45].

3.2.2 Surgery model

Common bile duct ligation is a model of secondary biliary cirrhosis. The surgical resection of the bile duct induces bile accumulation in the liver and thus leads to damage, inflammation, fibrosis, portal inflammation, and cirrhosis after 2 weeks [46, 55]. An increase in AST, ALT, and total bilirubin levels can be observed after surgery in rats and mice. Recent reports suggest that the use of a single dose of LPS can induce decompensated disease [56]. This model is used for drug discovery and omics data.

3.2.3 Genetically engineered models

The above-mentioned models have been in use for more than 30 years and allow for a deeper understanding of the pathogenic mechanisms involved in chronic liver

disease and malignant transformation. The better known genetically engineered models include HBV-transgenic, HCV-transgenic, *c-myc*-transgenic, *c-myc*-/TGF-α transgenic, E2F-1 transgenic, *c-myc*-/E2F-1 transgenic, *Apc* knockout mice, β-catenin/H-ras mutant, and cMyc+shp53 mice. Transgenic technology was initiated by inserting exogenous viral DNA into mice to induce the expression of different components of the viral particle [57]. Afterward, several important protein-coding genes and transcriptional enhancers associated with cancer were micro-injected into single-cell embryos of specific mice strains. Most transgenic mice show micro- and macro-fat vacuoles (at 6 and 11 months of age). Although inflammation is not commonly observed between 6 and 15 months of age, most mice develop preneoplastic hepatic lesions and adenomas at 6 and 11 months [100]. Those changes are accompanied by alterations in glycolysis and lipogenesis, which play a key role in the early (preneoplastic) stages of hepatocarcinogenesis. A high incidence of adenomas and carcinomas appears between 40 and 136 weeks. The advantage of these models is that they are highly reproducible and resemble human disease.

3.2.4 Humanized mouse model

Humanized mouse models are widely used to mimic the human immune system in mice [101]. This mouse model is based on transplanted human cells and tissues that have been uniquely engineered to produce combinations of human cytokines in immunocompromised mice and avoid rejection of implanted cells. This kind of model results in a more physiologically relevant model system for evaluating new cancer therapies, immuno-oncology, and effective treatments targeting the tumor microenvironment. Proteomics studies of humanized models have shown that there is an increase in proteins related to apoptosis at 12 months, the defense of fatty acid metabolism against oxidative stress [102]. Other studies have shown that, in combination with DEN, there can be an increase in the size of neoplastic nodules [103]. The mouse is the most widely used animal model in biomedical research because it is versatile, inexpensive, and genetically very similar to humans. However, success depends on animal age, sex, and strain.

4. Regulatory issues regarding the use of animal models in CLD research

Biotechnological advances in medicine and pharmaceutical sciences during recent decades have provided us with tools for better diagnosis and treatment of CLD. This has gone hand in hand with the exponential rise in the use of animal models for research. Animal models in preclinical trials have largely served as the scientific basis for the data that has enabled a better understanding of the pathophysiology of human disease. However, researchers around the world are currently expressing concern about the use of animals for scientific experimentation [104].

To make use of animal models in the field of hepatology, as well as in other medical areas, we must essentially consider various aspects regarding the rational use and management of animals for their use in research protocols. Whenever animals are used as research models, we must keep in mind that the main purpose of the study is to obtain feasible, reproducible, and reliable results inasmuch CLD comprises an important health problem that requires prompt study and attention. One way to achieve this is to reduce the stress to which animals are subjected throughout the study. Aforesaid, the

induction of the different stages of CLD requires handling the animals for prolonged periods of time, which can affect and compromise animal welfare. On the other hand, we already know that stress influences affective behavior and stress hormone release may alter the pathophysiology of the CLD [105–108]. For this reason, the scientific community has been made aware of the importance of ensuring the socio-environmental welfare of experimental animals. In Europe, for example, there are regulations regarding the use of experimental animals and a series of standards have been established regarding protection criteria and ethical issues. The scientific community has always been encouraged to implement them insofar as this is possible [109].

Discussions regarding the use of animal models are not recent and have increased over the years, involving the scientific community across different countries [110–112]. Today, there are standards such as Animals in Research: Reporting In Vivo Experiments (ARRIVE), which, since 2010, has become a useful guideline in biomedical science and related areas and is meant to improve the use and management of experimental animals from an ethical, social, and animal welfare-based perspective so as to obtain reliable results [113]. In fact, it has become common practice that, in order to publish scientific research, publishers request letters indicating that the research and ethics committees authorized the use of animals. The legal agreements, regulations, and guidelines for each country, as well as international standards, recommend that each study adheres to the most effective type of test and the most appropriate species for the study in order to reduce the number of animals to the bare minimum. Additionally, the use of more complex animal models, such as animals obtained *via* genetic engineering or humanized animal models, has required the expansion of these regulations.

One of the regulatory standard documents with international recognition is the Guide for the Care and Use of Laboratory Animals (NIH, Guide), which promotes the care and humane use of laboratory animals *via* a comprehensive program of publications, scientific protocols, and opinions based on the scientific experience of researchers using methodologies and practices that enable the desired results [114]. Another body handling international agreements is the Organization for Economic Cooperation and Development (OECD), which is responsible for regulating scientific protocols, tests, and analyses in order to guarantee quality results that adhere to human pathophysiology [115–119].

The implementation, development, and use of more complex animal models requiring special care will necessitate new international standards and agreements to develop better strategies and standardized protocols for scientific research. However, animal replacement is still far from becoming a viable alternative for the comparative study and development of models of human pathophysiology, at least in regard to CLD.

5. Conclusion and future outlook

Clinically speaking, the management of patients with CLD is difficult because, as mentioned above, the disease goes through several stages, each of which requires a different kind of therapeutic intervention. Clinicians and researchers still face many challenges regarding the management and study of CLD. However, there are possible ways of overcoming these challenges. More and more animal models have become available for the study of diseases, parallel to the discovery and development of new drugs. However, it is essential that we continue to improve and validate these models, particularly with regard to the molecular mechanisms that trigger and perpetuate the disease. This will ensure that they truly reflect each of the histopathological stages of

human disease and will increase their predictive validity, as well as their use in the discovery of new therapeutic options.

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Conflict of interest

The authors declare no conflict of interest.

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